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PHYSIOLOGICAL HISTOLOGY

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PHYSIOLOGICAL HISTOLOGY

METHODS AND THEORY

BY

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'Les efforts que font les hommes pour découvrir la vérité sont donc une espèce de jeu de hasard, dans lequel la probabilité de tomber dans l'erreur est très-grande, et celle de trouver la vérité est très-petite'
FONTANA, 1781

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PREFACE

THERE was a time when British histologists were in the forefront both as regards the nature and the extent of their researches, but now—possibly owing to the developments which have taken place in investigations relating to the physical and mechanical laws concerning nerve and muscle preparations, in experimental researches into the function of the central and peripheral nervous systems, as well as the increase and character of the inquiries relative to internal secretion, the mechanics of the circulation, and the chemistry of the tissues and fluids of the body—there has been a tendency of recent years to alienate the physiologist from the study of Histology.

Anatomy deals with the development and the mature structure of the different organs composing the body. It also includes the functions of these organs as far as they may be explained on mechanical lines. Physiology, built on the foundations of anatomy, devotes itself essentially to chemical and physical considerations. The common object which the anatomist and the physiologist ought to have in view is the elucidation of general laws governing biological phenomena.

Histology comprises investigations into the structure, the chemical composition, and the functions of healthy and diseased animal and vegetable cells, and therefore the ordinarily accepted view that Histology concerns itself merely with micro-anatomy seems to me a narrow one.

Owing to the brilliant work done abroad, the study of mammalian micro-anatomy, as distinct from micro-chemistry and micro-physiology, is approaching completion, and it is time for the physiologist to awaken to the fact that work requires to be done along micro-physiological lines, and that such work would be of immense value, since it would serve as an aid to the pathologist in the elucidation of the processes with which he is more particularly concerned.

The physiologist should study the life-cycles of the various cells composing the body; determine, for example, the chemical

changes which connective tissues undergo during development, show how they are interrelated, along what paths they have been evolved. He should investigate the factors calling forth or diminishing activity in the nucleus as contrasted with the rest of the cell. He should put to himself such questions as these: Which chemical substances, organic and inorganic, have power to accelerate or to diminish cell-division? What is the cause of the formation of giant cells? Or to put it differently, why do certain cells not divide when their nucleus does do so? What is the ratio between the surface of the cell and that exposed by the nuclei? How far can definite inorganic and organic compounds be located in the cell? To what extent are the evolutionary ideas of Kossel borne out by microscopic investigations?

Scientific endeavours to answer these and similar questions can only be made after a thorough realization of the difficulties which beset our inquiries at every step. We cannot in every case examine cells in their living condition, and if we could, and did thus restrict our investigations, we should not learn more anatomy and physiology than he who simply watches an animal move and feed.

Without in any way desiring to diminish the importance of investigating the living organism, it is necessary for purposes of biological research to kill, and to do this in such a manner as to preserve as far as possible the physical and the chemical state existing during life.

With this end in view the book has been written, and it represents a first attempt to explain the principles underlying histological methods.

Special attention has been paid to the question of preserving tissues by fixation, and hence to physical chemistry, to the chemistry of fixing reagents, to coagulation, and colloids; further to micro-chemistry, and hence to macro-chemical methods; to the chemistry and the methods of application of staining reagents, and lastly to the theory of staining.

Some earnest workers, I know, have been deterred from histological research because of the many uncertainties and difficulties which present themselves. In many respects the problems encountered by the histologist are shrouded in darkness, and co-operation is needed to throw light on them. Let us combinedly overcome the difficulties as they arise, and

gradually move forwards. Nobody realizes more than I do what labour there is before us, and I hope that this book, if it serve no other purpose, will bring out deficiencies in theories and in methods.

Much as I should have liked to deal with the problems of staining and examining living tissues, I feel that as yet I could not have done justice to them, and therefore I have not touched these subjects, other than by giving a full account of the chemistry of the dyes used in the *intra vitam* technique.

If any idea or method described as my own should be claimed by any one else, I shall gladly attribute it to him as soon as I am informed. I make no claims either as to priority or as to originality.

Most of the work embodied in this volume has been done in the laboratory of Professor Gotch, F.R.S., of the University of Oxford, and I am pleased to express my appreciation of the facilities which have been afforded to me.

I also desire to express my sincere thanks to many who have given me kind help: to Professor Arthur Thomson, M.A., of the University of Oxford, who persuaded me to publish my views and who revised my book; to D. H. Nagel, M.A., of Trinity College, who helped me with good advice generally, and specially in connexion with physical chemistry; to W. W. Fisher, M.A., and J. E. Marsh, M.A., for help in chemical matters; to Miss Mabel Purefoy FitzGerald for reading a great part of my manuscript and proof-sheets; to Miss L. H. Huie for the great interest she took in my work and for revising my index before it was printed.

To Professor C. S. Sherrington, F.R.S., whose kind criticism encouraged me in adopting the ambitious title chosen for this book, and to Sir John Burdon-Sanderson, Bart., F.R.S., for suggesting the title '*Physiological Histology*'—to these I also express my warmest thanks.

This book I have dedicated to the memory of one who endeared himself to his students by treating all with the same courtesy, and educated them by sparing neither pains nor trouble to make his lectures attractive; to one who aroused my interest in histological research and taught me patience.

TO THE MEMORY OF

THE LATE PROFESSOR OF BOTANY

IN THE UNIVERSITY OF EDINBURGH

ALEXANDER DICKSON, M.D.

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ERRATA.

Page 33, line 22, *for* 1872 *read* 1892

79, l. 26, *for* HO_2 *read* H_2O

79, ll. 34, 35, *for* potassium cyanide, in KCN *read* potassium cyanide, KCN

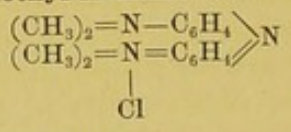
166, table, last col. but two, *insert* 50·6 *at bottom*

191, l. 18 infra, *for* intercellular *read* intracellular

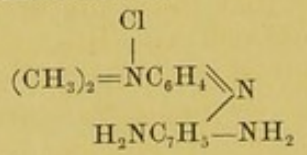
290, l. 18, *for* Fischer *read* Fisher

386, l. 5, *for* benzoquinone = phenyl-hydrazone *read* phenyl-hydrazone of benzoquinone

402, under **Tetramethyl-indamin** *read*



402, under **Toluylene-blue** *read*



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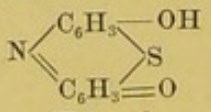
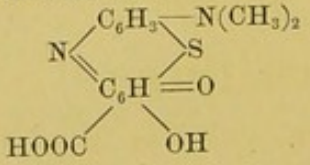


Thionol.

406, under **Gallo-thionin** *read*



HISTOLOGY

Histology comprises microscopical investigations into the structure, the composition and the functions of healthy and diseased animal and vegetable tissues.

Tissues are composed of cells and cell-derivatives and should be investigated along physical, chemical, and morphological lines; further, whenever possible they should be studied in their living condition as well as when dead, death having occurred either spontaneously or having been induced purposely by fixing reagents.

The term fixation comprises all those processes, physical and chemical, by which we regulate the changes occurring at and after death with the view of preserving the structure and the composition of tissues in a state approaching that found during life. Fixation, however, need not comprise a simultaneous 'hardening.' Thus after fixing in weak alcohol, picric acid or formaldehyde, tissues are still so frail, that to render them less susceptible to subsequent manipulations, they have to be hardened by special processes, as for example by treatment with solutions of the salts of heavy metals, or with strong alcohol.

Experience has taught us that the study of the living cell is very difficult because the various cell constituents possess during life approximately the same refractive index. For this reason, before successful endeavours can be made to interpret the ill-defined structures seen in living cells it becomes imperative to have a thorough knowledge of the appearances presented by such cells after death has supervened, and at once the question arises: To what extent is it legitimate, from the morphological and the chemical standpoint, to compare the dead cell with the living?

PART I.

CHAPTER I.

Discussion as to the Value of Fixation.

Before giving a summary of the views on the structure of the cell it is necessary to define what others have called 'protoplasm,' a term which is constantly used in the most loose manner, and also to define in what sense the word 'plasm' is used in this book.

If from cells in a state of complete inanition we take away their nuclei, centrosomes, ducts, cilia, fibrillar or foam-like supporting structures and zymogen granules, nothing will be left but the cell-membranes. What generally goes under the name of 'protoplasm' comprises the structures which were just enumerated, but impregnated with or permeated by lymph containing nucleo-proteids. 'Protoplasm' is thus a generic and not a specific term, and had best not be

employed at all, or if a generic term is needed, then the word 'plasm' serves better.

If amongst histologists 'protoplasm' is a vague expression, then amongst chemists the word 'proteid' is still more so. The question whether normally proteids do or do not contain salts seems to vex many, for they have not realized that so-called pure ash-free proteids are chemically inert and, in the true sense of the word, dead bodies. What puts life into them is the presence of electrolytes, either unorganized or organized. But this is discussed later in the chapter on colloids.

How diverse the opinions of histologists have been in regard to the supposed nature and structure of living matter, and also in regard to the methods which are best suited for fixing purposes, will be set out in the following pages.

Ever since the discovery of the cell, endeavours have been made to exclude the fictitious appearances produced by reagents. With this view, originally, substances were chosen which preserved the shape of the cell, but, as the internal cell-structures attracted more and more attention, the diverse appearances which result from the use of different fixatives made control absolutely necessary.

Two methods naturally suggested themselves, namely, to study the living cell before and while the reagents produced their effect, and also to investigate the behaviour of fixatives on isolated cell-derivatives such as egg-albumin and gelatin.

Flemming[1], whom we must regard as the pioneer in this research, has constantly laid stress on the point that we are not justified in regarding as normal all the structures to be seen in cells which have been treated with reagents. Knowing the fallacies accruing from 'fixing' cells, he, notwithstanding, published his osmo-chromo-acetic mixture, because he considered it the least liable to produce artefacts, and the best for preserving the appearances seen in perfectly normal cells.

As the result of his researches he arrived at the conception that 'protoplasm' has a threadlike or filar structure, which view in his later communications[2] he combines with the trabecular theory, started by Frommann, Heitzmann, and Klein, and supported in recent times by the observations of van Beneden, M. Heidenhain, Solger, Kromayer, von Nathasius, Dogiel, Reinke and others.

The original view of Flemming was, however, vigorously attacked by two botanists, namely, Berthold and Schwarz.

Berthold[3] in 1886 rediscovered, what has since been again discovered and extended by Ramsden[4], that a 'mechanical coagulum' can be produced by shaking white of egg with distilled water, and that this coagulum has the fibrillar appearance described by Flemming, while

[1] Flemming: *Zellsubstanz, Kern und Zelltheilung*, Leipzig, 1882.

[2] Flemming: 'Zelle' in *Ergebnisse der Anat. ü. Entwickelungsgesch* (Merkel and Bennet, 1893, and subsequent years).

[3] Berthold: *Studien ü. Protoplasmamechanik* (1886). Berthold was not the original discoverer, see Ramsden.

[4] Ramsden: see p. 50, On mechanical conglutination.

the same substance treated with a watery solution of iodine in potassium iodide gives a finely granular precipitate.

Schwarz[1] in 1887 studied the effects of alcohol, picric acid and Flemming's solutions on white of egg, gelatine and peptone, and showed that colloidal bodies are precipitated as minute droplets and granules, the size of which varies directly in proportion to the strength of the fixing solutions.

An entirely different conception as to the structure of protoplasm was arrived at by Altmann[2], who in a number of papers from 1886 to 1893 put forward the view that each cell consists of a large number of spherical or threadlike individuals, the bioblasts, held together by a glue-like substance ; in short, that a cell resembles the zoogloeae masses formed by certain bacteria which live together in a jelly. This theory he based on the appearances obtainable with his osmo-bichromate fixing method and acid fuchsin picric-acid stain. Flemming in 1893 regarded Altmann's granules as the homologues of such cell granules as are found in ordinary secreting cells, and stated that being 'formed' material they could not be regarded as representing part of the 'protoplasm.'

Bütschli[3] in 1891 published his view that all protoplasm has a chambered or foam-like arrangement. He subsequently extended his researches by studying not only the protoplasm of protozoa, plants, and the different organs of all classes of animals, but also by investigating the structure of gelatin, starch-paste, collodium and minerals; and with artificial foams consisting of soap and oil, he endeavoured to explain such phenomena as the mitotic division of cells. Thus the first attempt was made to give a mechanico-physical explanation of the appearances seen in histological specimens, and Bütschli was greatly helped in this endeavour by the work of the physicist Quincke[4], who experimented on plasmolysis and on the movement of protoplasm.

Ianošik[5] (1893) again studied the effects produced on white of egg by alcohol, 'osmic acid,' and the solutions of Flemming and Müller, and arrived at the conclusion that networks and granules, not at all unlike those seen in fixed cells, may be thus obtained. That this, however, is not the case if osmium tetroxide is used, will be shown later on (p. 69).

[1] Schwarz : 'Die morphol. u. chem. Zusammensetz. d. Protopl.,' *Cohn's Beitr.* 5 (1887).

[2] Altmann : (*a*) *Studien ü. d. Zelle*, Leipzig, 1886. (*b*) *Die Elementarorganismen*, Leipzig, 1890, 1893. (*c*) *Arch. f. Anat. u. physiol. Anat.*, Abth. 1892, p. 223.

[3] Bütschli : (*a*) 'Über d. Struktur d. Protoplasmas,' *Verh. d. deutsch. zool. Ges.* (1891). (*b*) 'Über d. feineren Bau d. kontraktilen Subst. d. Muskelzellen v. Ascaris, &c.,' *Leuckardt's Festschrift*, 1892. (*c*) *Unters. ü. mikroskopische Schäume u. d. Protopl.*,' Leipzig, 1892. (*d*) 'Über d. feineren Bau d. Stärkekörner, Über d. künstliche Nachahmung d. karyok. Figur.' (*e*) 'Über Strukturen künstlicher u. natürlicher quellbarer Substanzen,' *Verhandl. d. naturw. Vereins*, Heidelberg, 1892–5. (*f*) 'Meine Ansicht ü. d. Struktur d. Protoplasmas und einige ihrer Kritiker,' *Arch. f. Entwickelungsmechanik d. Organismen*, 11, 499 (1901).

[4] Quincke : Abstract in O. Lehmann's *Molekularphysik*, 2, 490, &c.

[5] Ianošik : 'Über d. Struktur d. Säugethiereizelle,' *Czechisch. Akad. d. Wiss.*, Prag, 1893.

Unna (1894), who has done so much for the minute investigation of the skin, worked out an elaborate system of specific stains[1], which led him to the following conclusion: Flemming's and Bütschli's views are not necessarily opposed to one another; for a cell, according to its state of health, may either show a filar or a foam-like protoplasm, and fibrils may even run along and through the foam-like remainder of the protoplasm[2].

In 1894 Alfred Fischer[3] commenced his researches, which he has continued up till now. He originally took up his investigations in order to determine whether the granules which Altmann obtained with his fixative were artefacts. Subsequently he extended his researches to all classes of chemically pure proteids[3d]. It is Fischer's chief merit to have shown that from an intimate 'homogeneous' mixture of albumose and albumin, the former may be precipitated in a granular form by certain reagents, including Altmann's mixture, while the albumin is thrown down as a net-like coagulum.

Hardy, in 1899, attacked the microscopical appearances from a physical point of view, in a similar manner to that of Bütschli and Quincke. His results may be briefly summarized thus: Coagulation, whether it take place spontaneously at death, or whether it be induced by fixatives in living cells or colloidal structureless substances such as filtered white of egg or gelatin, is always accompanied by the separation of a fluid part from a solid net-like structure, 'which differs in most cases in kind, in some cases in degree, from the initial structure.'

During the two years 1888–9 I[4] continued the work of Flemming, Berthold, and Schwarz, and made a systematic study of the various reagents used by physiological chemists for precipitating proteids. The material experimented on was Spirogyra nitida, the hairs of Tradescantia during rest and division, the growing apices of leaves of liliaceous plants, and white of egg. I confirmed the observations of Schwarz regarding the effects produced on white of egg by the fixatives he employed, but I did not attribute much importance to them, because I considered egg-white to be a dead substance which need not behave at all like a living cell. Living tissues, whilst under microscopic observation, were subjected to the action of a number of fixatives in dilute and concentrated watery and alcoholic solutions. As the result of two years' work I came to the conclusion that saturated alcoholic and watery solutions of picric acid in combination with corrosive sublimate and tannin were the most vigorous precipitating agents, and that for this reason they ought to be employed. With saturated solutions of picric acid and sublimate in absolute alcohol more homogeneous precipitates were obtained

[1] Unna, 'Über Protoplasma-Färbung nebst Bemerk. ü. d. Bindegewebsz. d. Cutis,' *Monatsschr. f. prakt. Dermat.* 19, 226 (1894).

[2] Unna, 'Über d. neueren Protoplasmatheorien u. d. Spongioplasma,' *Deutsche Medizinalzeitung* (1895).

[3] Fischer, (*a*) 'Zur Kritik d. Fixirungsmethoden,' *Anat. Anz.* 9 (1894). (*b*) 'Neue Beit. z. Kritik d. Fixirungsmethoden,' *Anat. Anz.* 10 (1895). (*c*) *Über d. Bau. d. Cyanophyceen u. Bakterien*, Jena, 1897. (*d*) *Fixirung, Färbung u. Bau d. Protoplasmas*, Jena, 1899.

[4] Mann, *Trans. and Proc. Bot. Soc.*, Edinburgh, 18th session 1889–90, pp. 429, 443.

than with any other fixative, the cytoplasm in plants[1] appearing to consist of closely packed granules, the microsomes of M. Heidenhain, with the intermediate substance reduced to a minimum. About 1893 I commenced to realize that those reagents, which most thoroughly precipitate proteids in test-tubes, need not of necessity be the most suitable for preserving the delicate structures occurring in the cell. Picro-corrosive is the best mixture for completely and readily precipitating all proteid substances, but such vigorous precipitation causes many of the fine details in cells to disappear. To remedy this defect, which I attributed to exosmosis being caused by the high concentration of salts in the fixing solutions, I passed from saturated solutions to those having approximately the specific gravity of tissues, namely, 1,020.

Since 1895 I have used combinations of the picro-corrosive mixture with osmium tetroxide and formaldehyde, because I believed these substances, by forming additive compounds with proteids, would render the cell more resistant. Now I consider all electrolytes, it being immaterial whether they be used singly or in combination with non-electrolytes, to have a tendency to produce artefacts, and for purely morphological purposes, if chemical substances have to be employed, I rely on the action of the non-electrolytes osmium tetroxide and formaldehyde dissolved in isotonic solutions of sodium chloride.

From the above short summary of the results obtained by different observers, it would almost appear as if the task of investigating the structure of the cell was a hopeless one, and that we should never get any nearer the truth. Such a view, however, does not seem to be warranted.

When investigating the anatomy of an animal, after studying first its outer aspect, we examine the general arrangement of its internal viscera, and do not start, for example, the study of human anatomy by contemplating the net-like arrangement of lines on the surface of the liver, and by speculating as to their probable cause.

Similarly, in dealing with cells we should study in the first instance the coarse anatomy of the cell, namely, its nucleus, centrosomes, secretions, ducts, and specialized plasms as found in muscle, connective tissue, or epidermal structures, for all these elements may readily be seen in suitable, perfectly normal, living cells.

Subsequently, by the addition of a fixing reagent we endeavour to preserve the appearances seen during life. If, in doing so, new structures are revealed, we have to determine whether these pre-exist, or whether they have been produced by the reagent employed. To settle this question a systematic use must be made of normal death, and that caused by the most diverse physical and chemical means, such as heat, cold, dehydration ; by acid, neutral, and alkaline media ; by oxidation and reduction, and the action of reagents forming additive compounds. All these means must further be tried not only on resting cells, but also on those which are in various phases

[1] Mann, 'The Embryo-sac of Myosurus minimus. A Cell Study.' *Trans. and Proc. Bot. Soc.*, Edinburgh, 1892.

of functional activity, such as division and secretion, or while absorbing food.

If we have ascertained by an exact study of the most diverse fixatives that they all agree in producing in the cells under investigation certain effects in common—for example, a definite number of chromatin segments—we have every right to suppose that the common feature is the outcome of something which was preformed in the living cell, but which for optical reasons we could not discern as long as life lasted.

Now an entirely new question arises, namely, to what extent is a cell-organ, revealed by different methods, preserved in its original state? By what changes in its physical or chemical composition does it become visible? In respect of gross external characters, as maintenance of shape or mutual arrangement of cell-organs, it is not difficult to come to a conclusion; for example, alcohol—which shows chromatin segments as do other fixatives—distorts them by its diffusion currents; other reagents, again, by their slow action, do not fix all the cell-contents simultaneously, in consequence of which a general disarrangement becomes evident as compared with similar cells fixed by quickly acting solutions. Further, some reagents, because of differences in their general precipitating power, show certain structures attenuated and shrivelled, which after the use of other fixatives appear in a more bulky and denser form.

Which amongst these varied appearances are we to choose as the one nearest to nature? The one which suits our fancy most, either because of its shape or its colour reactions? I think not. Let each cell-organ be examined in a systematic way, not by one but many reagents; do not describe only the effects produced by what some call the best fixative, leaving out the effects produced by 'bad' ones; give other investigators the benefit of 'failures,' and thus gradually help to build up a systematic way of investigating cells.

Only after a complete and separate investigation of the cell-body and the nucleus, by fixatives especially suited to each of them, is it permissible for purposes of reconstruction to combine into one scheme those features which were obtained repeatedly by diverse means[1].

Some people, on reading Hardy's results, may think, If coagulation always produces a foam or net-like structure in colloidal homogeneous substances, what right have we to make deductions as to the original condition of cells? To me it seems that such a person is assuming in the first instance that the structures just mentioned are always produced, and in the second instance that the substances under investigation, and also the cell, are homogeneous.

Hardy himself has stated that the surfaces of conjugate fluids and the advancing edge of a white of egg solution do not show a net-like arrangement, and reference to my slide and test-tube experiments (p. 125) will further show that absolutely uniform, granular membranes

[1] Following this plan, I have during the last five years employed close on 200 distinct fixing methods on nerve-cells.

and masses are to be obtained, with no indication whatever of a net or foam, when colloids are subjected to special conditions of stress or strain. Monckeberg and Bethe have further found white of egg fixed for forty-eight hours in the vapour of osmium tetroxide to be perfectly homogeneous, an observation bearing out my own.

Formaldehyde free from formic acid also fixes homogeneously.

It being thus possible to fix colloids in a homogeneously granular or foam-like manner, it is natural to choose primarily those reagents which produce the least visible alteration in media we know to have been homogeneous before fixation, because the less violent the change in a uniform medium the more completely also will be preserved those delicate differentiations existing normally in a cell. Just now it was assumed that homogeneous mixtures do exist, but when we see produced a net, or foam, or granules, in what appears to us before fixation a homogeneous mixture, have we any right to suppose that such a mixture is in reality without structure? I doubt it. When gelatin jelly sets at any given temperature, as soon as the percentage of water is reduced to an amount proportional to the temperature, we may assume either that an aggregation (polymerization) of the gelatin molecules has taken place, or that each gelatin molecule having become less mobile maintains its individuality, and that it touches its neighbour. As the gelatin molecules are larger than those of water, it is legitimate to suppose (not taking into account the intramolecular absorption of water) that the gelatin in the jelly state forms a double net consisting respectively of gelatin and water molecules, a view also supported by the rate of diffusion and ionic migration being the same in gelatin as in water.

If this conjecture be true, then the appearances which we see in 'fixed' gelatin represent an exaggerated picture of the normal condition. Further, as the network which is formed by reagents is in most instances so close as to necessitate, for its clear demonstration, sections not exceeding 1·5 μ in thickness, we may readily, by cutting a section 2·5–5 μ in thickness, reproduce the 'homogeneous' appearance of unformed matter to the advantage of the formed or structural elements in the cell, which under these conditions will stand out clearly.

Although for the general fixation of a cell those coagulants are best which give a homogeneous fixation, I believe it justifiable to use also reagents which do not preserve every cell-constituent. When we study the configuration of bones, we remove the muscles and other soft parts as completely as possible; when we desire to study the brain, the skull-bones are sacrificed, and we should proceed analogously with the study of the cell. After having studied the appearances in tissues fixed by reagents which act homogeneously, do not let us be afraid of using fixatives which cause artificial appearances in homogeneous solutions of proteids, as long as we know definitely what effects each reagent produces in a homogeneous mixture.

Hitherto far too little attention has been paid to the chemical and the physical aspects of cell-investigation; substances capable of acting as

fixatives have been used in the most indiscriminate manner, for acids and salts are employed in combination without the histologist knowing how these substances in solution interact upon one another, or on the tissue with which they are brought into contact. The teachings of both inorganic and of organic chemistry have been thrown away on the micro-anatomist, and so far only few investigators have touched the physical side of fixation.

Let me make my meaning clear by a short reference to two substances which are in daily use, namely, corrosive sublimate and formaldehyde, the former an electrolyte, while the latter is a non-electrolyte.

Sublimate or bichloride of mercury, $HgCl_2$, in watery solutions has an acid reaction, because it dissociates hydrolytically into HgOH and 2HCl, which latter electrolytically breaks up into the hydrogen kat-ion, which causes the acid reaction, and the chlorine an-ion, thus :

$$HgCl_2 = HgOH + 2HCl\ ;\ HCl = H^{\cdot} + Cl'.$$

As long as sublimate is in a watery solution it coagulates quickly ; now add sodium chloride to the sublimate solution, and if sufficient is added, the acid reaction of the original sublimate solution will disappear, because the sodium chloride, by its ready dissociation into sodium ions and chlorine ions, saturates the water acting as a solvent with chlorine ions, in consequence of which no call is made on the chlorine ions of the sublimate, and hence it does not dissociate ; non-dissociation of the sublimate means that no hydrogen ions will be set free, therefore the watery solution will no longer be acid, and the non-dissociated sublimate in the salt solution is unable to act as a fixative, because in the non-dissociated state it does not coagulate proteids.

Yet, daily use is made of sublimate solutions in 0·5 to 0·75 per cent. salt solutions ; the object of using the salt being to increase the amount of sublimate in the solution, on the assumption that increase in the concentration of the sublimate is equivalent to greater activity : but physical chemistry teaches us the very opposite ; the stronger the solution the less it is dissociated, and therefore the less effective, as just pointed out.

The direct bearing of this question on histology is that organs rich in chlorides will be fixed less efficiently by sublimate than will tissues poor in chlorides.

It has been known for close on seventy years that salts of the heavy metals, in causing a precipitate of proteids, do so by the metal of the salt forming an insoluble compound with the albumin ; thus the mercury radical of corrosive sublimate joins the albumin to form an albuminate of mercury, while the chlorine takes no part in the formation of the coagulum. Given such an albuminate of mercury, we can readily dissolve it by treatment with sodium chloride, for the sodium is a stronger kat-ion than the mercury ; it turns the latter out of its combination with the albumin and converts it into sublimate, because the mercury liberated from the tissues combines with the chlorine ions derived from the sodium chloride,

and having been formed, it cannot dissociate, provided sufficient of the sodium salt is present. The sodium albuminate being soluble, the coagulum disappears.

Yet it has been recommended to treat tissues, fixed with sublimate, only with normal saline solution and not with distilled water, to prevent the haemoglobin from diffusing out of the red corpuscles; and many histologists treat material fixed in sublimate with watery solutions of iodine in potassium iodide, not knowing that hereby the fixation is completely undone.

Formaldehyde, sold commercially under the name of formol or formaline, is a non-electrolyte, and when pure is neutral in its reaction, but the commercial article is always acid because of the oxidation of formaldehyde into formic acid. Now formic acid is an electrolyte, and should be carefully removed when we desire to study the effects of pure formaldehyde.

Placing on the same slide, side by side, sections of tissues fixed in sublimate and in formol, and staining them first in an acid substance such as eosin, and then in a basic dye, for example toluidin-blue, we find, after dehydrating with alcohol, clearing in benzene, and mounting in balsam, that the sublimate sections are deep red, while the formaldehyde sections show hardly any trace of red. This result is not difficult to explain if we know, as will be shown later, that the pseudo-basic radical of albumin was changed into the real base by the acid hydrogen ions of the sublimate solutions, while no such conversion took place in the formol-fixed tissue.

These short allusions to matter which will be treated more fully in later chapters, will show the importance of keeping the physico-chemical aspect of cell investigation constantly before our minds, and will, I hope, justify me in devoting the following chapter to electro-chemistry.

CHAPTER II.

Physical Chemistry.

For a fuller account of electro-chemistry the reader is advised to study the following works and papers:

Ostwald: *Electrochemie*, 1896; and *Grundlinien d. anorganischen Chemie*, 1900.
W. Nernst: *Theoretical Chemistry* (English translation, Macmillan).
James Walker: *Introduction to Physical Chemistry*. 2nd edition, 1901.
Abegg and Herz: *Chemisches Practikum*, Göttingen (Vandenhoek and Ruprecht), 1900. English translation, Macmillan.
H. Ley: 'Studien ü. d. hydrolytische Dissociation d. Salzlösungen,' *Zeitsch. f. physik. Chem.* 30, 193 (1899).
Kohlrausch and Holborn: *Leitvermögen d. Electrolyte*. Teubner, Leipzig, 1898.
Kohlrausch: *Kleiner Leitfaden d. praktischen Physik*. Teubner, Leipzig, 1900.

Faraday in 1833 showed that certain substances are split up into their components by means of the electrical current; and hence called such substances electrolytes. The atoms or groups of atoms resulting from the splitting up or dissociation were termed ions; thus

common salt or sodium chloride is an electrolyte which dissociates into the ions sodium and chlorine.

Certain ions were found to go towards the positive pole or anodal electrode, and were called an-ions, while others travelled towards the negative pole or kathodal electrode, and hence were designated kat-ions. In the case of sodium chloride, sodium is the kat-ion, while chlorine is the an-ion.

Faraday also showed that a definite amount of electricity on being passed through a solution of electrolytes does a definite amount of work, or, to put it differently, that the amount of electrolytic decomposition of a substance is directly proportional to the amount of the current which has passed through the substance, neither the size of the electrodes nor the concentration of the electrolyte in any way modifying the result. Thus 96,500 coulombs of electricity[1] liberate one gram molecular weight or 'mol' of hydrogen, or its chemical equivalent, viz. 35·4 grams of chlorine, 23 grams of sodium, or one-half the weight of such divalent radicals as oxygen (16) or 8; SO_4 (96) or 48, &c.[2] Therefore 1 Na = $\frac{1}{2}$ Hg = $\frac{1}{3}$ Au = $\frac{1}{4}$ Pt, or, as Biedermann[3] puts it, the electrochemical molecule is the chemical molecule divided by the number of valencies which are set free by electrolysis.

According to Faraday's conception, an electrical current, in passing through a solution of common salt in water, tears the electrolyte asunder, separating in this way the kat-ion sodium from the an-ion chlorine. Therefore an electrical change or movement of electricity in a solution of electrolytes is only possible with a simultaneous movement of the ions, and, vice versa, every movement of ions must lead to a shifting of the amount of electricity from one place to another.

The rate at which ions travel towards the poles of a cell, or their rate of migration, has been determined experimentally by Hittorf, who stated, if only the positive or only the negative ions travel, that these alone are responsible for the carriage of the whole amount of electricity; while if both ions travel, but at different speeds, that they will transport the electricity in direct proportion to the speed with which they migrate, the amount of charge carried by the different ions being the same.

The actual velocities of migrating ions in dilute watery solutions at 18° C., expressed in centimetres per hour, are:

+ *Kat-ions*		− *An-ions*	
H	10·8	OH	5·6
K	2·05	Cl	2·12
NH_4	1·98	I	2·19
Na	1·26	NO_3	1·91
Ag	1·66	CH_3COO	1·04

The relative velocities in dilute solutions at 18° C., in round figures, are as follows (Kohlrausch):

[1] See p. 460.
[2] See table on p. 27.
[3] Rudolf Biedermann, *Chemischer Kalender*, Beilage, 1901, 272.

+ *Kat-ions*		— *An-ions*		
H	320	OH	174	
K	65	$\frac{1}{2}SO_4$	70	
NH_4	64	Br, I	66	
Ag	56	Cl	65	
$\frac{1}{2}Ca$	53	H_2PO_4	42	
Na	44	CH_3COO	34	(ion of acetic acid)
Li	33			

It will be seen that the fastest ion is the kat-ion hydrogen and the next quickest the an-ion hydroxyl, in short the two ions resulting from the hydrolytic dissociation of water (p. 21). — Isomeric ions travel at the same rate.

Kohlrausch was the first to observe that the electrical conductivity of a salt increases with its dilution: thus if one gram molecular weight of common salt (NaCl) is dissolved in one litre of water it conducts less than if diluted with 10,000 litres.

1 grm. of sodium chloride in	1	litre	=	69·5	Siemens' units of conductivity[1].
	10	litres	=	86·5	
	100	,,	=	96·2	
	1000	,,	=	100·8	
	10000	,,	=	102·9	

The reason that dissociation proceeds at first at a quicker rate than subsequently is because the amount of non-dissociated substance is greater to begin with, and because the products of dissociation have not as yet accumulated and thereby saturated the solution (compare Ostwald's table, p. 15, and Kohlrausch's table, p. 20).

Kohlrausch deduced the following conclusions from his experiments:—(1) electrolytes undergo a dissociation into ions on passing into solution; (2) ions have the power of independent wandering when an electrical current is passed through a solution containing them; and (3) the 'molecular' conductivity of a binary electrolyte is equal to the sum of the conductivities of its two ions. The term 'molecular' conductivity expresses that one gram molecule of a substance has been dissolved in any given number of litres, and that the conductivity for that particular dilution, expressed in electrical units, has been multiplied by the given number of litres.

Arrhenius then brought forward the view that all substances imparting to water, which itself is a non-conductor, the power of allowing an electrical current to pass through it, do so in virtue of being in a state of electrical dissociation or ionization, there being formed, while no current was passing, two sets of ions, the one having an electro-negative, the other an electro-positive charge.

Sodium chloride, therefore, on dissolving in water dissociates into the sodium-ion carrying a positive electrical charge, and the chlorine-ion with a negative electrical load. In writing chemical formulae Ostwald has indicated the + and — electrical charges as follows:— A dot ˙ is placed for the positive and a dash ′ for the negative charge. Sodium chloride in a watery solution is represented in this way: $Na^{\cdot} + Cl' + H_2O$.

[1] See p. 460.

It must be clearly understood that sodium chloride requires for its complete dissociation very large quantities of water, and that it consists, as long as it is not dissociated, of the electrically neutral compound (NaCl). It is the mass action of water which induces a separation of the sodium from the chlorine radical, rendering the sodium electro-positive ($^{\cdot}$) and the chlorine electro-negative ($'$). Similarly, hydrogen chloride (HCl) or anhydrous 'hydrochloric acid' is an electrically neutral compound, but, on being mixed with water, splits into the electro-positive hydrogen ($H^{\cdot}$) and the electro-negative chlorine (Cl'), while a strong base, for example caustic potash, when dissociating in water forms the positive ion $K^{\cdot}$ and the negative ion OH'.

What happens, therefore, on passing an electrical current, for example through a watery solution of hydrochloric acid, is to set the positive or kat-ions ($H^{\cdot}$) wandering towards the kathode, while simultaneously the negative or an-ions (Cl') travel towards the anode. Having reached the kathode the hydrogen kat-ions lose their positive electrical load and become the non-electrical, non-ionized hydrogen atoms, which then escape at the kathode in the shape of hydrogen gas. Similarly the chlorine an-ions at the anode lose their negative electrical charge, and pass into the non-ionized state of free chlorine gas. This free chlorine gas may behave in one of two ways, according to the strength or concentration of the hydrochloric acid solution through which the electrical current is being passed. If the solution is strong the free chlorine gas will first saturate the hydrochloric acid solution, and whenever this saturation has been established, then escape as free chlorine gas. Should, however, a dilute solution of hydrochloric acid be taken, then the chlorine gas liberated at the anode is not given off as free gas, but acts on the water in which the hydrochloric acid is dissolved, there being formed hydrochloric acid and free oxygen, which latter escapes at the anode, according to the formula $2H_2O + Cl_2 = 4HCl + O_2$.

The following table shows a number of substances dissociated into their kat-ions and an-ions, and how these go to their respective poles :

	+ *anodal electrode* — *an-ions* ′	— *kathodal electrode* + *kat-ions* ˙
Water	OH (basic)	H (acid)
Hydrochloric acid	Cl	H
Ammonium hydrate	OH	NH_4
Sodium sulphate	SO_4	$_2Na$
Potassium hypochlorite	ClO	K
Cupric nitrate	$_2NO_3$	Cu
Platinum chloride	$PtCl_6$	$_2H$
Alcohol	OH	CH_3
Acetic acid	CH_3COO	H
Phenol	C_6H_5O	H

The question why ions with positive and negative charges do not come together in such a solvent as water, and why therefore these charges do not mutually neutralize one another, is readily answered, if we regard water as a medium having a high dielectric constant, that is a substance without any electrical charge of its own, but capable of having induced in it an electrical charge.

The positive ion will induce in the water molecules surrounding it a negative charge, while the negative ion will induce a positive charge, and hence the effect of the negative and positive ions upon one another is weakened, as now they are attracted by that induced charge to which they themselves previously have given rise.

Charge of kat-ion.	*Induced charge in solvent.*	*Charge of an-ion.*
+	− +	−

What leads to the original dissociation of an electrolyte is difficult to say, but owing to the slight dissociation of water which exists normally (see p. 21), it may be assumed that the hydrogen kat-ion or the hydroxyl an-ion starts the dissociation of the electrolyte, and this change having once started, spreads to the non-dissociated molecules of the electrolyte, owing to the induced charges in the solvent.

The conditions under which ions are formed have been classified by Ostwald as follows :

(*a*) An electrically neutral body splits up into equivalent quantities of positive and negative ions, as happens, for example, if anhydrous acids, bases, or salts come into contact with water ; thus HCl becomes $H^{\cdot} + Cl'$, and NaCl splits into $Na^{\cdot} + Cl'$.

(*b*) A neutral body abstracts from other ions present their electrical charge, to break up itself into free ions. When this change occurs the free ions, from which the electrical charge has been withdrawn, cease to be ions, as will happen if zinc displaces another metal, such as copper, from a salt, or if an acid radical, such as iodine, is displaced from an iodide by the stronger radical chlorine.

(*c*) A neutral body passes into positive ions while simultaneously another neutral body is changed into an equivalent number of negative ions, as happens, for example, if gold dissolves in chlorine water. Chlorine has normally a great tendency towards ion formation, but cannot satisfy this desire till it meets with another neutral body such as gold, which can become loaded with a positive charge.

(*d*) Changes, as just described under *a*, *b*, and *c*, take place with neutral bodies containing radicals capable of holding different or multiple charges of electricity. Thus, if chlorine is passed through a solution of ferrous chloride, it can become a negative ion, because the divalent ferro-ions take up an additional charge of positive electricity, becoming changed into the trivalent ferri-ions.

A diminution of the negative amount of electrical charge will have the same effect as an increase in the positive amount; for if chlorine be led into a solution of potassium permanganate, the ions of which are $2K^{\cdot}$, and the divalent MnO_4'', chlorine ions are formed because the divalent MnO_4'' loses a negative unit, and is changed into the monovalent ion MnO_4' of permanganic acid ($HMnO_4$).

What is meant by the term hydrolysis is explained on p. 21, and the bearing of the changes described above under *d* on the phenomena of oxidation and reduction is discussed on pp. 68–70.

It was mentioned on p. 10 that the electrical charge of a gram-ion of hydrogen amounts to 96,500 coulombs, and that the same amount

of charge can be held by all other monovalent radicals, while double the amount is carried by a divalent radical and so on. When the valency of a metal alters, for example, when the divalent ferro-ion becomes the trivalent ferri-ion, an extra charge of electricity is given to the ferro-ion, changing its nature thereby completely. It is readily seen that the greater the charge of an ion, the greater will also be its tendency to part with some of its load, hence the great readiness with which osmium or ruthenium tetroxides become reduced by organic bodies.

The valency of electrically active ions, as already mentioned above, p. 11, is expressed in monovalent an-ions by a dash, and for monovalent kat-ions by a dot; for divalent ions two dashes or two dots are given, and so on. Thus: $Fe^{\cdot\cdot}$ means the electrically charged divalent ferro-ion, while $Fe^{\cdot\cdot\cdot}$ indicates the trivalent ferri-ion, one gram molecular weight of which carries three times 96,500 coulombs.

Although ions of the same valency have the same carrying power as regards electricity, yet their affinities for electricity vary; thus the monovalent hydrogen will rob the monovalent mercury of its load, and in its turn has to give up its charge to potassium, which has a still greater electro-affinity.

The following table of electro-affinities is taken from the very clear account found in Abegg and Herz's book[1].

Electro-affinities of some kat-ions arranged in order of their strengths:

$K^{\cdot}$, $Na^{\cdot}$, $Li^{\cdot}$, $Ba^{\cdot\cdot}$, $Sr^{\cdot\cdot}$, $Ca^{\cdot\cdot}$, $Mg^{\cdot\cdot}$, $Al^{\cdot\cdot\cdot}$, $Mn^{\cdot\cdot}$, $Zn^{\cdot\cdot}$, $Cd^{\cdot\cdot}$, $Fe^{\cdot\cdot}$, $Co^{\cdot\cdot}$, $Ni^{\cdot\cdot}$, $Pb^{\cdot\cdot}$, $H^{\cdot}$, $Cu^{\cdot\cdot}$, $Ag^{\cdot}$, $Hg^{\cdot}$, $Pt^{\cdot\cdot\cdot\cdot}$, $Au^{\cdot\cdot\cdot}$.

Electro-affinities of some an-ions:

(F', NO_3', ClO_3') (Cl', SO_4'') Br', I', PO_4''', CO_3'', CrO_4'', SiO_3'', SH', H_2BO_3', OH', CN', O'', S''.

Solutions of electrolytes containing many ions per unit of volume are obtained with compounds of the strong kat-ions $K^{\cdot}$, $Na^{\cdot}$, and $Li^{\cdot}$ with all an-ions; and further with the soluble combinations of

$$\left.\begin{matrix} Ba^{\cdot\cdot}, Sr^{\cdot\cdot}, Ca^{\cdot\cdot}, Mg^{\cdot\cdot}, Mn^{\cdot\cdot} \\ Zn^{\cdot\cdot}, Fe^{\cdot\cdot}, Co^{\cdot\cdot}, Ni^{\cdot\cdot}, Pb^{\cdot\cdot}, H^{\cdot} \\ Hg^{\cdot}, Ag^{\cdot} \end{matrix}\right\} \text{ with } \left\{\begin{matrix} NO_3' \\ F', Cl', Br', I' \\ SO_4'' \end{matrix}\right.$$

Only a few ions per unit of volume are formed by $HgCl_2$, $Hg(CN)_2$, $Fe(CNS)_3$, $Fe(C_2H_3O_2)_3$, NH_4OH, H_2S, HCN, H_2CO_3.

As long as no electrical current flows through a solution containing equivalent numbers of negative and positive ions, these will be in a state of intimate mixture; but the passage of an electrical stream will set the $-$ions travelling towards the $+$pole, and the $+$ions on their way towards the negative pole. The rate of migration has already been given on pp. 10 and 11.

[1] Abegg and Herz, *Chemisches Practikum*, Vandenhoek and Ruprecht, Göttingen, 1900. (English translation, Macmillan.)

Acids, Bases, and Salts (inclusive of pseudo-acids and pseudo-bases).

The term 'salt' in a wider sense may be given to any substance the components of which, on passing into solution, can exert their influence individually and independently of one another (Ostwald). Components reacting in this manner must be ions; thus in hydrochloric acid, HCl, the $H^{\cdot}$ renders litmus paper red, and the chlorine ion gives with silver nitrate the white precipitate of silver chloride. Water, according to this definition, is also a salt, as its radicals $H^{\cdot}$ and OH' are independent of one another under certain conditions as explained later on. For our present purpose the term 'salt' does not include those substances commonly called acids, bases, or water.

Acids.

All the typical reactions of acids depend on the presence of hydrogen ions; thus hydrogen chloride HCl, by coming into contact with water, dissociates into the electro-positive hydrogen ion $H^{\cdot}$, and the electro-negative chlorine ion Cl'. Similarly acetic acid dissociates into $H^{\cdot} + CH_3COO'$, and phenol or carbolic acid into $H^{\cdot} + C_6H_5O'$. It is the hydrogen ion which turns litmus paper red, which decolorizes red phenolphthalein or yellow nitrophenol solutions; it causes the acid taste, and it also is able to transfer its positive electrical charge to such metals as zinc and magnesium, and thereby to pass into the non-ionized state of free hydrogen gas. The hydrogen ions also coagulate electro-negative colloids (see p. 46).

Arrhenius has shown that the conductivity of a solution is proportional to the amount of ionic dissociation an electrolyte in the solution has undergone, and that, further, the strength of an acid and its conductivity are also proportional. It follows that the strength of an acid is determined by the number of ions which are liberated in a normal solution (1 gram molecule per litre of water; see p. 18). As all acid reactions depend on the liberation of the electro-positive hydrogen ion $H^{\cdot}$, those acids which in normal solutions dissociate into many $H^{\cdot}$-ions are strong, while those in which only a limited dissociation into $H^{\cdot}$-ions takes place are feeble (p. 17).

The potential acidity of equivalent amounts of different acids, when completely ionized, must be the same; thus one gram of acetic acid when completely dissociated will have the same strength as one gram of completely dissociated hydrochloric acid; but it must be borne in mind that a large quantity of water is required to induce a complete ionization of acetic acid, while only a small amount is needed to dissociate hydrochloric acid.

The total and relative amounts of dissociation of the acids in common use may be gathered from Ostwald's table, in which the dilution is expressed in litres, and the amount of dissociation 1·01 grm. of hydrogen undergoes is represented by fractions:

Dilution in litres.	HCl	HBr	HI	HF	H_2SO_4	*Acetic acid.*
10	0·95	0·95	0·95	0·10	0·57	0·013
100	0·98	0·98	0·98	0·26	0·74	0·050
1000	0·99	0·99	0·99	0·56	0·92	0·125

While the three first acids, to which nitric acid may be added, undergo only a slight change on dilution, the other four acids dissociate greatly.

To put it differently; when using hydrochloric acid we are dealing with a concentrated solution of H-ions, while, when employing acetic acid, we make use of a dilute solution of H-ions, owing to the fact that the electro-negative an-ion Cl′ in hydrochloric acid does not interfere with the electrical dissociation, as does the electro-negative an-ion CH_3COO in the case of acetic acid. Therefore the greater the ease with which an acid dissociates in water, the greater will be the number of H-ions, the stronger the acid reaction.

The strongest acids in aqueous solutions are the monobasic ones, namely, hydro-chloric, -bromic and -iodic acids, also nitric acid (an oxyacid); while all dibasic acids are weaker, the strongest member of this group being sulphuric acid (see Table, p. 17).

Dibasic acids, in strong solutions, generally dissociate into one hydrogen ion and the remainder of the molecule, thus $H_2SO_4 = H^{\cdot} + HSO_4'$; but on being still more diluted the second hydrogen atom is also split off: $HSO_4' = H^{\cdot} + SO_4''$. Dibasic acids on coming in contact with neutral salts lead to the formation of acid salts, which then dissociate as such.

A typical example of a weak acid is acetic acid (see Table, p. 17).

Not only do strong acids undergo dissociation more readily than weak ones, but they also directly inhibit the dissociation of weak acids; thus hydrochloric acid will prevent or greatly retard the ionization of acetic acid, for this reason: Dissociation of an acid means the liberation of the acid hydrogen ion; and if a substance (the strong acid) dissociates readily, it will set free a sufficiently large number of hydrogen ions to saturate the solution, and therefore no more hydrogen atoms can be added from the weak acid, which at the best has only a slight tendency to dissociate.

The same effect which strong acids have on the dissociation of weak acids is also produced by adding to a weak acid one of its neutral salts. A solution of acetic acid contains the greatest number of molecules in the non-dissociated state, as $HC_2H_3O_2$, while a certain percentage is dissociated into acet-ions, $C_2H_3O_2'$, and hydrogen ions, $H^{\cdot}$. On adding to such a solution of acetic acid some sodium acetate, the latter dissociates freely into the acet-ion $C_2H_3O_2'$ and the sodium ion $Na^{\cdot}$. As by the ready dissociation of the sodium acetate the solution becomes saturated with acet-ions, no call is made on the acet-ions of the acetic acid and therefore it does not dissociate at all. But non-dissociation of acetic acid means that hydrogen ions cannot be liberated, or only to a slight extent, and therefore the acid reaction of acetic acid disappears or is greatly reduced on adding to its solution some sodium acetate. Strong acids have their ionization only slightly affected by the addition of neutral salts. Strong acids may also displace weak ones from their union with strong bases, for reasons which will become apparent from the following example:— Sulphuric acid is a comparatively strong while acetic acid is a feeble acid (see Thomsen's Table, below), and hydrogen ions have less

positive electro-affinity than have sodium ions. If we therefore start with a solution of sodium acetate, we are dealing with an almost completely dissociated salt, the ions being $Na^{\cdot} + C_2H_3O_2'$. On adding to these ions sulphuric acid, which also readily undergoes dissociation into $H^{\cdot}$ and SO_4'' ions, the strong kat-ions $Na^{\cdot}$ of the sodium acetate and the strong an-ions SO_4'' of the sulphuric acid remain, while most of the hydrogen ions, having little electro-affinity, join the feeble acet-ions to form non-dissociated molecules of acetic acid.

If a feeble an-ion and a feeble kat-ion are joined, as in the case of mercuric cyanide, $Hg(CN)_2$, no dissociation takes place in water, and the addition of a strong acid, e. g. HCl, will not liberate hydrocyanic acid.

The following table shows the relative strengths of various acids and bases referred to equivalent solutions:

Acids (semi-normal).		*Bases ($\frac{1}{10}$ normal).*	
Nitric	100	Lithium hydroxide	100
Hydrochloric	100	Sodium	98
Sulphuric	49	Potassium	98
Oxalic	24	Ammonium	2
Orthophosphoric	13		
Tartaric	5		
Acetic	3		

Bases.

The term base was originally given to the non-volatile constituent of a salt, in the belief that it formed the foundation or base of the salt; it is now given to substances which neutralize a solution of an acid, forming thereby a salt. In the case of solutions of electrolytes such as caustic potash, KOH, the basic reaction depends on the presence of the electro-negative radical OH; the so-called organic bases, such as anilin, which do not react alkaline to litmus, yield, with acids, solutions of salts exactly analogous to those given by inorganic bases.

Just as monobasic acids dissociate almost completely in water, so do the strong alkalies, for example the hydroxides of lithium, sodium, and potassium, while ammonia, according to Hantzsch and Sebaldt[1], is a feeble base, because the greater part of it continues to remain in water as the anhydride NH_3, a small percentage only being converted into the hydroxide which dissociates thus: $NH_4^{\cdot} + OH'$.

Weak bases, for example ammonia, behave analogously to weak acids, as they dissociate only slightly in the presence of their salts. It is therefore easy to reduce the basic character of ammonia by adding to its watery solution some ammonium chloride.

Strong bases, such as potassium or sodium hydroxide, on the other hand, dissociate freely and are analogous to strong acids, being only slightly affected by the addition of neutral salts containing the same metal.

Neutralization of an acid by an alkali, for example of hydrochloric acid by caustic soda, resolves itself into a union between the electro-positive, acid hydrogen ion $H^{\cdot}$ and the electro-negative, basic hydroxyl

[1] Hantzsch and Sebaldt: *Zeitsch. f. physik. Chem.* 30, 258 (1899).

ion OH′, there being formed electrically inactive, neutral water. In this particular case the two remaining radicals, namely sodium and chlorine, remain as electrical ions in solution:

$$(H^{\cdot} + Cl') + (Na^{\cdot} + OH') = (H^{\cdot} + OH') + Na^{\cdot} + Cl' = H_2O + Na^{\cdot} + Cl'.$$

To measure the amount of acid or base present in a solution means to determine the number of hydrogen or hydroxyl ions. For this purpose 'normal' solutions are employed.

A 'normal' solution is prepared by dissolving the gram molecular weight or 'mol' of any monovalent substance in 1,000 cc. or 1 litre of water. A normal caustic soda solution is therefore obtained by dissolving $Na + O + H = 23{\cdot}05 + 16 + 1{\cdot}01$ or 40·06 grms. in 1,000 cc. of water. Similarly a normal hydrochloric acid solution contains 36·46 grms. of HCl (Cl = 35·45 and H = 1·01). As a litre of normal caustic soda solution contains 17·01 grms. of the basic hydroxyl group OH′, and as a litre of normal hydrochloric acid contains 1·01 grm. of the acid hydrogen ion H˙, it follows that by the union of 17·01 grms. of OH and the 1·01 grm. of hydrogen the substance water, H_2O with a gram molecular weight of $[1{\cdot}01 + 1{\cdot}01 + 16]$ or 18·02, will be formed. As water is a neutral compound we know that neutralization must mean the union of the basic OH′ with the acid H˙.

To neutralize means to form water.

If 1,000 cc. of normal soda solution neutralize 1,000 cc. of normal hydrochloric acid solution, it follows that 1 cc. of NaOH will also neutralize 1 cc. of HCl, however much they may be diluted with water. This fact is made use of for preparing deci- and centi-normal acid and basic solutions by diluting the normal solution 10 or 100 times. In histology normal solutions diluted 1,000 and even 10,000 times have still very marked effects, and are required for determining the relative acid and basic affinities between dyes and tissue constituents.

The estimation of acidity or basicity by means of neutralization experiments is called titration.

Salts.

The ions of an electrolyte as regards their electro-affinities may be both strong, both weak, or one of the radicals may be feeble while the other is strong. Thus in sodium chloride the an-ion Cl′ and the kat-ion Na˙ are both strong; in mercuric cyanide the an-ion CN′ and the kat-ion Hg˙ are both feeble; in corrosive sublimate the an-ion Cl′ is strong while the kat-ion Hg˙ is feeble; and in sodium carbonate the an-ion CO_3' is feeble while the kat-ion Na′ is strong[1].

When the salts just mentioned dissolve in water, no action on or by the latter will take place if the an-ion and kat-ion radicals are both equally strong, as in sodium chloride. It is different, however, with such ill-matched radicals as are met with in corrosive sublimate or sodium carbonate, as is explained later.

[1] Compare the table of electro-affinities on p. 14.

Salts in relation to water may be classified as either insoluble[1], such as barium sulphate,—or soluble. The latter on passing into solution either do not become ionized and do not conduct the electrical current, as for example mercuric cyanide, or they become dissociated into ions and offer therefore little resistance to the flow of an electrical current. All substances belonging to the last-mentioned group may be redivided into those which are dissociated only electrolytically, such as sodium chloride [$NaCl + H_2O = Na' + Cl' + H_2O$], potassium sulphate, and other neutral salts; and those salts which after a preliminary electrolytic dissociation undergo a secondary hydrolytic decomposition, as do for example the chlorides and nitrates of aluminium, copper, zinc, mercury, anilin, pyridin, and urea, all of which give an acid reaction because they contain a strong 'acid' radical joined to a feeble 'base.' Reversely, all salts which have a feeble acid joined on to a strong base, give an alkaline reaction, as for example the carbonates, borates, cyanides, and soaps of sodium and potassium. This hydrolytic decomposition is explained on p. 21.

On p. 11, taking sodium chloride as an example, it was pointed out that the dissociation of a substance is increased by dilution: but how greatly the dissociation varies in the case of different salts may be gathered from the accompanying table, representing the relative conductivities of some salts in common use, the concentration in each case amounting to one gram molecular quantity in two litres of water at 18° C.:

Magnesium sulphate	54
Sodium chloride	75·7
Potassium nitrate	83·9
Ammonium nitrate	88·4
Sodium sulphate } Ammonium chloride }	94·8
Ammonium sulphate	128·6
Potassium sulphate	134·4

Excepting in the case of sodium chloride practically nothing is known regarding the conductivity of saturated solution; but the table on p. 20, taken from Kohlrausch's *Leitfaden*, shows that electrolytes possess a maximum point of conductivity, on either side of which the concentration of electrolytically dissociated ions per unit of volume diminishes. Magnesium sulphate, for example, has the greatest conductivity in concentrations of 17·4 per cent., while 10 and 25 per cent. solutions have approximately the same conductivity, there being present the same number of ions in a given quantity of the solution.

Generally speaking it may be said[2] (1) that the dissociation of an electrolyte is diminished by adding to it one or more of the products of its dissociation, namely ions; (2) provided two substances have a common product of dissociation, that each substance will dissociate

[1] Solubility and insolubility are only relative terms, as no substance is completely insoluble.

[2] Walker: *Introduction to Physical Chemistry*, 2nd edition, 1901.

to a less extent than if it were by itself; and (3) that of two substances, both capable of dissociation, the one normally breaking up most will also do so in the presence of the other [1].

KOHLRAUCH'S TABLE SHOWING MAXIMUM POINTS OF CONDUCTIVITY.

Percentage of Electrolyte.	NaCl	$MgSO_4$	H_2SO_4	HNO_3
5	67	26	209	258
10	121	41	392	461
20	196	48	653	711
25	214	42	717	770
30	—	—	740	785
40	—	—	680	733
50	—	—	541	631
60	—	—	373	513
70	—	—	216	396
80	—	—	111	267
Maximum at		17·4 % = 49·2	30 % = 740	29·7 % = 785

So far it has been assumed that the conductivity of a solution is dependent only on the dissociation of an electrolyte, but a second factor must also be taken into consideration, namely, the amount of resistance which is offered to the movements of ions by the viscosity of the solvent. The denser the solvent the greater will be the friction between its molecules and the ions of the electrolyte. Thus if water be taken as the solvent, and secondly an electrolyte in so dilute a solution as to ensure a dissociation of all its molecules, then the water containing a definite number of ions will conduct electricity to a lesser extent at low temperatures than at high temperatures, a fact only explainable on the hypothesis that heating gives greater mobility to the particles of the water and to the ions in it.

It would appear from the literature that an increase of temperature does not in itself lead to increased electrolytical dissociation of a salt; but the fact of ions travelling much faster in a warm or hot fixing solution than in a cold one must be remembered in all histological investigations, as with increased mobility of ions there goes hand in hand a more ready coagulation or precipitation of the colloidal tissue-elements, quite apart from the direct effects of the temperature on the cell.

Water.

According to the definition of a salt given above, namely, a substance formed by the union of a kat-ion and an an-ion, we must regard water as a salt, for it is capable of splitting up into $H^{\cdot} + OH'$, either component being able to exert its own influence.

We saw above that pure hydrogen chloride does not conduct, and the same holds good, practically speaking, for pure water, which offers an enormous resistance to the flow of the electrical current.

[1] See, however, p. 63.

Inasmuch, however, as the purest water yet obtained did conduct the current to a very small extent, we are justified in believing that it was slightly dissociated. This state of dissociation is even more marked in ordinary distilled water, and therefore we have a right to consider water as a potential base or acid, as by its dissociation there are formed acid hydrogen ions and basic hydroxyl ions.

The effect of electro-positive bodies, such as sodium, on water is to liberate the electro-negative OH-ion, which renders the solution alkaline; the hydrogen ion, formed at the same time, transfers its charge to an atom of sodium, which goes into solution as a sodium ion, while the hydrogen is liberated as electrically neutral gas. In the presence of strongly negative radicals, such as chlorine, the electro-positive H-ion is liberated by sunlight; the solution becomes acid, a chlorine atom goes into solution as a chlorine ion; the OH-ions not capable of forming stable molecules condense to form molecules of water H_2O, and oxygen atoms are set free.

Thus water can react like an ordinary salt in which the positive or negative radical is turned out by a group possessing stronger electro-affinities.

Alcohol behaves in this respect like water, becoming dissociated thus: $C_2H_5OH \rightleftarrows C_2H_5O' + H^{\cdot}$.

It decomposes salts of weak acids, as, for example, sodium phenolate

$$C_6H_5ONa + C_2H_5OH \rightleftarrows C_6H_5OH + C_2H_5ONa.$$

Hydrolysis.

The chief papers bearing on hydrolysis are those by Walker [1], Arrhenius [2], Bredig [3], Ley [4], Cohnheim and Krieger [5], Erb [6], Bruner [7], von Kowalewsky [8], and Kohlrausch [9].

Salts containing weak acids or weak bases undergo in the presence of water a change called hydrolysis, which differs in character from the electrolytic dissociation described above. By referring to the table of electro-affinities on p. 14, it will be seen that mercury possesses a weak positive electro-affinity, while chlorine is strongly electro-negative.

Mercuric chloride is chemically satisfied as regards the equivalence of the an-ion and kat-ion composing it, yet on being dissolved in water, the mercury is turned out of its combination with the chlorine by the hydrogen ions of the water, as these possess greater positive electro-affinity than mercury, and thus $H^{\cdot} + Cl'$ is formed. The hydroxyl ions of the water then unite with the mercury, probably to form (HgOH). Mercury being a feeble kat-ion, its hydroxyl com-

[1] J. Walker: *Zeitsch. f. physik. Chem.* 4, 319 (1889).
[2] S. Arrhenius: *ibid.* 5, 1 (1890).
[3] G. Bredig: *ibid.* 13, 289 (1894).
[4] H. Ley: *ibid.* 30, 193 (1899).
[5] O. Cohnheim and H. Krieger: *Zeitsch. f. Biol.* 60, 95 (1900).
[6] W. Erb: *ibid.* 61, 309 (1901).
[7] Ludwik Bruner: *Zeitsch. f. physik. Chemie*, 32, 133 (1900).
[8] Wl. von Kowalewsky: *Zeitsch. f. anorg. Chemie*, 23, 1 (1900).
[9] Friedrich Kohlrausch: *Zeitsch. f. physik. Chemie*, 33, 257 (1900).

pounds dissociate only to a slight extent, while hydrogen chloride readily dissociates into $Cl' + H^{\cdot}$ ions, which latter bring about the acid reaction of watery sublimate solutions. Substances containing strong kat-ions joined to feeble an-ions behave analogously; thus sodium carbonate reacts with water in the following way:

$$Na_2CO_3 + 2H_2O = 2NaOH + H_2CO_3$$
$$Na_2CO_3 + H_2O = NaOH + NaHCO_3.$$

The sodium hydrate dissociates freely into $Na^{\cdot} + OH'$, the hydroxyl causing the alkaline reaction of sodium carbonate solutions, while the carbonic acid, being a feeble an-ion and therefore dissociating only slightly, does not set free acid hydrogen ions.

If A stands for the electro-positive and B for the electro-negative radical of a salt, and H for the acid and OH for the alkaline radical of water, then hydrolysis may be represented in this way (Ley):

$$(AB) + (H^{\cdot} + OH') = AOH + BH.$$

The table of salts undergoing hydrolytic dissociations on p. 24 is taken from the work of Abegg and Herz.

The amount of hydrolysis in different substances varies greatly.

According to Bruner, as a rule the chlorides are hydrolysed to the greatest extent, then follow the nitrates, and finally the sulphates. The chlorides of quadrivalent metals are considerably hydrolysed; thus stannic chloride is almost completely decomposed hydrolytically. Then follow in this order: ferric chloride, aluminium chloride, aluminium nitrate, aluminium sulphate (mercuric chloride), while in solutions of ammonium, potassium, calcium and magnesium chlorides no hydrolysis occurs, or the amount is too small to be measured.

Stannic chloride, according to von Kowalewsky, undergoes hydrolysis according to the formula $SnCl_4 + 4H_2O = Sn(OH)_4 + 4HCl$. The hydrolysis of this salt, produced by the course of time, reaches ultimately a point of equilibrium, owing to the presence of the chlorine ions of the hydrochloric acid preventing a complete hydrolysis. Whilst in this state an increase in the temperature also diminishes the extent of hydrolysis[1]. The chlorides of iron and aluminium dissociate hydrolytically to the extent of about 5 and 3 per cent., while mercuric chloride only dissociates to about 2 per cent. into $HgOH + 2H^{\cdot} + Cl'$, the remaining 98 per cent. being still in the undissociated state of $HgCl_2$. Kohlrausch considers platinum chloride to be an oxy-acid of the formula H_2PtCl_4O. Sunlight causes rapid hydrolysis of this compound, and when the limit of hydrolysis is reached in dilute solutions, the greenish-yellow colour changes to a bright orange, a fluorescence showing itself simultaneously, due probably to the formation of finely divided particles of platinic hydroxide, the whole of the chlorine being in the form of $H^{\cdot} + Cl'$. Gold-chloride is not affected by light, nor is stannic chloride, but the latter with time undergoes considerable hydrolysis.

[1] Non-electrolytes seem also to interfere with the dissociation of hydrochloric acid, probably by altering the amount of the solvent at the disposal of the electrolyte.

The percentage hydrolysis of the hydrochlorides of weak bases and of weak acids is as follows:

Hydrochlorides ($\frac{1}{32}$ *normal*).		*Salts of weak acids* (*decinormal*).	
Anilin	2·6	Potassium phenolate	3·1
Paratoluidin	1·5	Potassium cyanide	1·1
Orthotoluidin	3·1	Sodium biborate	0·5
Urea	76·0	Sodium acetate	0·01

The chlorides of egg-white, according to Erb, dissociate from 60 to 80 per cent. and more.

Sjöqvist has used the amount of hydrolytic dissociation to measure the relative strength of bases, and the great hydrolytic dissociation of albumin chloride shows how exceedingly feeble a base the organic albumin must be as compared with sodium or other fixed alkalies.

The amount of hydrolytic dissociation of albumin is greatest when sufficient acid has been added to just give a faintly acid reaction; any further addition of acid diminishes hydrolysis more and more, and this diminution takes place the more readily the more dilute the egg-albumin is (Erb). The following observations are also intimately connected with hydrolysis:—Spiro and Pemsel found that up to a certain point albumin will bind more acid, if the acid be used strong, and this has been confirmed by Erb, who found that 10 cc. of 0·5 per cent. pure vitellin solution absorbed when treated with

0·1 cc. of normal HCl	63 mg.
0·5 „ „ „	176 „
1·5 „ „ „	212 „

The maximum amount of hydrochloric acid which 1 grm. of various proteids in $\frac{1}{2}$ per cent. solutions can bind, is given by Erb as follows:

Serum albumin	204 mg.	HCl.
Vitellin	212 „	„
Egg-albumin	234 „	„
Hetero-albumose	314 „	„

How the concentration of a feeble base greatly influences its capacity for binding acids was first pointed out by Sjöqvist, who states that 1 grm. of egg-albumin, if diluted, will only combine with 36 mg. of HCl, while the same amount of egg-albumin in the solid state unites with 120–130 mg. Erb found that, using the same amount of acid, the greatest capacity for holding hydrochloric acid was shown by $\frac{1}{2}$ per cent. solutions of proteids.

Substances which have undergone hydrolytic dissociation cannot be titrated in the usual way, as the addition of caustic soda solution to albumin chloride, for example, only leads to further hydrolytic changes and the liberation of free hydrochloric acid, so that ultimately the acidity is found as great as if no albumin had been present at all. For this reason recourse must be had to special methods, such as (1) the determination of differences in the conductivity of solutions, or (2) the study of the amount of sugar-inversion caused by the presence of acids according to Ostwald's plan (Arrhenius and Ley), or (3) the salting-out method of Spiro and Pemsel [1], or (4) the precipi-

[1] *Zeitsch. f. physicl. Chem.* 26, 233 (1898).

tation method of Cohnheim and Krieger[1], by means of neutral calcium-phosphotungstate. Erb gives the following directions for making this salt:—A boiling 4 per cent. solution of phosphotungstic acid (Keller and Co., in Heidelberg) is treated with calcium carbonate, till a drop of the solution, after cooling, gives a neutral reaction with rosolic acid. Great care must be taken not to add too much carbonate, for if the point of neutralization is once exceeded, a subsequent addition of acid will not give permanently neutral solutions. Calcium-phosphotungstate + albumin-chloride = albumin-phosphotungstate + calcium-chloride. After precipitating the albumin with the calcium-phosphotungstate and filtering, the remaining fluid may be titrated with an alkali in the usual manner, to determine how much acid was taken up by the albumin. Studying the hydrolysis of different proteids in this way, Erb has arrived at the result that increased dilution of a proteid need not of necessity produce an increase in its hydrolysis.

TABLE OF SALTS UNDERGOING HYDROLYTIC DISSOCIATION ACCORDING TO ABEGG AND HERZ.

The salt undergoing dissociation.	*Its ordinary ions.*	*Radicals other than the components of the salt contained in the solution.*
Ammonium carbonate	$(NH_4)_2^{\bullet} + CO_3''$	OH', NH_3, HCO_3', CO_2
Ammonium sulphide	$(NH_4)_2^{\bullet} + S''$	OH', NH_3, SH', H_2S
Ammonium chromate	$(NH_4)_2^{\bullet} + CrO_4''$	OH', NH_3, CrO_3 [2]
Barium carbonate	$Ba^{\bullet\bullet} + CO_3$	OH', CO_2 [3]
Potassium or Sodium carbonate	K_2 or Na_2 + CO_3''	$OH^{\bullet}$ + CO_2
Potassium or Sodium sulphide	+ S''	+ SH', H_2S
Potassium or Sodium pyroborate (borax)	+ B_4O_7''	+ $B(OH)_3$
Potassium or Sodium chromate	+ CrO_4''	+ CrO_3
Potassium or Sodium metasilicate	+ SiO_3''	+ SiO_2
Potassium or Sodium zincate	+ ZnO_2''	+ $Zn(OH)_2$, $Zn^{\bullet\bullet}$
Potassium or Sodium cyanide or aluminate	$K^{\bullet} + CN'$ or $Na^{\bullet} + AlO_2'$	OH' HCN or $Al(OH)_3$
Potassium or Sodium phosphate or arsenate	$3K^{\bullet} + PO_4'''$ or $3Na^{\bullet} + AsO_4'''$	OH' HPO_4'' or $HAsO_4''$
Aluminium or Chromium or Iron chloride or sulphate	$Al^{\bullet\bullet\bullet}$ or $Cr^{\bullet\bullet\bullet}$ or $Fe^{\bullet\bullet\bullet}$ + Cl' or SO_4''	$H^{\bullet}$ $Al(OH)_3$ or $Cr(OH)_3$ or $Fe(OH)_3$ or basic salts
$Bi^{\bullet\bullet\bullet}$, $Sb^{\bullet\bullet\bullet}$, $As^{\bullet\bullet\bullet}$, $Sn^{\bullet\bullet\bullet\bullet}$, $Hg^{\bullet\bullet}$, $Cu^{\bullet\bullet}$ with all an-ions		$H^{\bullet}$ $BiO^{\bullet}$, $Bi(OH)_3$ or $SbO^{\bullet}$, $Sb(OH)_3$ or $As(OH)_3$, H_2AsO_3' or $Sn(OH)_4$, $HSnO_3'$, SnO_3'' or $Hg(OH)^{\bullet}$ or $Cu(OH)^{\bullet}$

[1] Cohnheim and Krieger: *Zeitsch. f. Biol.* 60, 95 (1900).

[2] See pp. 72 and 80.

[3] The OH'-ions in a barium carbonate solution will convert ferri-ions into the insoluble ferri-hydroxide.

On pseudo-acids and pseudo-bases.

In addition to the true acids and bases just described there exist certain substances which have been termed pseudo-bases and pseudo-acids by A. Hantzsch, who has formulated and, in collaboration with M. Kalb and G. Osswald, developed the following view:—Pseudo-ammonium bases are isomers of true bases, but they form with water neutral solutions, which remain neutral on the addition of an acid. The explanation offered is that by the addition of acids an intra-molecular change is set up in the pseudo-base by which it is converted into a true base, the true base uniting with the acid to form a neutral compound. Thus pseudo-bases can form salts only indirectly.

The intra-molecular change just referred to depends on the migration of the hydroxyl-group OH, from the carbon atom of a polyvalent radical R to which it is joined in the pseudo-compound, towards the nitrogen atom of an ammonia radical, thus:

$$HO—\overset{IV}{R}\equiv\overset{III}{N} \text{ becomes } \overset{IV}{R}\equiv\overset{V}{N}'—OH'.$$

A pseudo-ammonium base can therefore be regarded as an indifferent organic hydrate, for example a carbinol, which on the addition of an acid changes into an organic base—

$$HO—\overset{|}{\underset{|}{C}}—\overset{III}{N}= \text{ becomes } \overset{|}{\underset{|}{C}}=\overset{V}{N}'—OH'.$$

Simultaneously with the conversion into true bases, Hantzsch has found solutions of electrically indifferent pseudo-compounds to become conductors because of the dissociation of the resulting salt.

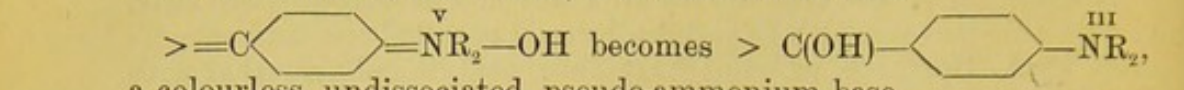
Pseudo-acids, analogous to pseudo-bases, are bodies, in themselves indifferent or very slightly acid, incapable of forming salts or of conducting electricity without having undergone previously an intra-molecular change.

The conversion of true bases into pseudo-bases is accompanied by a loss of dissociation, in consequence of which these bodies cease to act as electrolytes, and therefore offer great resistance to the flow of the electrical current[1]. Making use of this electrolytic factor Hantzsch and Kalb showed that the halogen-alkylates of the acridin series of dyes (see p. 431), on being dissolved in water, behave at first like the strongly electrolytic ammonium bases, but that soon they are converted into the insoluble isomeric non-electrolytes, because of the migration of the hydroxyl group (OH) from the nitrogen of the ammonia radical to the carbon atom.

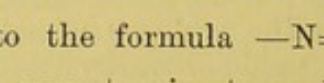
The same law holds good also for many dyes of the quinone series, containing the NH_3 group in the quinone ring. These substances on being dissolved in water undergo an intra-molecular change, as the result of which the hydroxyl group (OH) leaves the nitrogen and

[1] Hantzsch: *Berichte d. Deutsch. Chem. Gesellsch.* 32, 575 (1899); *ibid.* p. 3066. Hantzsch and Kalb, *ibid.* p. 3109; Hantzsch and Osswald, *ibid.* 33, 278 (1900). Since writing the above account the following papers have appeared: Hantzsch and Barth, *ibid.* 35, 210 (1902); Hantzsch and Dollfus, *ibid.* 35, 226 (1902).

goes to the carbon, converting the quinone into a carbinol derivative; thus the coloured, dissociated, true ammonium base

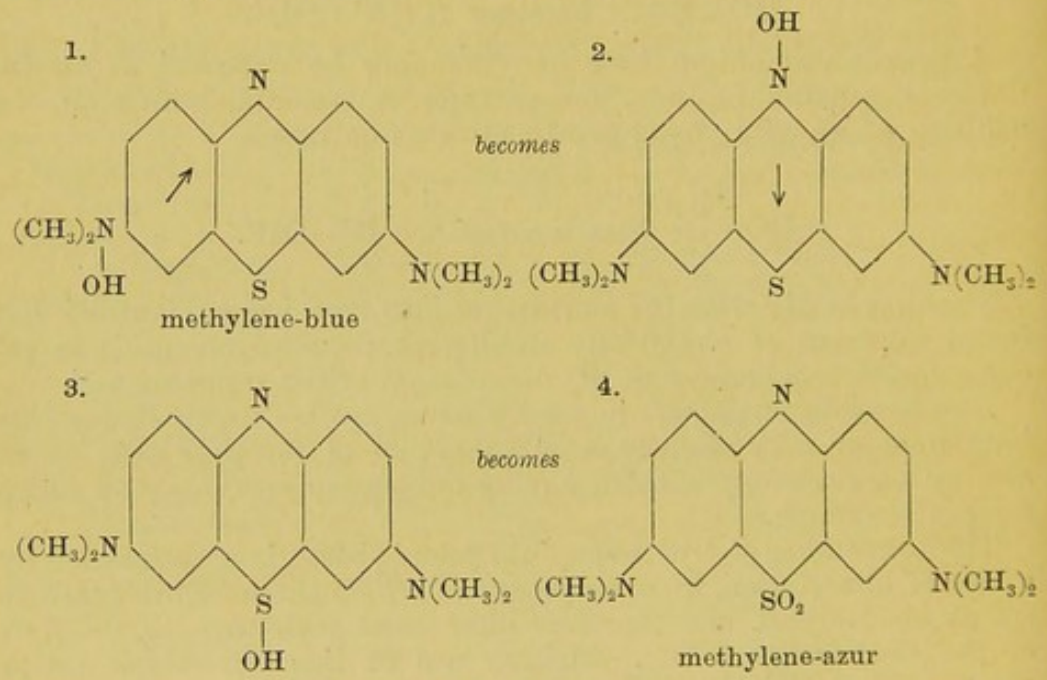


a colourless, undissociated, pseudo-ammonium base.

If in a dye, as met with for example in the azonium group (see p. 414), no free carbon atom is available, but only a nitrogen atom according to the formula 

then the OH radical cannot migrate under ordinary conditions. Yet the nitrogen, occupying a paraposition to that linked on to the hydroxyl, may play an intermediate part in handing on the (OH) by intramolecular interchange to a sulphur group, to which it stands in the paraposition. Having arrived at the sulphur-atom the hydroxyl (OH) is converted by oxidation into O_2.

In this way methylene-blue is oxidized into methylene-azur.



The researches of Hantzsch have also been applied to the study of 'proteids.' Spiro and Pemsel (see p. 287), having determined the capacity for acids and bases possessed by a number of 'proteid' substances, also found that 'proteid' solutions showed very slight electrical conductivity, and arrived at the conclusion that 'proteids' are 'bodies which, although charged electrically, do not ionize, and which possessing two different electrical charges cannot play the part of either acids or bases, but yet have the power of forming addition-compounds.'

Sjöqvist, Bugarzky, and Liebermann[1] have further shown that albumin combines with acids and bases to form true salts, obeying the laws laid down by van 't Hoff and Arrhenius for ordinary salts.

[1] For references consult p. 287.

This apparent discrepancy has been solved by Cohnheim and Krieger[1], who, applying the observations of Hantzsch to albumins, observed (1) that neutral solutions of albumoses are not precipitated by neutral alkaloidal precipitants such as phosphotungstic acid or tannin; (2) that on the addition of acids to neutral albumose solutions, these latter remain neutral, but that now they are precipitated by the neutral alkaloidal precipitants.

They offer the following explanation: 'Proteids' in neutral solutions are pseudo-bases, which on the addition of acids are converted into true bases, and therefore become precipitable.

Albumin chloride + phosphotungstic acid = phosphotungstate of albumin + hydrochloric acid.

Albumin chloride + sodium phosphotungstate = phosphotungstate of albumin + sodium chloride.

The authors also believe that 'proteids' may play the part of 'pseudo-acids,' which means that neutral solutions of 'proteids' on the addition of bases are converted into true acids, which by uniting with the appropriate amount of base may give rise to neutral solutions.

I believe the study of pseudo-compounds to be an exceedingly important one, not only in connexion with questions of coagulation or staining, but also from the biological standpoint, as owing to the peculiar intra-molecular change induced by acids or alkalies, the living organism is enabled to still remain, within certain limits, a neutral compound. Its chances of surviving in inimical circumstances, as far as acids and bases are concerned, is simply dependent on its pseudo-acid and pseudo-basic nature.

The two following tables will be referred to in connexion with the problems of coagulation.

TABLE OF ATOMIC WEIGHTS AND VALENCIES OF THE MORE IMPORTANT ELEMENTS.

Aluminium	(27)	3, 4
Arsenic	(74·9)	3. 5
Barium	(136·9)	2, 4
Bismuth	(207·5)	3, 5
Boron	(10·9)	3, 5
Bromine	(79·8)	1, 3, 5, 7
Calcium	(39·9)	2
Carbon	(12)	2, 4
Chlorine	(35·4)	1, 3, 5, 7
Chromium	(52·5)	3, 4, 6
Cobalt	(58·6)	2, 4
Copper	(63·2)	2, 1
Fluorine	(19·1)	1
Gold	(196·2)	1, 3
Hydrogen	(1)	1
Iodine	(126·5)	1, 3, 5, 7
Iridium	(192·5)	2, 4, 6
Iron	(55·9)	2, 3, 4, 6
Lead	(206·4)	2, 4
Lithium	(7)	1
Magnesium	(23·9)	2
Manganese	(54·8)	2, 4, 6, 8
Mercury	(199·8)	1, 2
Molybdenum	(95·9)	2, 4, 6, 8
Nitrogen	(14)	3, 5
Osmium	(195)	2, 3, 4, 8
Oxygen	(16)	2, (4)
Palladium	(106·2)	2, 4, 6
Phosphorus	(31)	5
Platinum	(194·3)	2, 4, 6
Potassium	(39)	1
Ruthenium	(103·5)	2, 4, 6, 8
Silver	(107·7)	1
Sodium	(23)	1
Strontium	(87·3)	2
Sulphur	(32)	2, 4, 6, 8
Tin	(117·3)	2, 4
Tungsten	(183·6)	4, 6
Uranium	(239·8)	4, 6
Vanadium	(51·1)	3, 5
Zinc	(64·9)	2

[1] O. Cohnheim and H. Krieger: *Zeitsch. f. Biol.* 40, 95–116 (1900).

TABLE OF MOLECULAR WEIGHTS AND SATURATIONS OF SALTS IN COMMON USE.

Name of salt.	*Its chemical formula.*	*Molecular weight.*	*Cold saturated.*	100° C. *saturated.*
Ammonium sulphate	$(NH_4)_2SO_4$	132	76·8	97·8
Magnesium sulphate (Epsom salt)	$MgSO_4 + 7H_2O$	246·3	25·8	71·43
Potassium sulphate	K_2SO_4	174	12·5	25
Sodium sulphate (Glauber's salt)	$Na_2SO_4 + 10H_2O$	322	5	42·5
Mercuric sulphate	Hg_2SO_4	496	slightly	decomposed.
Sodium chloride	NaCl	58·4	35	39·5
Mercuric chloride	$HgCl_2$	270·6	7	54
Zinc chloride	$ZnCl_2$	136	300	very soluble.
Stannous chloride	$SnCl_2 + 2H_2O$	225	271	decomposes.
Stannic chloride	$SnCl_4$	260	readily decomposes in water.	
Strontium chloride	$SrCl_2 + 6H_2O$	266·5	44 at 0°	117 at 118°
Barium chloride	$BaCl_2 + 2H_2O$	244	33·4 at 10°	60 at 104°
Potassium chloride	KCl	74·5	32	56·6
Aluminium chloride	$Al_2Cl_6 + 12H_2O$	265	400	very soluble.
Mercurous nitrate	$Hg_2(NO_3)_2$	524	slightly, + excess of water forms the basic salt.	
Mercuric nitrate	$Hg(NO_3)_2$	324	decomposes to basic salt; 10 per cent. solution obtained in 3 per cent. nitric acid.	
→Potassium acetate	$KC_2H_3O_2$	98	97·844 if made with hot water and allowed to cool.	
Copper acetate	$Cu(C_2H_3O_2)_2 + H_2O$	199	0·76336	20
Lead acetate	$Pb(C_2H_3O_2)_2 = 3H_2O$	384·4	apt to decompose into basic salt.	
Potassium chromate	K_2CrO_4	194·5	50	60
Potassium bichromate	$K_2Cr_2O_7$	295	10·4167	100
Potash alum	$K_2SO_4 + Al_2(SO_4)_3 + 24H_2O$	948	9·5	357
Ammonia alum	$Al_2(SO_4)_3 + (NH_4)_2SO_4 + 24H_2O$	904·4	9	422
Potassium permanganate	$KMnO_4$	158	6·4516	very soluble.

CHAPTER III.

ON COLLOIDS AND COAGULATION.

Short Historical Account.

THOMAS GRAHAM[1] in 1861 was the first to make a thorough investigation of certain substances which are unable to pass through animal bladders or vegetable parchment, because of the large size of their molecules. A substance having this property Graham termed a colloid, and showed that it may occur in one or more of the following states :

[1] Graham : *Philos. Trans.* 151, 183 (1861).

1. As a fluid mixture, or 'sol'; a watery mixture being called, for example, a hydrosol.

2. As a firm mixture, or 'gel'; thus ordinary gelatin-jelly is a hydrogel of gelatine.

3. As a solid; for example, as dry silicic acid or glass.

Graham having noticed that colloidal solutions are disintegrated by the addition of salts, that is by electrolytes, also observed that they were altered by heat. He called the solid constituent of gels, which contracts on heating, the clot, and the exuding liquid, the serum.

Hans Schulze[1] in 1882, when experimenting with colloidal solutions of arsenic trisulphide and antimony sulphide, found that in addition to acids and salts the coagulation of the colloid was also greatly influenced by temperature, mechanical movements, and time. Some of the substances which were tried failed to produce coagulation, for example, carbonic-, boric-, arsenious-, tartaric-, benzoic-, salicylic-acids, chloral hydrate, cane-sugar, glycerin, and absolute alcohol; while others coagulated, but with greatly varying intensity.

Thus 1 milligram of ferric chloride coagulates 1,000 milligrams of arsenic trisulphide, while 1 milligram of potassium chloride can throw down only 2·74 milligrams of As_2S_3. As there exists no relationship between the molecular weight of the coagulant and its power of causing coagulation, Schulze comes to the conclusion that the coagulation of a colloidal substance does not depend on chemical interaction.

But notwithstanding the absence of chemical action a definite relationship between the effect produced and the nature of the coagulant was apparent; thus strong inorganic acids with great avidities brought about the greatest changes, while nothing resulted from adding carbonic acid, boric acid, or arsenious acid. Similarly, organic acids, almost without exception, did not coagulate As_2O_3.

With these data, Schulze was forced to come to the conclusion that 'the coagulating power of a salt is determined primarily by the nature of the metal, and only occasionally and to a slight degree by the nature of the acid'; for 'the salts of one and the same metal show as regards their coagulating power only slight differences, immaterial whether the acid be organic or inorganic, whether it possess strong or only feeble coagulating powers.'

The relative coagulative power of mono-, di-, and trivalent metals was as 1 : 30 : 1650. Alkali salts, being monovalent, for this very reason coagulated least; double salts, containing metals of different valency, behaved as if only the metal with the higher valency had been present; thus ammonium ferrous sulphate reacted like ferrous sulphate, and alum solutions behaved as would do the trivalent metal contained in them if it were in the form of a simple salt. The ferri- and ferro-compounds of potassium cyanide did not act, however, as double salts, but behaved as potash salts. Chlorides were found to have a higher precipitating power than nitrates and sulphates, and

[1] Schulze: 'Schwefelarsen in wässriger Lösung,' *Journ. f. prakt. Chemie*, 25, 431 (1882), and 26, 320 (1883).

the oxalate and tartrate of potassium to be even more powerful coagulators than the sulphates.

Franz Hofmeister [1] in 1886 found that the precipitating power of salts to a certain extent diminished with increasing molecular weight, and that for accurate comparison of salt solutions it was necessary to have for each molecule of the salt an equal number of water molecules and in the same volume. The former requirement is fulfilled by dissolving 1 gram molecular weight of the salt in 1,000 grams of water, while the latter requirement is attained by dissolving 1 gram molecular weight of the salt in 1,000 cc. of water. A salt solution made up according to the former principle commenced to produce coagulation of albumin, globulin, and gelatin, when used in the following strengths:

	Serum globulin.	*Egg-albumin.*	*Gelatin.*
Sodium chloride . .	4·02	3·90	5·16
Potassium chloride .	3·91	3·94	
Sodium nitrate . .	6·73	6·64	7·81

The nitrates and chlorates precipitated less than the chlorides.

It was suggested by Hofmeister that coagulation with neutral salts is due to an abstraction of water from the albumin molecules, as the salt has a greater affinity for the water than has the proteid.

Prost [2], in 1887, working with cadmium sulphide, confirmed Schulze's observations. He pointed out that the coagulating salt was dissociated to a small extent and that the metal became included in the coagulum; that no relation exists between the molecular weight of acids and salts and their coagulative power; that the metallic radical is the determining factor, and that, generally speaking, the acid radicals do not produce any effect, but that acid salts coagulate more vigorously than neutral salts.

Michailow [3], in 1887, distinguished, firstly, between true coagulation due to ferment action and heat, after the type of etherification, or what amounts to the same, the type of formation of polyhydrosilicates, polyhydroaluminates, and polyglycols, and, secondly, pseudo-coagulation after the type of degelatination, owing to the loss of the water of crystallization and the water of hydration (constitutional water). The coagulating action of salts on proteids is dependent on the strength of the acid and the alkalinity of the base contained in the salts. In any given salt the acid radical is least affected by NH_4 and most by Na and K, and therefore, using different acids joined to Mendeljejeff's first group of metals and its analogues, the absolute maximum effect is produced by $(NH_4)_2SO_4$, the relative maximum by NH_4NO_3, the absolute minimum by KCl, and the relative minimum by K_2SO_4. The action of the salt is quite independent of its solubility.

Lewith [4], in 1888, determined the coagulating power of different

[1] Hofmeister: 'Zur Lehre v. d. Wirkung d. Salze,' *Arch. f. experim. Path. u. Pharm.* (1886).

[2] Prost: *Bull. d. l'Acad. Roy. d. Sc. de Belg.* (3) 14, 312 (1887).

[3] Wlad. Michailow: *Žurn. russk. fiz. chim. obšč.* 19, i. 331, 332 (May 7); Abstract in *Chem. Centralbl.* 18, 1088 (1887).

[4] Lewith: 'Zur Lehre v. d. Wirkung d. Salze,' *Arch. f. experim. Pathol. u. Pharm.* 24, 1 (1888).

salts, and showed at what concentration globulin and albumin commence and cease to be precipitated (see table below). He pointed out that the solubility of salts and their coagulating power do not run parallel, and that the solubility of a salt is only of importance inasmuch as certain salts cannot develop their coagulating capacity because of their insolubility. 'The two exceptionally powerful salts, namely ammonium sulphate and potassium acetate, are inferior to other salts as regards globulin precipitation. In this connexion it is surprising that the sulphates and acetates are amongst salts the most vigorous precipitants, that next come the chlorides, and finally the nitrates. This seems to point to the acid component of a salt being the determining factor as regards the coagulation of albumin.'

It will be seen that Lewith arrived at the diametrically opposite view to that of Schulze (p. 29).

TABLE INDICATING WHAT SALTS DO (+) OR DO NOT (−) COAGULATE PROTEIDS (CONSTRUCTED FROM LEWITH'S ACCOUNT).

	Potassium.	*Sodium.*	*Ammonium.*	*Magnesium.*	*Calcium.*	*Barium.*
Acetate	+	+	−	−	−	−
Chloride	+	+	−	−	+	−
Nitrate	−	+	−	−	+	−
Phosphate	.	+[1]	.	.	.	.
Sulphate	−	+	+	+	.	.
Sulphocyanate	.	.	−	.	.	.
Iodide	−	−	−	−	−	−
Bromide	−	−	−	−	−	−
Chromate	.	.	−	.	.	.
Bicarbonate	.	−	.	.	.	.

Lime salts occupy amongst the alkalies and alkaline earths a peculiar position, as they lead to the formation of insoluble precipitates.

The effect of saturated salt solutions on proteids is as follows:

Salt.	*Solubility.*	*Effect on proteid.*
Potassium acetate	97·844	complete precipitation of all proteids.
Ammonium sulphate	76·8	complete precipitation of all proteids.
Sodium chloride	35	incomplete globulin precipitation.
Magnesium sulphate	25·8	complete globulin precipitation.
Potassium sulphate	12·5	no precipitation.
Sodium sulphate	5	incomplete globulin precipitation.

It is evident that saturated solutions behave quite differently according to the nature of the salt. If we multiply the solubility by the molecular weight (see table on p. 28), we find that ammonium sulphate, potassium acetate, and magnesium sulphate agree in giving high figures as compared with sodium chloride; but potassium sulphate does not coagulate, although it gives a higher figure than sodium sulphate.

The limits of concentration necessary to precipitate globulin and albumin from a one per cent. white of egg solution, or in other words

[1] Sodium phosphate and chlorate coagulate only slightly, while potassium chlorate does not coagulate.

the percentage of salt at which, in an approximately 1 per cent. proteid solution, precipitation commences and terminates, has been ascertained by Lewith:

	Globulin precipitation		*Albumin precipitation*	
	commences.	*terminates.*	*commences.*	*terminates.*
Ammonium sulphate	14·2	23·1	33·6	47·2
Potassium acetate .	17·6	35·2	64·6	+ 82·2
Magnesium sulphate	16·9	25·7		

Ostwald[1] in 1885, and Paternò[2] in 1889, first pointed out that colloidal solutions were in fact not solutions at all, but mere suspensions.

Barus and Schneider[3], in 1891, when experimenting with colloidal silver solutions, observed, on adding to the solution small amounts of hydrochloric acid, that metallic silver was deposited, while the addition of larger amounts of HCl led to the formation of silver chloride. They also found one molecule of the acid to exert a measurable effect in the presence of 10 to 50,000 molecules of water. Generally speaking, the rate of sedimentation of a colloid depends on the amount of its dilution, for the greater the dilution the more quickly does the colloid settle, and also on the temperature, as heat diminishes the viscosity of the fluid[4].

Colloids are, however, not kept in suspension merely by the viscosity of the solvent, as was shown by suspending the same substance (tripoli powder) in different substances, the viscosities of which varied, and then comparing the rate of sedimentation in these fluids with their ratio of viscosity, thus:

	Ether.	*Alcohol.*	*Water.*	*Glycerin.*	
Rate of sedimentation .	7500	1300	3	0·09	$\left[10^{-6} \times \frac{\text{cm.}}{\text{sec.}}\right]$
Ratio of absolute viscosities	0·002	0·012	0·010	5	

In studying the laws of mechanical sedimentation[5] Barus and Schneider use the formula:

$$x = \frac{2}{9\,\eta} r^2 (\rho - \rho^1)\, g \text{ where}$$

x = rate of subsidence of the particle
η = viscosity of the suspending medium
ρ = specific gravity of the particle
ρ^1 = specific gravity of the suspending medium
r = radius of particle
g = acceleration of gravity.

In other words, the rate of settlement increases as the square of the dimensions of the particle, and as the difference in density between the particle and the medium, and inversely as the viscosity.

[1] Ostwald: *Lehrb. d. allg. Chem.* 1, 527 (1885).
[2] Paternò: *Zeitsch. f. physik. Chem.* 4, 457 (1889).
[3] C. Barus and E. A. Schneider: *Zeitsch. f. physik. Chem.* 8, 278 (1891).
[4] A detailed account of changes produced in the viscosity of water: 1, 5, 10 and 20 per cent. sodium chloride, and 1, 5, 10, 20 and 40 per cent. cane-sugar solutions by alterations in temperature from 0° to 90° C., is given by R. Hosking: *Phil. Mag.* (5), 49, 274–286 (1900).
[5] Kirchhoff: *Math. Physik*, 26. Vorlesung, § 4 (1876).

It has been shown above that x does not stand in any relation to η; pycnometric determinations of tripoli and clay suspended in water and ether showing that $\rho - \rho^1$ is constant, the only variable is r. Hence Barus and Schneider conclude that the same number of particles aggregate in different solvents into masses differing greatly in size, the mean radius ρ for each solvent 'depending on the mutual chemical relationship of any particular solvent and the suspended solid.'

It is suggested that colloidal particles may consist of only 1,000, or 100, or even 10 molecules, and that each particle is surrounded by a definite water-jacket or mantle, which latter, if the concentration is sufficiently great, will touch one another or allow their spheres of action to intersect, there being formed in this manner a kind of framework of water-jackets, each jacket containing in its interior a solid particle, and thus keeping the particles separate and suspended. Any factor leading to a withdrawal of this mantle will allow the particles to come together, and thereby to form still larger aggregates. An electrolyte, for example, by surrounding itself with a mantle of water is supposed to withdraw water from the suspended particles, which thereby become surrounded by a thinner jacket, and in consequence are more apt to aggregate.

Picton in 1872[1] divided arsenic sulphide As_2S_3 solutions according to their physical state into four classes, which he called α, β, γ and δ. The α solution is termed a pseudo-solution, because under a magnification of 1,000 diameters, the fluid is seen to contain crowds of minute suspended particles in rapid Brownian movement. The $\beta - As_2S_3$, forming the transition to the $\gamma -$ variety, is composed of particles so small as to be microscopically invisible. The $\gamma - As_2S_3$ differs from the α and β variety in diffusing and exerting osmotic pressure, but it cannot be filtered through a porcelain filter without the solid separating, while $\delta - As_2S_3$ contains sulphide particles of so small a size as to pass readily through the filter.

Picton and Linder (1892) state that such pseudo-solutions as those just referred to, namely $\alpha - As_2S_3$, could be converted into true solutions 'by carrying the subdivision further and further, till finally, perhaps, we may have the substance dissociated into ions.' The authors used Tyndall's experiment for determining whether particles were in suspension in the fluids. The experiment consists in sending a beam of limelight through a solution, when if particles be present the beam of light stands out vividly in the solution, and the light, being refracted when examined with a Nicol's prism, is found to be completely polarized[2].

The chief points brought out by the paper are:

(1) Silicic acid in the presence of HCl gives negative results with Tyndall's experiment, but in the absence of acid the silicic acid soon undergoes some change by means of which an aggregation into

[1] Harold Picton: *Journ. Chem. Soc.*, 61, 137 (1892).

[2] Prange (*Rec. des trav. chim. des Pays-Bas*, 9, 125) was, I believe, the first to apply Tyndall's test to colloids.

particles is brought about, and now Tyndall's experiment gives positive results.

(2) On passing an electric current through water containing substances varying in their character, some (for example freshly prepared, strongly diffusible arsenic sulphide) are repelled 'as an unaltered whole' from both poles, but much more from the negative than from the positive electrode. By this repulsion the fluid near the negative pole becomes quite clear, but on interrupting the current the colloids again diffuse into the clear liquid. A solution of shellac in alcohol dropped into water behaves similarly to As_2S_3, while ferric hydrate and haemoglobin are repelled from the positive pole. An analogous phenomenon was previously observed by Porret, namely that on passing a current through acidified water divided into two parts by a septum, the level of the water rises on that side of the septum at which the negative electrode is immersed.

(3) The following crystallizable solutions reveal molecular aggregates large enough to be detected by Tyndall's method: Ferric hydrate in ferric chloride (said by Ramsay to crystallize as $9Fe_2O_3$, $FeCl_3$), oxyhaemoglobin, and carbonic oxide haemoglobin.

Bodländer in 1893[1] was the first to point out the great difference in the behaviour of electrolytes which do, and non-electrolytes which do not, 'coagulate' colloidal solutions.

Picton and Linder in 1895[2] confirmed Schulze's general conclusions (p. 30). They also found trivalent metals to have the highest coagulating power, while bivalent metals had about $\frac{1}{10}$ and monovalent ones less than $\frac{1}{500}$ the efficiency of the trivalent metals. An analogous difference was observed if the valency of the same metal varied, as it does, for example, in iron salts in which the ferro-ion is divalent, while the ferri-ion is trivalent. Silver and thallium (in thallous salts) however fall, although trivalent, into the same group as copper and the bivalent metals, while the divalent mercury and lead behave as trivalent metals along with aluminium and iron. Thus 1 molecule of aluminium chloride, $AlCl_3$, has the same coagulating power as 5·32 molecules of sublimate, 16 molecules of cadmium chloride, or 750 molecules of sulphuric acid. Expressed generally this means that the molecular coagulating power of a salt is inversely proportional to the number of molecules which are required to produce coagulation.

If to produce coagulation, different salts of the same group are added successively, then their effect is additive, but such is not the case with salts of different groups. According to these authors, it is probable that the power which metallic salts have of producing coagulation depends entirely on the metal or positive radical.

Further, that the coagulative power depends on the amount of dissociation of the coagulating agent seems to be shown by the fact that acids which undergo a ready dissociation, namely HCl, HBr, HI, HNO_3 and H_2SO_4, have a considerably greater coagulative power than, for example, oxalic, phosphoric and arsenic acids, which dis-

[1] Bodländer: *Zeitsch. f. physik. Chem.* 12, 685 (1893).
[2] Picton and Linder: *Chem. Journ.* 67, 63 (1895).

sociate less, while some acids, such as tartaric, succinic or acetic acid, do not coagulate arsenious sulphide at all.

Thus the 'equivalent coagulative power' (c) seems to be proportional to the number of free ions x in the solution from which a second substance coagulates and to some constant θ, dependent on the nature of the positive radical in the salt employed. Therefore $\frac{c}{c^1} = \frac{x\,\theta}{x^1\,\theta^1}$, where c and c^1 are the equivalent coagulative powers of two salts, while θ and θ^1 are constants depending on the metals, and x and x^1 are proportional to the number of salt molecules dissociated into free ions (x and x^1 being calculated from the electrical conductivities). For salts of the same metal $\theta = \theta^1$ and the above equation becomes $\frac{c}{c^1} = \frac{x}{x^1}$.

The molecular conductivity of a salt, according to Kohlrausch, is of the form $\mu = x\ (u+v)$ where μ = molecular conductivity, x = proportion of the electrolyte dissociated into ions, and u and v = velocities of the migrating ions. Therefore the molecular conductivity of two salts is expressed thus $\frac{\mu}{\mu^1} = \frac{x\ \ (u+v)}{x^1\ (u^1+v^1)}$, and the coagulative power is controlled by the number of free positive ions in the solution of the colloidal arsenious sulphide.

TABLE COMPARING THE RELATIVE EQUIVALENT MOLECULAR CONDUCTIVITIES, MC, AND COAGULATIVE POWERS, CP (AFTER PICTON AND LINDER).

	Potassium.		*Hydrogen.*		*Sodium.*		*Ammonium.*	
	MC	CP	MC	CP	MC	CP	MC	CP
Chlorine . . .	1·07	1·07	0·94	0·98	1·05	1·07	1·02	1·19
Bromine . . .	1·08	1·04	0·99	1·03	1·06	1·05	1·10	1·00
Iodine . . .	1·08	1·04	1·01	1·00	1·05	0·93	1·09	1·00
NO_3	1·00	1·00	1·00	1·00	1·00	1·00	1·00	1·00
SO_4	0·85	0·85	0·62	0·62	0·83	0·81	0·82	0·77

Picton and Linder also demonstrated by optical tests that an increase in the size of colloidal particles takes place when the point of coagulation is neared, although the hydrosol may not be decomposed, and they say: 'There is a reaction other than mechanical between solvent and solid even in these cases of colloidal solution.'

Von Bemmelen in 1897 [1] continued his earlier work [2] on the composition and properties of inorganic colloidal oxides, such as the hydrogels of SiO_2, SnO_2, MnO_2, Al_2O_3, Fe_2O_3, Cr_2O_3, BeO, MgO, CuO; their powers of absorbing acids, bases and salts, and their transition into crystalline true hydrates.

He considers all gels as precipitation membranes, which agree with organic tissues in being composed of a micellar meshwork [3]

[1] J. M. v. Bemmelen: *Zeitsch. f. anorg. Chem.* 13, 233 (1897).
[2] J. M. v. Bemmelen: *ibid.* 5, 466 (1893).
[3] Naegeli's term of micella means an aggregation of molecules.

of amorphous particles, which imbibe and enclose fluid. Such colloids may be anhydrous substances or chemical hydrates, as is, for example, magnesia, which in its colloidal state is MgO, H_2O[1]. The water which has been imbibed von Bemmelen believes to be not in chemical union with the colloid but simply absorbed, the hydrosol being not a true solution, subject to gaseous laws, and the gel not a 'solid solution[2].' A gel consists thus of colloidal particles, each of which is surrounded by its own water-jacket; the radius of each mantle varying with temperature, dilution and composition of the gel.

A gel which has been dehydrated may either be able to pass again into the gel and sol conditions by the absorption of water, or the properties of the gel may be altered permanently by dehydration, and then traces of either acids or alkalies are needed to 'peptonize' the gel.

In 1898 von Bemmelen[3] stated that gel formation is not comparable to the hardening of a gum-solution which depends on the evaporation of water, but that it has a great resemblance to the foams made by Bütschli, who mixed olive oil, water and potassium carbonate, or to the foams made by Krafft, who combined the colloidal watery solutions of an amide of the higher fatty acids with an inorganic acid (for example, the hydrochloride of hexadecylamin, $C_6H_{13}\cdot NH_2\cdot HCl$), or who united a higher fatty acid with an organic base (for example, methylammonium palmitate).

No transition existing between the sol and the gel state comparable to a semi-solid gum-solution, the phenomenon of gel formation or coagulation is likened to the separating out of the colloid as a more or less insoluble foam, which includes in its interstices and cavities the solvent. The separation of the solvent and the colloid is termed 'Entmischung' or demixing, or, as Minchin[4] translates it, 'desolution.'

By a careful comparison of the amount of water contained in jellies with the vapour pressure they exert, he arrived at the conclusion that the fluid and the solid portions of a gel cannot 'be considered as two phases in the sense of the phase rule, since there is no sharp line between them, and he therefore concludes that the phase rule cannot be employed to elucidate the phenomenon.' 'The curves of the equilibrium points are gradually bending lines if the dehydration of the gels is sufficiently slow; but if dehydration is relatively rapid there is a sudden change of direction, when the water content is very much diminished[5].'

Bredig (1898)[6] prepared colloidal solutions of gold, silver, and

[1] See also W. Pascheles (Pauli) *Pflüger's Arch.* 77, 219 (1897); O. Bütschli: *Verhandl. d. naturhist.-med. Vereins z. Heidelberg*, N.F., 5, and *Abh. d. Königl. Ges. d. Wiss. z. Göttingen*, 40; Hofmeister: *Arch. f. experim. Pathol. u. Pharm.*, 28; Pierre Duhem: *Journ. Physical Chemistry*, 4, 65–122 (1900).

[2] See p. 330. [3] J. M. von Bemmelen: *Zeitsch. f. anorg. Chem.* 18, 24 (1898).

[4] E. A. Minchin: *Investigations on microscopic foams and on protoplasm.* Translation of Bütschli's work, 1894. See preface.

[5] Hardy: *Proc. Roy. Soc.* 66, 110 (1900).

[6] Georg Bredig: *Zeitsch. f. angew. Chem.* 951–954 (1898).

platinum, not by the usual chemical method of adding reducing substances, but by making electrodes of these metals, immersing them in water, and then passing electric discharges through the fluid. By this means, the metal at the cathode is disintegrated and passes into colloidal solution, and when electrolysed, the metal is deposited at the anode as a black slime. Gold solutions prepared in this way are either reddish-purple or dark-blue, while silver and platinum give dark-brown solutions. Bredig assumes the colloidal particles to have a diameter of about a thousand times their molecular dimensions, and the colours produced to be due to the fine state of division.

Stoeckl and Vanino[1] have shown that light is polarized elliptically by colloidal solutions of metals, because light reflected from metallic surfaces also produces elliptical polarization. The movement of colloidal particles in a solution through which an electrical current is passing, they explain as due, not to electrolysis, as does Zsigmondy[2], but as due to the electrical current having the power of carrying solid particles with it through the solution[3]. The following substances go with the negative stream to the positive pole, if suspended in water: gold, platinum, copper, iron, graphite, asbestos, kaolin, sulphur, shellac, and silk. If turpentine is used as the 'solvent,' then these bodies go with the positive stream to the negative pole, except sulphur, which in this case also goes to the positive electrode. 'By streaming electricity fluids are driven on. The particles of the fluid in motion rub against the suspended particles. By this friction, electricity is generated, the particles becoming electrically positive or negative according to the medium in which they are suspended. If we take water, the most positive of all substances, then the particles become negative and go to the positive pole. In turpentine the particles become positive and go to the negative electrode, with the exception of sulphur, which also becomes negative if rubbed against turpentine.'

Hardy[4], through whose work my attention was first drawn to the purely physical aspect of coagulation, has divided colloids into those which form reversible jellies, for example ordinary gelatine dissolved in water (p. 49), and those which do not (p. 132). He thus makes his criterion, the reversibility of the solid state or gel into the fluid state or sol, by means of heat, and classes together those gels which do not change on heating, considering it immaterial whether the solid state has been induced by purely physical or by chemico-physical means.

Hardy has experimented with the following colloidal solutions: (1) gold, made by adding a couple of drops of a solution of phos-

[1] K. Stoeckl and L. Vanino: *Zeitsch. f. physik. Chem.* 30, 98 (1899).
[2] Zsigmondy: *Lieb. Ann.* 301, 33-36 (1898).
[3] The literature on this subject is collected by L. Grätz: 'Elektrische Endosmose und Strömungsströme' in Winkelmann's *Handbuch d. Physik*, 3, 1, 493.
[4] Hardy: (1) *Journal of Physiology*, 24, 172 (1899); (2) 'The coagulation of Proteid by Electricity,' *ibid.* 288; (3) 'On the conditions which determine the stability of irreversible hydrosols,' *Proc. Roy. Soc.* 66, 110 (1900).

phorus in ether to about a litre of a very dilute solution of gold chloride, dialysing the ruby-coloured fluid for fourteen days against distilled water freed from dissolved carbonic acid, and concentrating the gold solution by boiling; (2) gum mastic, prepared by adding a very dilute solution of the gum in alcohol to distilled water, and then dialysing it for fourteen days against distilled water; (3) heat-modified egg-white, prepared by dissolving white of egg in nine times its volume of distilled water, filtering, boiling[1] and dialysing against distilled water for some days. 'If the solution is boiled in a test-tube, a milky fluid is formed and a film of proteid is left on the glass; a second quantity boiled in the same test-tube comes out less milky, until, when the proteid film is sufficiently thick to eliminate all action by the glass, the solution after boiling contains the proteid dispersed as particles so small that they scatter pure blue light.'

According to Hardy, these colloids in a watery solution form a system which is composed of solid colloidal particles floating in an exceedingly dilute solution of the colloid in water, and therefore 'each particle in a hydrosol is surrounded by a zone in which the components are in a condition of chemical instability.'

'The stability of colloidal systems is related to the contact difference of potential which exists between the solid and the fluid phases, and which forms round each particle a double electric layer. Such double electric layers round particles of any kind immersed in a fluid would resist any movement of the particles through the fluid, because, as Dorn's experiments show, electric work is done in displacing the particles[2].'

These electric layers producing, as J. J. Thomson put it to Hardy, an effect equivalent to having the particles suspended in a viscous fluid[3], it follows that any factor leading to a disappearance of the electric charges in the particles and in the fluid, must convert the 'viscous' fluid into a mobile one and thus facilitate the aggregation of the colloidal particles.

'Free acid, added to a hydrosol in which the particles are negative to pure water, will diminish the relative difference of potential of the water. In this case the reagent acts directly on the water, and the coagulation activity of unit mass of the substance varies directly with its chemical activity when dissolved in water. The same relation seems to hold when free alkali is added to a hydrosol in which the particles are electro-positive.' 'The stability of the system may also be destroyed by induction, the active agents being free ions carrying a static charge[4]. In this case the action may be said to be on the particles, or rather on the electric layers immediately around them, and the active ions are those whose electric sign is the opposite of that of the charge on the surface of the particle. In this case coagulation power does not vary directly with variations in

[1] That egg-white diluted with nine times its bulk of water does not coagulate was first observed in 1880 by William Roberts: 'Lumleian lectures on digestive ferments and artificially digested food. (London: Smith, Elder and Co., 1880.)

[2] Dorn: *Wiedemann's Ann.* 10, 70 (1880).

[3] Compare with the view of Barus and Schneider, p. 33.

[4] Whetham: *Phil. Mag.* (November, 1899).

chemical activity. It rises exceedingly rapidly with a rise in the valency of the active ion, so that the relation

$$I' : I'' : I''' = n : n^2 : n^3$$

is approximately satisfied.'

The factors determining the stability of hydrosols vary greatly according to the specific colloid we are dealing with; thus the slightly basic ferrihydroxide $Fe(OH)_2$ is only stable in the absence of free acids, free bases or neutral salts; gum mastic is stable in fairly concentrated monovalent bases but is precipitated by acids, while the heat-coagulated proteid-hydrosol is only stable in the presence of either free acids or free bases.

As the colloidal proteid, just alluded to, is precipitated from its acid solution by the addition of alkalies and from its alkaline solution by the addition of acids, there must exist somewhere a point of neutrality at which precipitation occurs. Coagulation in this case is therefore equivalent to the establishment of an iso-electric state between the colloid and its solvent.

If the amount of a coagulant which has been added to a colloid is not sufficient to cause complete coagulation, then an effect is produced which is proportionate to the amount of coagulant added. The solid colloidal particles by increasing somewhat in size lead to the formation of a new state of equilibrium accompanied by a diminution in the extent and curvature of the surfaces of contact between the solid colloid and its solvent. It is during this stage that the originally blue proteid solution (p. 38) becomes white. Compare pp. 33 and 35.

The behaviour of hydrosols in an electric field has been studied especially by the following observers:—Zsigmondy[1] noticed how gold in colloidal solutions moves against the current; Picton and Linder[2] in 1897 found the direction of movement of the particles to be either with or against the stream according to their chemical nature (p. 33). See also Stoeckl and Vanino, p. 37.

Hardy in 1899 established the fact that heat-modified egg-white, under certain conditions, may go either with or against the electrical current according to the reaction of the solvent. This proteid precipitated by neutralization, made into a fine mud in an agate mortar,

[1] Zsigmondy: *Liebig's Ann.* 301, 29. For the preparation of red colloidal solutions of gold Zsigmondy gives these directions: Obtain perfectly pure, twice distilled water, and pure crystalline gold chloride, which is got by evaporating a solution of gold in aqua regia.

Make a 0·6 p.c. solution of the gold chloride; take of this 25 cc.; dilute with 100 to 150 cc. of water; add 2 to 4 cc. of a 0·2 normal potassium carbonate or bicarbonate solution (the amount required depends on the condition of the water; blue-violet solutions require stronger dilution and more alkali); bring to the boiling-point, but do not boil for any length of time, as otherwise CO_2 is given off and the solution becomes reddish-violet; as soon as the fluid is boiling remove it from the flame and add in small quantities, but quickly, 4 cc. of a 1 p.c. solution of formaldehyde, which latter must be freshly distilled, the distillate between 97 and 100° C. being taken. The gold solution ought soon to acquire a carmin-red colour; it contains about 5 mg. of gold in 100 ccm., but by careful dialysis may be concentrated to 0·12 p.c. Gold solution prepared in this manner is not decomposed by boiling.

[2] Picton and Linder: *Journ. Chem. Soc.* 70, 568 (1897).

and suspended in water in a U-shaped tube, rapidly sinks to the bottom and the sediment does not move, even in forty-eight hours, either towards the negative or the positive electrode, on establishing an electric field having a potential gradient of 100 volts in 10 cm. On adding, however, the merest trace of an acid the proteid 'becomes electro-positive' and travels with the positive stream, while by the addition of an alkali to the sediment it is 'rendered electro-negative' and goes with the negative current.

Colloidal proteid is thus a potential base or acid. The general conclusions of Hardy given on pp. 38 and 39 were arrived at after studying the action of electrolytes on colloidal substances according to the following plan.

1. *The action of salts.*

The coagulative power of a salt is determined by the valency of that prepotent (i.e. more active) negative or positive ion, which has an electrical load the opposite of that carried by the colloid-particles. Experiments were made with neutral salts (sodium chloride, NaCl); salts neutral to litmus but acid to phenolphthalein (magnesium sulphate, $MgSO_4$, and barium chloride, $BaCl_2$), and salts acid to litmus (aluminium sulphate, $Al_2(SO_4)_3$, cadmium nitrate, $Cd(NO_3)_2$, copper chloride, $CuCl_2$, and copper sulphate, $CuSO_4$). All these substances were made up in the concentration of 1 gram molecule in 2,000 cm. Working with coagulating salts in concentrations of 1 gram molecule in 120,000 cm., the colloid used being the electro-negative silica, dialysed free from chlorides, the following results were obtained at 16° C.:

Coagulation at once.	*In 10 minutes.*	*In 2 hours.*	*In 24 hours.*	*Still fluid.*
$Al_2(SO_4)_3$	$CuSO_4$	$MgSO_4$	K_2SO_4	NaCl
	$CuCl_2$		Na_2SO_4	Control
	$Cd(NO_3)_2$			
	$BaCl_2$			

With proteid rendered electro-negative by a trace of alkali, and with the coagulating salts in strengths of 1 gram molecule in 80,000 cc., at 16° C.:

Coagulation at once.	*On slightly warming.*	*No effect.*
$Al_2(SO_4)_3$	$MgSO_4$	Na_2SO_4
$Cd(NO_3)_2$	$BaCl_2$	K_2SO_4
$CuSO_4$	$CaCl_2$	NaCl
$CuCl_2$		

With proteid rendered electro-positive by a trace of acetic acid:

Instant coagulation.	*No effect.*
$Al_2(SO_4)_3$	$CuCl_2$
$CuSO_4$	$Cd(NO_3)_2$
K_2SO_4	$BaCl_2$
Na_2SO_4	NaCl
$MgSO_4$	

In confirmation of the observations of Schulze, Picton and Linder, Hardy found the coagulating power of a salt to be not directly proportional to the amount of its dissociated molecules and the sum of the mobilities of its ions, but to be determined by an additional factor, which varied as the square or cube, according as to whether the precipitating ion was mono-, di-, or trivalent. If K be

the number of gram molecules of a substance just sufficient to bring about coagulation; na = the percentage of the dissociated molecules of the coagulant; $u + v$ the sum of the mobilities of the two ions set free by electrical dissociation; and A^x the effect produced, x being positive and increasing quickly with the valency of that ion which has a charge opposite to the charge possessed by the substance to be coagulated, then

$$K = na\,(u + v)\,A^x.$$

Thus the coagulative power of an active ion 'varies with its valency approximately according to the square and cube':

$$R' : R'' : R''' = K : K^2 : K^3.$$

'If two electrolytes are present, then the action is additive if the metals are of the same valency, and subtractive if of different valency. Therefore there is interference between e.g. salts of the form $R'Ac^n$ and $R''Ac^n$; the one inhibits the other.'

'The effect of the acid or basic reaction of the salt on the hydrosol is as a rule small as compared with the effect of the metal-ion. Thus the stability of a hydrosol of electro-positive proteid is increased by free acid, yet the acid salts find their proper place in the scale of valency. Again, ferric hydrate is coagulated by nitric acid when the concentration reaches 1 gram molecule in 2,500 cc.; yet the cadmium salt of this acid is not much more potent than are the "neutral" salts $MgSO_4$, $BaCl_2$.'

2. *Action of acids and alkalies.*

The action of acids on electro-negative colloidal matter and that of alkalies on electro-positive colloids 'is determined by the laws which govern ordinary chemical equilibrium,' because solutions of acids which are equi-coagulative with respect to electro-negative colloids agree in their electric conductivity, that is in the amount of electric dissociation. The action of acids on electro-negative colloidal matter is brought out by the following table, in which K represents the relative coagulative power (the value of K being referred to Al_2Cl_6 as unity) and C stands for the specific conductivity (i.e. the conductivity of 1 gram equivalent in 1,000 cm.). The table shows the action of different acids on the electro-negative hydrosol of arsenious sulphide, and was calculated by Hardy from Picton and Linder's results:

Acid.	*K*	*C*
HBr, HI, HCl, HNO_3	0·001	2950
H_2SO_4	0·0006	1935
Oxalic	0·0005	578
H_3PO_4	0·00007	230

Should the colloid-particles be, however, electro-positive, then the conductivity of equi-coagulative strengths of acids varies to a remarkable extent:

Acids.	*H'*	*H''*	*H'''*
Mastic, electro-negative	12·6	14·4	13·9
Ferric hydrate, electro-positive	1650	6·8	0·7

The coagulating power, K, of salts, acids, and alkalies may be summed up in the following scheme, which is based on Hardy's researches, detailed above (+ means coagulation, ○ means no effect):

Coagulant.		*Colloid.*	
		electro-positive.	*electro-negative.*
Neutral salt	=	○	○
Salt: prepotent ion negative	=	+	○
Salt: prepotent ion positive	=	○	+
Acid	=	○, or K varies, or $K = x$ or x^2 or x^3	K varies directly with chemical activity.
Alkali	=	K varies directly with chemical activity and not in ratio of square and cube	○, or K does not vary in any simple way with valency.

Whetham[1] has applied the law of chances to the phenomena of coagulation. Linder and Picton (see above) by acting on arsenious sulphide, taking aluminium chloride as unity, found mono-, di-, and trivalent metallic ions of different sulphates to possess equi-coagulative powers when they were used in the concentrations of 930 : 26 : 0·9 respectively, which means that the relative coagulative power of these different sulphates would be proportional to the reciprocals of these numbers, according to the ratio 1 : 35 : 1023. Schulze had previously also found mono-, di-, and trivalent chlorides to possess coagulative powers in the ratio of 1 : 30 : 1650. The electrical charge on an ion being proportional to its valency, 6 monads equalling 3 diads and 2 triads, it follows that the valency of the metallic ion has an effect on the coagulative power which is very different from the effect on properties for which the usual 1 : 2 : 3 ratio holds good.

Coagulation being produced, firstly, whenever a certain minimal electrical charge is brought within reach of the colloidal particles, and, secondly, whenever the effect on the colloid produced by the charge occurs throughout the solution with a certain minimum frequency, it follows that if by experiment it has been determined that two substances are active in the ratio as $1 : x$, then by the law of chances, three substances will be active in the proportion of $1 : x : x^2$. Thus if $x = 32$, then the ratio will be as 1 : 32 : 1024, which agrees very well with the results of Picton and Linder, namely 1 : 35 : 1023; or if $x = 40$ we get 1 : 40 : 1600, or numbers comparable to Schulze's values 1 : 30 : 1650. Similarly equal electrical charges would be obtained by the conjunction of 3 tetrads, 4 triads, 6 diads or 12 monads, or $1 : x : x^2 : x^3$. Therefore if $x = 32$, then the ratio is as 1 : 32 : 1024 : 32800, or if $x = 40$, the ratio is as 1 : 40 : 1600 : 64000.

Bredig and Coehn[2] (1900) show that the theory of Starke, according to which colloidal material is kept in solution by gases dissolved in the water, does not hold good, because colloidal solutions of gold,

[1] Whetham: 'Coagulative power of electrolytes,' *London Phil. Mag.* 47, 474 (1899).

[2] Georg Bredig and A. Coehn: *Zeitsch. f. physik. Chem.* 32, 129–132 (1900).

silver, and platinum, prepared by Bredig's electrical method [1], when freed from all air by the gas-pump did not coagulate. Starke used zinc chloride solutions, which are very apt on dilution with water to form the insoluble zinc hydroxide, especially if the solution also contain some basic chlorides.

Bruni and Pappadà [2] distinguish between colloids proper (such as silicic acid, ferric hydroxide, chromic hydroxide, ferric ferrocyanide, egg-albumin, gelatin) and semi-colloids (for example, dextrin and molybdic acid). These latter pass comparatively readily through a dialyser, and also give small depressions of the freezing-point, which are, however, quite appreciable and proportional to the concentration; dextrin, for example, gives a molecular weight, 1135, corresponding with the formula $(C_6H_{10}O_5)_7$. Semi-colloids when in solution have thus very high molecular weights.

My present views on 'Coagulation.'

Coagulation is a term which in its wider sense is applicable to any change affecting substances whereby they are rendered insoluble, either temporarily or permanently. I propose, however, to restrict the term coagulation to the formation of gels by physical means, and to call gels, produced by chemical action, precipitates, while the firming of gelatine, which results from a lowering of the temperature, will be described as 'setting.' Thus gelatin sets on cooling; it is coagulated by ammonium sulphate while precipitated by corrosive sublimate. Precipitation is therefore accompanied by a chemical change, while setting and coagulation are not, as far as we know at present.

We are apt to attribute a transparent appearance to a jelly and an opaque one to precipitates and coagulates, but this is purely arbitrary, because albumin may be changed into a perfectly transparent jelly by the action of strong alkalies such as caustic soda (Lieberkühn's jelly), or by salts such as ferric chloride (Rose's jelly) [3], or by glacial acetic acid (Neumeister's jelly) [4]. As far as it is possible to arrive at any conclusion, colloids when 'in solution' appear to have the following characteristics:—

1. They polarize transmitted light.

2. They move either with or against an electrical stream, which is being passed through them, and are therefore either electro-positive or electro-negative, but they offer a great resistance to the flow of the electrical current.

3. They are precipitable by electrolytes, the potent ion of which has an electrical sign opposite to that carried by themselves.

4. They have no chemical affinities as long as they are in the

[1] G. Bredig: *Zeitsch. f. angew. Chem.* 951 (1898); and *Zeitsch. f. Electrochemie*, 4, 514, 547.

[2] Giuseppe Bruni and N. Pappadà: *Atti Real. Accad. Lincei*, 9, i. 354–358 (1900). Abstr. *Journ. Chem. Soc.* 78, 2, 591 (1900), 'Nature and Properties of Colloidal Solutions.'

[3] Ferdinand Rose: *Poggendorff's Ann.* 28, 140 (1833).

[4] Pure egg-white with glacial acetic acid does not however form a clear jelly, but an opaque coagulum, see Exp. 3, p. 105.

colloidal state, except that certain of them, such as osmium tetroxide and formaldehyde, form addition compounds with non-electrolytes.

5. They do not raise the boiling-point, or affect the freezing-point, of water.

6. They are not coagulated by a rise of temperature, provided electrolytes are absent, and provided that they themselves do not become chemically altered by heat.

7. They do not as a rule pass through animal and vegetable membranes, and they are retained by carbon filters.

8. They have little or no tendency to crystallize.

The question whether colloids are in suspension or in solution seems to me not a difficult one to answer, provided we can define what we mean by 'solution.' The best account of this question is that of J. W. Brühl[1], who has discussed the part played by media in the process of dissolving substances. It will suffice to point out that the power of acting as a solvent seems to be intimately connected with the solvent possessing some atom which is potentially pluri-valent; for example, oxygen in water is divalent but capable of becoming tetravalent, the nitrogen of ammonia is trivalent but with a tendency to become pentevalent, and so on.

Krafft[2] is of the opinion that solubility of salts depends on the ease with which they undergo hydroxylation, or hydrolysis, as is shown by comparing hydrocarbons with carbohydrates, the anhydrides of monobasic acids and their hydrates. On this principle sodium stearate is soluble in water because in concentrated solutions it splits up hydrolytically into stearic acid and sodium hydrate

$$C_{18}H_{35}O_2Na + H_2O = C_{18}H_{35}O_2H + NaOH.$$

Strictly speaking the term solution should only be used when two or more substances have undergone a definite chemical change dependent on mutual interaction as in the case of hydrolysis. In a wider sense electrolytes undergoing electrolysis may also be said to pass into solution, owing to the development of electro-chemical affinities; but once in solution, true electrolytes form with water a treble (or quadruple) mixture, thus kat-ion + an-ion + solvent + (non-dissociated electrolyte).

In the light of Brühl's conception and also in that of the facts that pure water is partly dissociated and that it possesses a high dielectric constant (p. 12), the following possibilities suggest themselves:

1. The solvent undergoes with the substance a definite chemical change as in hydrolysis. Thus: corrosive sublimate and water:

$$HgCl_2 + xH_2O = HgOH + 2H^{\cdot} + 2Cl'.$$

2. The solvent induces a chemico-electrical change as in electrolytic dissociation. For example, sodium chloride and water:

$$NaCl + xH_2O = Na^{\cdot} + Cl' + H_2O.$$

3. The solvent forms with the substance additive compounds as in the case of colloids. For example, ferric oxide and water $= Fe_2O_3 + xH_2O$.

[1] *Zeitsch. f. physik. Chem.* 10, 1 (1899).

[2] Krafft, F.: 'Über d. Krystallisationsbedingungen colloidaler Salzlösungen,' *Ber. deutsch. chem. Ges.* 32, 1596 (1899).

4. The solvent and the substance mutually diffuse into one another as would gases, there being formed mixtures as in the case of non-electrolytes, for example sugar 'dissolving' in water.

As water is always partially dissociated into the electro-positive $H^{\cdot}$ and the electro-negative OH' ions it can act either as an acid or as an alkali towards any substance with which it is brought into contact. In the case of hydrolysis and electrolysis the chemical change induced by the solvent is accompanied by an electrical change, for positive and negative charges are distributed over the acid and basic radicals of the electrolyte, while the water acts as an insulator, because the charges induced in it by the two radicals of an electrolyte balance and thus neutralize one another.

The passage of an electric current through such a solution depends on three factors, namely, firstly, on particles, the ions, carrying negative and positive charges; secondly, on the particles being freely mobile; and thirdly, on the solvent being electrically neutral.

In the case of a non-electrolyte such as sugar dissolving in water, we are dealing with a phenomenon in every particular comparable to the mixing of two mutually inert gases; and through such a mixture an electrical current does not pass, because neither the sugar molecules nor the water molecules carry charges, or, if they carry charges, then not being distributed in a third medium in which they can glide past one another, they are unable to carry whatever charges they possess in any definite direction[1].

If substances undergoing hydrolysis and electrolysis form one end of a chain and non-electrolytes the other end, then substances which form additive compounds with water stand in the middle, because they have certain features in common with electrolytes and non-electrolytes. These addition-compounds in the non-dissociated state would have to be regarded as hydrates or H_2O-bodies, while after dissociation the colloid radical if electro-positive would have as its companion-ion the electro-negative hydroxyl-radical, OH', or reversely an electro-negative colloid would have as its electro-positive mate the hydrogen-ion, $H^{\cdot}$. There is, however, a great difference between ordinary electrolytes, which are hydrogen or hydroxyl compounds, and true colloids; for while the former break up, if they dissociate at all, into an equal number of individual units, the kat-ions or an-ions, we are dealing in the colloids with substances in which either the kat-ions or the an-ions are composed of many units aggregated together, each aggregate playing the part of a single ion and being kept in 'solution' by a unit-ion of the opposite sign. This assumption would explain Hardy's experiment with electrically neutral proteid, which is a perfectly insoluble compound, and with no tendency to go towards either the positive or negative electrode till traces of acid or alkali have been added (p. 40). As the proteid acquires the charge of the positive hydrogen-ion of acids and the negative charge of the hydroxyl-ions of alkalies we may assume the hydrogen- or hydroxyl-ions to unite with aggregates of proteid molecules and thus to form new ions consisting

[1] The same reasoning may be applied to non-dissociated electrolytes, it being immaterial whether they are in the solid state or mixed with a solvent.

of the (colloid + H)$^{\cdot}$ or (colloid + OH)$'$. The an-ion of the acid which was added (for example Cl$'$ or the acet-ion) or the kat-ion of the alkali (for example Na$^{\cdot}$) become the companion-ions to the (colloid + H)$^{\cdot}$ or the (colloid + OH)$'$-ions.

Provided my hypothesis is correct, we may consider the solution of an ordinary dissociated electrolyte as representing a double 'colloidal' solution, and the formation of insoluble hydrates during hydrolysis as the precipitation of one of the two colloidal solutions. But we have to remember that Picton and Linder are mistaken when they say (p. 33) that a colloidal solution would become dissociated into its ions, if we could break up the particles sufficiently fine; for what is characteristic of a colloid is that each aggregate of colloidal particles should contain a definite charge, and the only factor which will allow of a breaking up of an aggregate into smaller particles, and which will produce at the same time a true colloidal solution, is one giving to each unit of the original aggregate a definite charge, and then maintaining this charge by the presence of other ions having an electrically opposite charge. If the aggregates in a colloidal solution are completely broken up into the composing units by acquiring definite charges, then the colloidal solution loses its colloidal character and becomes a solution of a completely dissociated electrolyte, as, for example, in the case of silicic acid, mentioned on p. 33. Reversely, if to a solution containing a definite number of electro-positive (colloid + H)$^{\cdot}$-ions there is added an alkali containing the same number of electro-negative hydroxyl-ions, then the H$^{\cdot}$ of the colloid and the OH$'$ of the alkali unite to form electrically neutral water, and the colloid having lost its electrical charge is precipitated; if, however, not a sufficient number of OH$'$-ions were added to bind all the hydrogen-atoms, then the colloid-aggregates rearrange themselves into larger aggregates, which will remain in 'solution' as long as the H-atoms, joined to the colloid, enable its aggregates to maintain a definite charge. As the colloid particles become larger and larger and the number of electrical charges for a given volume of solution fewer and fewer, complete precipitation will probably be induced at a time short of perfect neutralization of the charges, because of the specific gravity of the colloid-aggregates overcoming the viscosity of the fluid.

Why the colloid does not unite in every case with the radical having the stronger electro-affinity, why, for example, after the addition of an acid the colloid unites with H, which has less electro-affinity than K, while after the addition of an alkali it does not unite with K but with the OH, which latter has also less electro-affinity than K, seems to be determined by the fact that H$^{\cdot}$ and OH$'$ are those very radicals which by their union form water, and which therefore may have special chemical affinities for the colloid, inasmuch as the latter owes its existence to having been formed in water.

The effect of H$^{\cdot}$ and OH$'$ on colloids may be regarded as analogous to their peculiar action on the colour of litmus and other indicators.

The aggregation of colloid matter into larger masses, and the arrangement of these latter into foam or mesh-like structures, is one

of the highest importance histologically, because during and by the process of aggregation a great deal of histological detail may be destroyed, or so altered in character as to make the final product resemble but little the appearance which the object had during life. Electrolytes acting on proteid substances always give rise to structural changes, but it is possible to prevent or counteract this result by means of non-electrolytes, as stated elsewhere (pp. 68–70, and on pp. 137 and 138).

If we assume with Hardy that a colloidal solution consists of colloidal particles floating in an extremely dilute solution of the colloid in the solvent, then we would have also to assume that the very minute particles in the dilute solution carry definite charges.

On the lines of my hypothesis it seems more probable, although Hardy's conception holds good for the time immediately following the addition of an alkali or an acid, that subsequently there is established a new balance in which the colloid aggregates into masses, all of which are approximately similar in size, the absolute size varying directly in proportion to the number of $H^{\cdot}$ or OH' radicals which were introduced.

Whether certain colloids may be held in solution simply by the hydrogen and hydroxyl ions of water is a question on which I have been unable to get any information. There is nothing improbable in such a conception, but it would appear that most colloids require some other substance to remain in solution; thus ferric oxide + water dissolves in ferric chloride forming oxychlorides, and provided the ratio of Fe_2O_3 : $FeCl_3$ is as 23 : 2 (Ordway) the oxychlorides will remain soluble in water. Picton and Linder[1] have also found, on dissolving freshly precipitated ferric hydroxide by the addition of ferric chloride, that a basic chloride is formed containing 1·5 parts of hydrochloric acid to 98·5 parts of ferric oxide. This solution, on being filtered through a porous cell, leaves the whole of the iron and the hydrochloric acid behind. The ferric oxide may also be obtained as a colloidal solution by dialysing ferric acetate, when ultimately a liquid results which contains only six parts of acetic acid to 94 parts of oxide of iron. It seems that in the one case the hydrochloric acid or chlorine ion, and in the other case the acetic acid or acet-ion, are the factors which keep the colloidal Fe_2O_3 in solution.

That substances in the colloidal state do not undergo chemical reactions till they cease to be colloids, seems to be proved by the observation of Barus and Schneider given on p. 32, who found that colloidal silver unites with hydrochloric acid to form silver chloride only if this acid be added in greater quantities than is necessary to precipitate the silver in a metallic state. Dyes in the colloidal state also do not stain tissues[2]. True additive compounds between proteid-colloids and osmium tetroxide and formaldehyde are, however, possible, and in this case no electrolytic changes take place.

If we adopt the Reuss-Wiedemann-Quincke theory that movement of colloidal particles in an electrical current is due to charges being

[1] Picton and Linder : *Journ. Chem. Soc.* 1, 152 (1892).
[2] Spiro, etc. Footnote 4 on p. 52.

generated on the colloid-particles by the friction between the latter and the solvent (see Stoeckl and Vanino, p. 37), and expand it, we would have to assume that colloid-particles receive their charge when they are first formed, and that they are able to keep the charge till by the introduction of electrolytes the character of the solvent is altered. Under these conditions colloids will be precipitated when friction with the solvent does not generate a charge; on the other hand, Hardy's proteid-colloid would become electro-positive whenever the solvent became electro-negative, and reversely, the proteid would be rendered electro-negative by an electro-positive solvent.

Instead of explaining the electro-positivity of the proteid-colloid, after the addition of an acid, as being due to a union between the positive hydrogen ion and the colloid, as was done above, we would have to assume one of the two following views: either, firstly, that the positive charge on the colloid is induced by the an-ion of the acid added—that, for example, by the addition of hydrochloric acid, the negative Cl′-ion will induce during its diffusion into the colloidal solution (see p. 53) a positive charge on the colloid by friction—or, secondly, that the positive hydrogen ions, by diffusing into the solvent water, will induce in the latter a negative charge because of its high dielectric constant (p. 12), and that now the electrically negative water induces in the colloid a positive charge.

The two possibilities just considered along with the view that a direct union between the colloid and the H˙ or OH′ radical takes place, may be represented thus:

Direct union between an acid and the colloid:

$$\text{Colloid} + \text{HCl} + \text{water} = (\text{colloid} + \overset{+}{\text{H}}) + \overset{-}{\text{Cl}} + \text{H}_2\text{O}.$$

Indirect action of kat-ion radical on colloid:

$$\text{Colloid} + \text{water} + \text{HCl} = (\overset{+}{\text{colloid}})\ (\overset{-}{\text{water}})\ \overset{+}{\text{H}} + \overset{-}{\text{Cl}}.$$

Direct action of an-ion radical on colloid:

$$\text{Colloid} + \text{HCl} + \text{water} = (\overset{+}{\text{colloid}})\ \overset{-}{\text{Cl}}\ \overset{+}{\text{H}} + \text{water}.$$

In this last case the chlorine-ion, which has greater negative electro-affinity than the hydrogen-ion has a positive affinity, may be assumed to satisfy its full negative affinity by evoking a positive response of the colloid, in which case the positive H˙ and the positive colloid˙ will together completely balance the negative Cl′.

Graham's classification of colloids into sols, gels, and solids was given on p. 29, and Hardy's division of colloids into reversible and irreversible compounds on p. 37.

The classification which I have adopted is as follows:

A. Changes in temperature leading to 'setting':
1. by lowering the temperature . . . . p. 49
2. by raising the temperature [?] . . . . p. 65
B. Mechanical shaking producing 'conglutination' . . p. 50
C. Physical factors inducing 'coagulation' by
1. dehydration . . . . . p. 52
2. alterations in the electrical tension between the colloid and its solvent . . . . . p. 53

D. Chemico-physical factors causing 'precipitation,' owing to
1. a withdrawal of the H' or OH' radical of the colloid p. 55
2. a removal of salts . . . . . p. 56
3. the formation of insoluble salts . . . . p. 58
4. heat action . . . . . p. 59
E. Chemical action unaccompanied by physical change, owing to the formation of additive compounds between colloids and non-electrolytes by the process of oxidation or reduction . . . . . p. 68

Before giving a more detailed account of the different factors altering the colloidal state I wish to state definitely in what sense I have used the word colloid when applied to proteids:

Through Cohnheim and Krieger (see p. 27) we have learned to regard proteid substances as pseudo-acids and pseudo-bases, in short as bodies which have a great tendency to pass from the electrolytic salt-like state into the non-electrolytic colloidal condition. For this reason we require to be constantly on our guard in applying the term colloid to proteid solutions: they are colloids only as long as their pseudo-basic or pseudo-acid character is not disturbed by the action of potent kat-ions or an-ions.

A. 1. *On the 'setting' of colloidal solutions (according to Hardy).*

As already pointed out (p. 37), jellies either melt when heated and revert to the fluid state, when they are said to be reversible, or they shrink, remaining insoluble or irreversible. Reversible jellies may form, according to the temperature, (*a*) a homogeneous fluid; (*b*) a mixture of two heterogeneous solutions; or (*c*) a mixture of a solid and a fluid.

(*a*) On raising the temperature of a 1 per cent. agar solution it will become perfectly fluid and homogeneous.

(*b*) On cooling it and subjecting it to pressure two fluids may be obtained, the first being a solution of water in agar, and the second a solution of agar in water, showing that these two form a binary system of partially miscible fluids. Below a certain temperature such binary systems either separate readily into two layers, or they remain in the state of an intimate mixture according to the mobility of the molecules. Thus in the case of the agar solution just mentioned, the molecules are exceedingly immobile; the watery-agar and the agary-water constituents do not separate from one another, and hence the surface of contact between the two solutions is very great. On the other hand, in binary solutions with mobile molecules, such as ether and water or phenol and water, the surface between the two mixtures is soon reduced to the smallest possible area, namely a plane. All similar mixtures, having a surface in common through which diffusion from one solution into the next may take place, are said to be conjugate.

(*c*) On cooling the agar mixture still more, the solution containing the higher percentage of agar begins to solidify on its surface, and this 'setting' produces the jelly-like state.

A microscopical study of the phenomena just described can readily be made with a ternary mixture, such as a solution of 13·5 grms. of gelatin in a fluid composed of 50 cc. water and 50 cc. absolute alcohol. Above 17° C. this mixture is homogeneous, but on lowering the temperature it becomes viscid, and simultaneously with this change there appear minute fluid granules which gradually grow to 3 μ in size. At 12° the viscous mixture sets and the fluid granules, which had arranged themselves into 'anastomosing' threads, become solid.

If solutions containing more than 13·5 per cent. gelatin be taken, then somewhere between this and 30·5 per cent. strength, a sudden inversion of the system occurs, for now the granules or droplets in the mixture no longer contain the higher percentage of gelatin, but less than that found in the remainder of the mixture, as is shown by the accompanying table:

	Mixture.	*Droplets.*	*Nexus fluid.*
Temperature 15° C.	6·7	17	2
	13·5	18	5·5
	36·5	8·5	±40

When therefore colloidal matter occurs in the state of a gel, which means a mixture of a fluid and a solid, then 'it may consist of a solid mass containing spherical fluid droplets, or of solid droplets, which by hanging one to the other form a framework, in the spaces of which fluid is held. These two types present important mechanical peculiarities. The former is firm and elastic, and it maintains its structural integrity even under high pressure. The latter is much more brittle and manifests a tendency to spontaneous shrinking, which is due to a continuous increase in the surface of contact or possibly union between droplet and droplet. These gels with an open solid framework therefore specially manifest that property of spontaneous shrinkage to which Graham applied the term "synaeresis."'

That, however, watery gelatin-jelly does not show any structure as long as it remains reversible, and also that it becomes irreversible and coarsely spongy by the action of formol and other fixatives, will be pointed out later (p. 133).

As regards the solidification on cooling, gelatin-jelly does not stand isolated, for a great many colloidal solutions may have the colloid separate out either completely or partially on cooling, this separation being equivalent to the colloid assuming a firmer state.

A. 2. *Heat-reversible colloids.*

Heat-reversible gels may also be formed, for Sydney Ringer[1] showed that casein in a slightly alkaline solution forms a solution, which on heating becomes a jelly and on cooling reverts again to the fluid state; but this type of jelly comes under the heading of precipitation, and is discussed on p. 65.

B. *On mechanical conglutination (coagulation).*

The production of mechanical coagula has been very carefully studied by Ramsden[2].

[1] *Journal of Physiology*, 11, 464 (1890).
[2] Ramsden: *Arch. f. Anat. u. Physiol.* (*physiol. Abtheil.*), 517–530 (1894).

Filtered, clear solutions of white of egg and other 'proteids' on being shaken in a test-tube soon form masses in the shape of long strands and flocculi. These mechanical 'coagula' probably differ physically rather than chemically from the original 'proteid,' as in many instances they disappear spontaneously.

The following explanation seems to me to account for the phenomenon :—The molecules of white of egg in a dilute watery solution may be supposed to be evenly distributed and spheroidal in shape. If in sufficient number to touch one another, regular geometrical figures will result, as are seen for example in foams, with this difference, that each space in the foam must be imagined to be filled by one molecule of proteid. Such solutions of 'proteid' appear clear because all the molecules being symmetrically arranged will form a homogeneous mass.

Where the solution is in contact with the air, as on its free surface, the proteid-molecules are subjected along with the water-molecules to the effects of surface tension, and may be supposed to form collectively an elastic proteid membrane. The latter again appears clear because the component molecules are all similarly arranged, forming a homogeneous layer. By shaking, the surface particles are, suddenly, partly released from tension and partly subjected to still greater stress, and being unable to resume at once the original shape, owing to their viscosity, masses of distorted and compressed molecules will be produced. On continuing the process of shaking, the already formed aggregates act as nuclei to which other surface molecules attach themselves, and thus strands and fibres are formed of sufficient size to become visible to the naked eye.

That the aggregated molecules become visible, depends on the fact that light is reflected and refracted from all heterogeneous mixtures, such as the distorted proteid particles floating amongst non-distorted ones. The spontaneous disappearance of the 'coagula' is reversely due to the gradual recovery, by each molecule, of its uniform state of rest [1].

It may be useful to point out that a molecule, and especially a proteid one, must not be imagined as a solid particle like a pebble on the shore, but as a porous structure resembling a sponge and permeated by the solvent or fluid in which it floats. Under certain conditions the solvent may be withdrawn from or be squeezed out of the molecule as shown below.

C. *On coagulation proper by physical means.*

Coagulation according to the definition given above is a process which leads more or less completely to the separation of a colloidal

[1] Starke, in the *Zeitsch. f. Biol.* 40, 419 (1901), explains these 'mechanical coagula' as due to a drying of the proteid, whenever air-bubbles are formed in proteid solutions. To prove his point he fills a bottle completely with proteid solution, drops in pieces of glass rods about one centimetre long, corks the bottle so as to exclude all air, shakes vigorously for some weeks, and obtains no 'coagula.' This experiment, however, excludes those very conditions as to surface tension which Ramsden considers are essential to his results; besides which Ramsden adopted special precautions to prevent the drying of the foam. Starke has not taken the laws of vapour pressure into consideration, otherwise he would not have published his criticism.

substance from its uniform suspension in a solvent; the separation resulting in the formation of a honeycombed, sponge-like structure or an amorphous mass.

C. 1. *The effect of dehydration.*

No allusion will be made here to the actual drying of proteid substances, as in making film preparations or in using Altmann's dehydration method (p. 139), but the throwing down of globulins and albumins from their watery solutions, by means of salts, has to be considered. The idea that salts may act as 'dehydrating agents' and thus lead to the separation of proteids started with Hofmeister[1], as mentioned on p. 30, and has been worked at by several of his pupils, in particular by Kander[2], who introduced the method of fractional precipitation, and Lewith[3]. The results obtained by the latter and his views have already been stated on p. 31. In addition to the substances mentioned there, calcium chloride is also interesting because, owing to its great hygroscopic power, in saturated solutions it will precipitate albumin (Hofmeister).

Precipitation of proteids by neutral salts amounts thus practically to a physical crowding out of the proteid-molecules by the molecules of the neutral salt, as pointed out by Spiro[4].

Cohnheim[5] distinguishes four groups of salts with regard to their coagulating power, namely (1) Sodium:—chloride,—sulphate,—acetate and nitrate, which salt out fibrinogen and casein completely, even if their solutions are not quite saturated. (2) Magnesium sulphate, which allows a ready separation between the globulins and albumins. (3) Potassium acetate, calcium chloride and nitrate, and Schäfer's mixture of sodium and magnesium sulphates, all of which in saturated solutions completely coagulate both globulin and albumin. (4) Ammonium sulphate and zinc sulphate, of which the former was introduced by Méhu[6] and the latter by A. Bömer[7] and E. Zunz[8]. These two reagents coagulate every proteid except peptones.

Why the four groups of salts just mentioned precipitate in the order given, is as yet quite impossible to say, but there is little doubt they act mainly by absorbing all the available water; by dehydrating the proteid-molecules in a manner analogous to the dehydration of blood corpuscles by strong sodium chloride solutions, which latter also change the optical properties of blood-solutions.

On the supposition that proteids are hydrates when in true colloidal solution (p. 45), we must further bear in mind that dehydration will prevent the proteid-molecules from maintaining their electrical charge, and that, for this reason also, they will tend to aggregate.

[1] Franz Hofmeister: 'Zur Lehre v. d. Wirk. d. Salze,' *Arch. f. experiment. Pathol. u. Pharm.* (1886).
[2] Kander: 'Z. Kenntniss d. Eiweissk. d. Blutserums,' *Arch. f. experim. Path. u. Pharm.* 20, 411 (1886).
[3] Lewith: 'Z. Lehre v. d. Wirk. d. Salze,' *ibid.* 24, 1 (1888).
[4] K. Spiro: *Physik. u. physiol. Selektion*, Habilitationsschrift, Strassburg (1897).
[5] Cohnheim: *Chemie der Eiweisskörper*, English translation, Macmillan.
[6] Méhu: *Journ. de Pharm. et de Chim.* (août, 1878).
[7] Bömer: *Zeitsch. f analyt. Chem.* 34, 562 (1895).
[8] Zunz: *Zeitsch. f. physiol. Chem.* 27, 219 (1899).

Whether some of the salts mentioned above do not also act chemically it is difficult to say, for magnesium sulphate, which is neutral to litmus, is acid to phenolphthalein, as has been pointed out by Hardy.

Hofmeister has shown that all salts which precipitate well do not for this very reason penetrate cells easily, and also that they are not readily absorbed from the intestinal canal. This I can confirm from experiments made while investigating their action on tissues. It is absolutely necessary, if we desire to procure good fixation, for example with ammonium sulphate solutions, to inject warm-blooded animals with solutions saturated at 40° C., and made neutral, if necessary, with ammonia.

From my test-tube experiments (p. 103) it will further be seen that methyl and ethyl alcohol and acetone (Experiment 2), glacial acetic acid (Experiment 3), and pure formol under certain conditions (Experiment 4), also throw down albumin as coarse membranous flocculi.

For all these different coagula I believe the same explanation to hold good, namely, that primarily we are dealing with dehydration.

C. 2. *Coagulation due to alterations in the electrical tension between the colloid and its solvent.*

In the chapter on electro-chemistry it was shown that an electrolyte by dissociating into its ions always gives rise to an amount of positive electricity which equals the amount of negative electricity, and that the electrical charges are distributed over ions. It was further shown (p. 10) that each kind of ion travels with its own velocity, the two fastest ions being the acid hydrogen and the alkaline hydroxyl ions.

If now, for example, hydrochloric acid is poured into water, the positive hydrogen ions, travelling much faster than the negative chlorine ions[1], diffuse more quickly into the surrounding water, and, carrying their positive charge with them, will render that part of the water where they arrive more positive than that part which contains the slowly wandering chlorine ions. If one electrode is placed where the hydrogen ions are abundant and another where the chlorine ions are more numerous, an electrical current can readily be demonstrated. The idea that differences of potential are due to differences in the rate of migration of ions was first suggested by Nernst (1888).

Similarly, if we start with a liquid chain composed, for example, of 5 per cent. sodium chloride solutions at the ends and 10 per cent. hydrochloric and 10 per cent. caustic soda in the middle,

5 per cent. NaCl	10 per cent. NaOH	10 per cent. HCl	5 per cent. NaCl

then by the interaction of the alkali and the acid a 5 per cent. salt solution will be formed in the middle:

[1] Hydrogen-ion = 320; chlorine-ion = 65.

A	B	C	D	E
5 per cent. NaCl	10 per cent. NaOH	5 per cent. NaCl	10 per cent. HCl	5 per cent. NaCl

On diluting A and E a current will pass from B to D, that is, from the alkali to the acid, because the hydrogen ions, $H^{\cdot}$, will pass from D into E more quickly than the chlorine ions, Cl', and similarly the hydroxyl ions, OH, enter A more freely than do the slower sodium ions; therefore an excess of negative chlorine ions being left in D and an excess of positive sodium ions in B, the latter becomes electro-positive to D and the current in the solution passes from B to D.

The same result will be obtained by strengthening the salt solution in C, and for the same reason.

On strengthening the salt solutions in A and E, or by diluting the salt solution in C, a current will be set up from the acid to the alkali, from D to B, because the fast hydrogen ions will carry their positive charges from D to C at a quicker rate than the slower hydroxyl ions will carry their charge from B to C. When the hydrogen ions meet the hydroxyl ions, neutral water is formed, but there being, owing to the rate of migration, more hydrogen ions than hydroxyl ions, a positive stream is set up from D to B[1].

Should precipitates be formed which do not act as conductors, such as are obtained on coagulating egg-white, but little interference with the strength of the current is set up.

In the liquid chain represented above by A to E, we have salt solutions at the end, while in the middle are equivalent solutions of alkalies and acids in equal volumes, and of such strength as to form by their union a salt solution of the same concentration as is present in the end-links A and E. Given such a chain, no current is set up, though a chemical union takes place.

If two solutions of an acid varying in their concentration are interpolated between the same salt solution, a current passes from the stronger to the feebler solution, the electro-motive force increasing with the difference in concentration. If the dilute acid is replaced by water, the current passes from the acid to the water, and with the dilution of the acid diminishes in electro-motive force. Here again the current is caused by the hydrogen-ions migrating rapidly from a place of greater to that of lesser osmotic pressure, although the hydrogen-ion is not subjected to a greater pressure than is its fellow ion.

The greatest difference of potential will be set up in a solution to which an electrolyte is added, the radicals of which differ greatly in their rate of migration, as for example in the case of hydrochloric acid [$H^{\cdot}=320$ and $Cl'=65$], while no differences of potential can be obtained, for example, from potassium chloride [$K^{\cdot}=65$ and $Cl'=65$]. The facts just stated will allow us to understand, if colloids owe their existence to carrying definite charges, how such charges will be influenced by electrolytes; how in some places the

[1] Worm-Müller: *Poggendorff's Ann.* 140, 114 (1870). See also Gustav Wiedemann: *Die Lehre v. d. Elektricität*, (1893-8), and Max Oker Blom: *Pflüger's Arch.* 84, 191 (1901).

colloidal charge will be diminished while in others it will be increased, or, what comes to the same, how in the former case bigger aggregates are formed, while in the latter instance the original aggregates will become smaller or even pass into solution (see p. 46).

Thus by the diffusion of electrolytes alone, the original state of the colloid becomes greatly altered, and if, in addition, new insoluble compounds are formed between the radicals of the electrolyte and the colloid, then the temporary structural changes due to a disturbance of the electrical equilibrium may become 'fixed.'

D. *Chemico-physical factors causing 'precipitation.'*

For descriptive purposes, the hydrogen and hydroxyl compounds of colloids are discussed by themselves, but it must be remembered that they differ from other compounds only in degree and not in kind.

D. 1. *Precipitation of the colloid due to the withdrawal of the hydrogen or hydroxyl radical or due to 'neutralization.'*

Pure albumins and globulins Starke[1] regards as acid or alkali albumins or globulins. They are insoluble in water and neutral salt solutions, but are soluble in very dilute acids or alkalies, because they unite with the $H^{\cdot}$ or the OH'-ions to form new compounds, as can be demonstrated by using tropaeolin as an indicator.

Alkali and acid albumins and globulins occur naturally; thus lentils contain an acid globulin, while an alkali globulin is found in blood, and these are kept in solution because they occur along with either the acid $H^{\cdot}$ or the alkaline OH'-ions. The relationship between the colloid and the acid $H^{\cdot}$ or the alkaline OH' has been expressed as follows:

(1) The acid or alkali and the proteid form a salt which is capable of dissociation, and therefore of remaining in solution (Starke).

(2) The proteid is partly in solution and partly in the colloidal state. The colloid as a whole is kept in suspension, because the acid or alkali which was added, establishes a difference of potential between the solid and the fluid phases of the colloid, giving rise to a double electric layer round each solid particle (Hardy).

(3) A neutral pseudo-basic proteid, by the addition of the acid $H^{\cdot}$, is converted into a real base, and the pseudo-acid colloid, by the basic OH' radical, is changed into a real acid. This possibility is based on the work of Cohnheim and Krieger (p. 27).

(4) The proteid forms, with the $H^{\cdot}$, a kat-ion, or with the hydroxyl ion, an an-ion; the an-ion in the former case is the an-ion of the acid which was added, while in the second case the kat-ion is the kat-ion of the alkali added (see p. 48).

Whatever view we adopt, if we are dealing with a (colloid + H)$^{\cdot}$, then, by the addition of the OH' group, neutral water is formed, $H^{\cdot} + OH' = H_2O$. The H on separating from the colloid takes on the positive charge and becomes $H^{\cdot}$, while the colloid radical loses its charge and aggregates into larger masses, and maybe becomes precipitated (p. 46).

[1] Starke: *Zeitsch. f. Biol.* 42, 187 (1901).

The precipitation in this case depends therefore on the chemical union between the hydroxyl- and the hydrogen-ions producing electrically neutral water, and thereby diminishing or completely destroying the electrical charges on the colloid. Another method of rendering the hydrogen-ion inert is to convert it into the non-ionic state by removal of the an-ion to which it was linked, as in the following case: Given faintly acid solutions—for example, an acid-albumin—coagulation may be induced by the addition of neutral salts of the alkalies or of alkaline earths, because this addition drives out the carbon dioxide contained in the water and thereby renders the solution less acid. The less acid the albumin solution is, to begin with, the less neutral salt will be required for complete coagulation.

Alkali-albumins in an alkaline solution are coagulated by the addition of CO_2, or other dilute acids; by small quantities of alkaline earths, probably because of the formation of insoluble hydrates; further, by dilution with water or dialysis against pure water; and lastly, by saturated solutions of sodium chloride or magnesium sulphate. All these reactions, except the last (see pp. 30 and 31), depend directly on the alkalinity or the acidity of the proteid compound being interfered with. When Starke states that the acidity of a dilute acid is diminished by a neutral salt, it must be remembered that this only holds good if the acid and the salt have a common ion. Starke also observed if the same amount of alkali is added to two test-tubes, one of which contains pure distilled water while the other contains an equal amount of pure sodium chloride solution, made up with the same distilled water, that in the latter case litmus paper is turned much bluer. The explanation he offered was that alkalies in the presence of neutral salts dissociate to a greater extent, and therefore give a more pronounced alkaline reaction. This view cannot be upheld, because by the addition of neutral salts the CO_2 normally present in water is discharged, and therefore the alkali which is added will not be partly bound by the acid, and in consequence will produce a stronger effect on the litmus. I arrived at this conclusion by adding neutral litmus solution to distilled water, and then boiling it to get rid of the CO_2. My conclusions are borne out by the criticism of Starke's paper in Wolff and Smits' article [1].

D. 2. *Precipitation due to a removal of salts.*

Proteids, which require the presence of neutral salts to remain in solution, are widely distributed amongst both plants and animals, and are represented by the globulins proper and the closely allied muscle-proteids (paramyosinogen and myosinogen). These substances are precipitated from their solutions either if the salts normally present are removed by dialysis, or if the concentration of the inorganic salts is greatly diminished by the addition of water. Various causes have been assigned for this precipitation.

W. Pauli [2] has studied the effect both of non-electrolytes and of

[1] K. Wolff and A. Smits: *Zeitsch. f. Biol.* 41, 437 (1901).

[2] Pauli: *Pflüger's Arch.* 78, 315 (1899)

electrolytes on globulin solutions. He found pure water containing grape-sugar in quantities varying from mere traces up to 3·25 normal (= 68·5), or containing pure urea up to three or four times normal strength, always precipitated globulin as if neither sugar nor urea had been present, and the globulin which separated out in these solutions could always be readily dissolved by the addition of neutral salts. He drew the conclusion that globulin requires for its solution the presence of a neutral salt which has dissociated into its ions, and that the non-dissociated molecules of a salt play no part. The ion-action depends on the number of ions present and on the quantity of the globulin, for only that amount of globulin passes into solution for which sufficient salt has been added, and the subsequent clearing of the globulin solution is proportional to the amount of salt added.

The fact that free ions render globulin soluble, leads Pauli to suppose that the negative and positive ions unite with the proteid-molecules in a loose, chemical manner, the kat-ions attaching themselves to certain radicals in the proteid while the an-ions join on to other groups. This supposition is supported by the following analogous case, which was pointed out to Pauli by Hofmeister :—Amido-acids unite simultaneously with both alcohol and hydrochloric acid to form beautifully crystalline, very permanent, water-soluble compounds, for example $HCl{\cdot}NH_2{\cdot}COO(C_2H_5)$. After removing the HCl of this body with Ag_2O, the remaining water soon decomposes by giving off the alcoholic radical.

On this principle globulin and sodium chloride would form the compound (HCl)—globulin—(NaOH)—H_2O, or more probably according to the formula, globulin + NaCl = (Cl—globulin—Na). In support of this last view Pauli refers to the investigations of Spiro and Pemsel, who showed that proteids can bind both acids and bases.

Cohnheim, however, has rightly pointed out that Pauli's view does not account for the precipitation of the globulin which results from diluting its solution, as in this case no ions are removed, and he suggests that globulins are rendered insoluble owing to their strong hydrolytic dissociation. This latter we may assume to be induced on the same principle as the hydrolytic decomposition of many salts of the heavy metals which form clear solutions as long as they are concentrated, but which on dilution at once undergo hydrolysis, forming insoluble hydrates.

Starke[1], in 1900, found that a substance giving all the characteristic reactions of ordinary globulins may be obtained by diluting egg-white with ten times its bulk of water and then dialysing the solution at a temperature of 75–85° C. There is formed by this process a substance which is quite insoluble in pure water, and also in neutral salt solutions, but which, when treated with very dilute alkalies, becomes soluble. Starke found, after adding the same amount of alkali to two identical quantities of globulin, that more globulin passes into solution if neutral salts are present, and explains his results on the assumption that alkalies undergo greater dissociation in the presence of neutral salts,

[1] J. Starke : *Zeitsch. f. Biol.* 40, 419, 494.

and that for this reason they produce a greater effect. As shown on p. 56, Starke's explanation is impossible; but, apart from the fact that neutral salts do drive out carbon dioxide from solutions, we must remember Pauli's work (see p. 62) and Hardy's observation (pp. 39 and 40), and therefore arrive at this conclusion: Globulins pass into solution only in the presence of free hydrogen- or free hydroxyl-ions,—and neutral electrolytes, if electrically dissociated, greatly augment the power of the $H^{\cdot}$ and OH' ions.

D. 3. *Precipitation due to the formation of insoluble salts.*

Proteids with the salts of heavy metals form insoluble compounds, provided that the metallic salt forms an acid solution owing to hydrolytic dissociation, as in the case of corrosive sublimate, $HgCl_2$ ($= HgOH + 2HCl$: see p. 21)—or if the salt of a metal be mixed with an acid, as is done in the case of ferrocyanide of potash and acetic acid (= ferrocyanic acid). Even those salts which give rise to an acid solution act better if some free acid be added, for the reason that normal proteids contain basic diamido-acids (see p. 281).

The formation of salts, between the heavy metals (such as mercury or iron) and the proteid, depends on the replacement of the normal hydrogen of the proteid by metals having greater electro-affinities (see p. 14), and the heavy metals in their turn are replaced by the metals of the alkalies which have still stronger electro-affinities. According to this view it is easy to interpret Experiment 8 on pp. 109–112: Sodium chloride dissolves an albuminate of mercury because a soluble albuminate of sodium and non-dissociated corrosive sublimate are formed. As already mentioned on p. 8, undissociated or electrically neutral sublimate molecules do not precipitate proteid.

Attention is specially drawn to the paper by F. Rose[1], in 1833, who was the first to show that only the metallic radical of salts, such as sublimate or copper sulphate, enters the proteid-molecule, while the acid radicals (Cl or SO_4) do not. He also points out that haemoglobin prepared by Berzelius' method is precipitated by concentrated sublimate, but that, on diluting the mixture, it passes into solution. It is, however, again precipitated by increasing the amount of sublimate.

The chlorides of gold, platinum, palladium, iridium, &c., are not true chlorides, but are hydrogen salts, as explained on p. 78.

D. 4. *Heat-coagulation.*

Natural albumins are always changed chemically by heat action, a fact which must be kept in mind in micro-chemical research[2]. They become more basic; thus distinctly acid solutions become less acid and neutral solutions turn alkaline. On the other hand solutions of muscle-proteids, which are essentially globulins, are rendered acid by heat-coagulation, as was first shown for myosinogen and para-

[1] F. Rose: *Poggendorff's Ann.* 28, 132 (1833).
[2] Cohnheim: *The Chemistry of Proteids*, English translation, Macmillan.

myosinogen by Halliburton, and as has been fully confirmed by G. N. Stewart[1].

That heat-coagulation is not accompanied primarily by any great chemical alteration seems to be shown by the work of Corin and Ansiaux[2], who found that the first traces of coagulation disappeared on quickly cooling and shaking the solution.

Aronstein[3], in 1874, described how albumin freed from inorganic salts by prolonged dialysis becomes uncoagulable by heat, but that the addition of salts restores the power of coagulation. We do not know, as yet, the precise nature of that chemical change which heat induces in proteids, provided salts are present.

Cohnheim believes that even in neutral solutions traces of acid may be present, and he assumes the increase in alkalinity after heating to be due to this acid uniting with a part of the albumin to form acid albumin, and becoming for this reason undetectable by litmus.

F. Michailow[4] originated the conception, that heat-coagulation is due to a dehydration of the proteid molecule and analogous to the change which occurs when inorganic substances give off their water of crystallization. This conception has been adopted by Starke[5] on the following grounds: Globulin is precipitated by diluting its solution; the precipitate, if thoroughly washed in water to remove all traces of salts and alkalies, is rendered insoluble in neutral salt solutions, while it is still soluble in dilute acids or alkalies. Such globulin, suspended in neutral salt solutions and boiled, is changed by heat into a substance which no longer dissolves on the addition of dilute acids and alkalies because it has become coagulated. Taking the same amount of globulin in each case, Starke found that it was completely coagulated by

0·5 grm. mol.	$MgSO_4$	0·0153 grm. mol.	$CaCl_2$
0·2 ,,	NaCl	0·015 ,,	$MgCl_2$
0·16 ,,	KCl		

These salts, arranged according to their coagulative power, may be divided into three groups, namely

1. $MgSO_4$, 2. NaCl, KCl, 3. $CaCl_2$, $MgCl_2$,

and this order represents also the water-absorbing power of these salts (de Vries).

Therefore, according to Starke, those salts coagulate best which have the greatest affinity for water, and coagulation amounts to a loss of the water of crystallization or water of constitution. Chemical union between the metal and the proteid he excludes because $MgSO_4$ and $MgCl_2$ stand at the two ends of the series, but he has not taken the an-ion radicals into account (see later, p. 66).

Schadee van der Does[6] has described how solutions of egg and serum albumin may be rendered uncoagulable, by being shaken up with freshly prepared metallic silver, or freshly prepared or not too

[1] G. N. Stewart: *Journ. of Physiol.* 24, 450 (1899).
[2] Corin and Ansiaux: *Bull. de l'Acad. Roy. de Belg.* No. 21.
[3] B. Aronstein: *Pflüger's Arch.* 8, 75 (1874).
[4] Michailow: *Chemisch. Centralbl.* (1887), 1088.
[5] Johannes Starke: *Zeitsch. f. Biol.*, Jubelband z. Ehren v. C. Voit, 206 (1901).
[6] Schadee van der Does: *Zeitsch. f. physiol. Chem.* 24, 351 (1897).

old silver oxide[1]. Silver chloride and silver sulphide do not act in this way. It is suggested by him that the silver may possibly replace the sulphur of the proteid-molecule. Another explanation is offered on p. 67.

Much has been written as to the value of recognizing different albumins by their specific temperatures of coagulation, but, as everything depends on the reaction and the amount of salts present, this method cannot be used for the histological determination of different albumins, although definite histological changes can be made out on gradually warming epithelial cells and muscle-fibres[2].

W. Pauli (Pascheles)[3] has made a careful study of those changes in the coagulation-temperature of globulin which are produced by mixing neutral salts with solutions of egg-white.

The egg-white was whipped and filtered, and the clear filtrate, stored in a dialyser surrounded by cold water, was kept exposed to the vapour of chloroform. Before use this stock solution was diluted 5 to 10 times with distilled water. The small amount of salts contained in such a solution is negligible because large amounts of salts have to be added to produce precipitation.

The salts experimented with were rendered in every case carefully neutral, and were generally added to 2 cc. of the stock proteid-solution in such quantity as to make the total amount of fluid equal 10 cc. All salts were further used in equi-molecular gram-weight solutions (1 : 1000 cc. of water), that is, in 'normal' strengths; plurivalent acids or bases were employed in equivalent solutions, and the water of crystallization was taken into account.

The occurrence and degree of precipitation were gauged by a printed paper just becoming invisible when placed behind the test-tube.

Pauli finds the time of heat-coagulation to be shortened by chlorides and acetates, while it is lengthened by bromides and nitrates. This phenomenon is quite distinct from the raising or lowering of the temperature of coagulation by a salt. Those salts which delay coagulation produce a faint or deep sky-blue colour, owing to the reflection of rays having short wave-lengths, while salts which hasten coagulation produce a yellowish-grey colour.

The higher the percentage of the proteid, the lower is the temperature of coagulation.

In no case does an increase in the amount of a salt produce a proportional rise of the coagulation-temperature, and with some salts, such as lithium and sodium chlorides, after a certain maximum effect has been produced, the coagulation-temperature is lowered on still further increasing the amount of these salts.

[1] To obtain metallic silver in a finely precipitated form, suspend silver chloride in dilute sulphuric acid; reduce it by a zinc rod; treat the reduced silver first with boiling dilute hydrochloric acid, then with a warmed solution of ammonia, or as Van der Does calls it, ozonize it, and finally wash thoroughly with water. To prevent oxidation by the air keep the silver under water. Silver oxide is most readily obtained by precipitating a silver nitrate solution with pure soda or potash.

[2] Details will be published later.

[3] Pauli (Pascheles): *Pflüger's Arch.* 78, 315 (1899).

Chlorides of ammonium, potassium, sodium, lithium, barium, and magnesium show, in the order given, a gradually increasing power of raising the temperature of coagulation or, what amounts to the same, of inhibiting the tendency to coagulate. Therefore the monovalent metals have the least effect, while the divalent metals are the most powerful. No relation exists between the molecular weight and the inhibitory power of Na, Li, Ba, and Mg. Lithium, although an alkali, resembles in its action the metals of the alkaline earths, with which it also shares the property of forming hydroxides and carbonates which are not readily soluble.

Bromides do not coagulate proteids by themselves. As amongst the chlorides, so here the coagulation-temperature depends on the basic ion (Pauli), but the dibasic magnesium acts like the monobasic alkali-metals.

Iodides, if pure, do not precipitate proteids (Hofmeister). Even in low concentrations they increase the coagulation-temperature considerably; they soon produce a maximal effect which then is diminished on further concentrating the salt.

Nitrates: only sodium nitrate in 5·5 normal strength coagulates globulin. The potassium, sodium, and ammonium salts are most efficient in raising the temperature of coagulation, while the magnesium and lithium salts form a special group, analogous to the chlorides.

Thiocyanates (rhodanates): the potash salt is like potassium iodide in its action.

Acetates resemble the chlorides; again the ammonia compound has the least, the magnesium compound the greatest, and the potassium and sodium salts intermediate inhibitory powers. Therefore in this case also any direct relation between coagulation and precipitation may be excluded, as in the case of the chlorides (Pauli).

Sulphates resemble the chlorides and acetates as regards the order in which the metals act. Even when used in concentrations approaching those apt to induce salting out, no marked effect as regards lowering of the coagulation point can be detected.

From the fact that globulins and albumins are coagulated by heat, while the albumoses derived from them are not, Pauli draws this conclusion: the albumin molecule owes its coagulability either to the fact that it contains several albumose molecules, or to these latter being linked together in the albumin molecule in some special way. Before dealing with the question how the neutral salts exert their influence, the author shows that changes in the coagulation-temperature are not dependent on the number of salt molecules present, because different salts when present in equi-molecular numbers do not produce equal changes in the coagulation-temperature. Thus the alkali salts of monobasic acids resemble one another as regards osmotic pressure, because they dissociate in equi-molecular solutions to the same extent; they also lower the freezing-point of water to the same degree, and yet they differ greatly in their inhibiting power as regards coagulation-temperature. For example, the same salt in two different concentrations (osmotic pressures) will show the identical

results on being heated with solutions of egg-white; thus 1 normal resembles 3·5 normal NaCl, and 1·5 normal equals 5 normal LiCl in its effect. Further, different salts, in the same molecular numbers, produce coagulation at quite different temperatures.

Therefore it is essential not only to study the effect from the molecular point of view, but also to take into consideration the amount of dissociation into ions which the salts undergo; to investigate in short the part played by the basic and acid radicals of salts. For this purpose Pauli gives the two following tables.

In Table A the basic radicals (kat-ions) are arranged according to their efficiency, the least effective being always mentioned first and the most effective last; for example, ammonium chloride has the least inhibitory power as regards coagulation, while magnesium chloride has the greatest.

TABLE A.

Chlorides.	*Bromides.*	*Iodides.*	*Nitrates.*	*Acetates.*	*Sulphates.*	*Chromates.*
NH_4	NH_4	—	Mg	NH_4	NH_4	—
K	K	Na	(Li)	K	K	K
Na	(Mg)	K	—	Na	Na	Mg.
(Li)	Na	NH_4 (?)	NH_4	—	—	NH_4 (?)
Ba		—	K	Ba	—	—
Mg		—	Na	Mg	Mg	—

In Table B the acids are arranged on the same principle as the bases are in Table A.

TABLE B.

K	*Na*	*NH_4*	*Mg*	*Ba*
chloride	chloride	chloride	—	—
acetate	acetate	acetate	—	—
sulphate	sulphate	sulphate	sulphate	—
oxalate	chlorate	—	acetate	acetate
chromate	nitrate	nitrate	nitrate	—
bromide	bromide	bromide	bromide	—
rhodanate	—	—	—	—
iodide	iodide	iodide	chromate	—
nitrate	—	—	chloride	chloride

These two tables show that salt solutions possess 'additive' (Ostwald) properties, which means that the effect of salt solutions depends on the sum of the actions of their kat-ions and their an-ions, for in Table A in most salts the kat-ions follow in this order: NH_4, K, Na, Ba, Mg. The curious position of the NH_4 in the nitrates and chromates depends on the ease with which these salts decompose and thereby set free the NH_4. Lithium occupies a peculiar position for reasons already mentioned (p. 61), and the divalent magnesium and barium resemble one another in their action, while the regular arrangement of the alkali metals proves that the part played by them in altering the coagulation-temperature is in no way affected by the acid radical to which they may be joined.

'As regards the alkali salts it may further be said that the action of the acid radical (the an-ion) is the predominating one,' for the changes in the coagulation-temperature produced by salts with a common base (kat-ion) move within wider limits than do those dependent on salts with a common acid (an-ion).

Table B shows an arrangement analogous to that in A, for the acids (except in the Mg and Ba group) are almost invariably arranged in the same order, proving that the changes induced in the coagulation-temperature are independent, within wide limits, of the basic radicals.

Special experiments were further made by Pauli to determine the mutual interaction of similar ions.

If two binary electrolytes be taken which do not possess a common ion there will be formed in a solution eight different 'molecules,' namely, four ions and four electrically neutral molecules. As the control of such solutions is exceedingly difficult, Pauli has used two salts having a common ion. Under these conditions, generally speaking, the amount of dissociation of the two salts is diminished, but on mixing equivalent solutions of two salts having the same dissociation-constant there results a mixture in which the two salts are not only equally strongly dissociated, but in which also each salt is ionized to the same extent as if the other salt were not present at all. The salts of the alkalies (and ammonium) with monobasic acids are good examples in point.

In the following Table C the concentration of the salts is expressed in fractions of a 'normal' solution. Both the horizontal and the vertical rows form definite groups in which one salt is constant while the other is changing in concentration. The diagonal line expresses the point of coagulation for equivalent concentrations of both salts.

TABLE C.

$NaCl + NaNO_3$.

→ $NaNO_3$	0·0	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	4·5	5·0
↓ NaCl											
0·0	—	69·8	71·9	72·6	72·8	71·8	70·5	69·2	67·7	—	66·4
0·5	61·8	**62·6**	66·5	67·9	68·2	68·4	66·8	65·6	63·8	62·8	61·5
1·0	62·6	62·5	**62·9**	63·5	64·4	64·6	63·8	63·0	61·6	60·8	Precip.
1 5	63·4	62·2	62·2	**62·2**	62·0	61·4	60·8	60.6	Precip.	—	—
2·0	64·2	62·1	61·5	61·1	**61·0**	60·4	59·6	Precip.	—	—	—
2·5	64·5	61·9	61.2	60·5	59·8	Precip.	—	—	—	—	—
3·0	63·6	—	—	—	—	—	—	—	—	—	—
3·5	62·4	—	—	—	—	—	—	—	—	—	—
4	60·0(?) Precip-itate	—	—	—	—	—	—	—	—	—	—

This table brings out the following points:

1. An increase in the amount of $NaNO_3$ up to 2·0 raises the coagulation-temperature to 72·8° C.

2. An increase in the amount of NaCl up to 2·5 raises the temperature to 64·5° C.

3. On gradually adding sodium chloride to a sodium nitrate solution containing 2·0 $NaNO_3$, in such quantities as to give a concentration of 2·5 NaCl, the coagulation-temperature is lowered to 59·8°, but already at a time when the concentration of the NaCl amounts only to 1·0, the effect of the sodium nitrate on the egg-white is practically completely counteracted, as the coagulation-temperature of the 2·5 $NaNO_3$ is lowered from 71·8° to 64·6°, that of 1·0 NaCl being 62·6°.

4. Sodium chloride solutions within the strengths of 1 to 1·25 normal are in no way affected by the addition of $NaNO_3$ in strengths of 0·5 to 3·5 normal.

5. Sodium nitrate solutions in concentrations less than 0·5 normal are in no way affected by an addition of NaCl differing in concentrations from 0·5 to 2·5 normal.

There exist, then, two points of stable interaction in the case of NaCl and $NaNO_3$, one for the sodium nitrate at about 0·47 normal, and the other for sodium chloride at 1·5 normal concentrations.

6. The curve for equivalent concentrations of both salts reaches its maximum when the concentration for each salt = 1·0 normal, and then rapidly falls beneath the sodium chloride curve (see the diagonal line printed in heavy figures in Table C, p. 63).

On investigating the interaction of ammonium bromide and ammonium chloride, Pauli found a specially stable zone for the bromide to correspond to a concentration of 0·75 NH_4Br, in which no change is produced by the addition of NH_4Cl varying between 0·0 to 2·0 normal.

A third example is $MgCl_2$ + NaCl, in which the two kat-ions differ. The fact of magnesium chloride dissociating much more than sodium chloride only allows general conclusions to be drawn, but magnesium chloride in concentrations of 0·5 to 0·6 strengths seems to possess special affinities for the proteid-molecule, as in these concentrations its effect on the coagulation-temperature is altered only to the extent of 0·5° C. by the addition of from 0·0 to 2·5 normal NaCl. The sodium chloride curve is lowered by the addition of $MgCl_2$ up to 3·0 normal, and a stationary interaction between NaCl (1·5 to 1·75 normal) and $MgCl_2$ (0·5 normal) seems to exist, as the corresponding coagulation-temperatures, namely, 62 and 62·2°, are not markedly influenced by raising the concentration of $MgCl_2$ to 1·5.

Pauli sums up his results thus: The change in the coagulation-temperature of proteids depends on the added effects of the two independent ion-actions, each kind of ion possessing for nearly every salt its zone of maximal action. In the case of a single salt its action on the proteid may be so pronounced within a certain zone that the addition of other salts produces no further effect. Thus NaCl within certain limits is practically not affected by the addition of any quantity of $NaNO_3$. If two different acid or metallic ions are present it is impossible to predict the result on the coagulation-temperature, as much depends on the nature of the ions; but the result generally depends on the effect of one salt predominating within certain concentrations, and then the other salt, if present up

to a certain minimum, may be increased five or six fold. Sodium chloride and sodium nitrate show two such phases, one for either salt, while the mixtures of NH_4Br and NH_4Cl or $MgCl_2$ and NaCl show only one phase.

That albumin coagulates while albumoses do not, Pauli ascribes to the presence of several albumose radicals in each albumin molecule, or to a special kind of union between the albumose groups in the albumin; and he believes the ions of a salt during heat-coagulation to attach themselves, more or less firmly, to different parts of the albumose radicals.

Heat-reversible gels (referred to on p. 50).

This jelly formation does not depend on purely physical causes, but is due to hydrolysis, as shown by W. A. Osborne [1], who finds that caseinogen is an acid substance forming two distinct groups of salts.

The first group is represented by the salts of calcium, magnesium, barium, and strontium, further by the salts of strong organic bases such as caffein and strychnine. All these are markedly opalescent in solution; they are unable to pass through the pores of a clay filter, and are precipitated from their solutions by the addition of insoluble finely divided substances. 'On warming their solutions, a remarkable turbidity occurs at a temperature between 35° and 45° C.; this turbidity disappears on cooling.' On prolonged heating in an open vessel they form a film or 'skin' on the surface, and they are also acted upon by the rennin ferment.

The second group contains the salts of potassium, sodium, and ammonium, all of which form comparatively clear solutions; pass through clay filters; are not precipitable by addition of finely divided substances; are not visibly altered on heating; do not form a skin, and are not acted upon by rennin. They are thus in every particular opposite to the salts belonging to the calcium group.

Osborne offers the following explanation for the increased turbidity which occurs on heating the calcium group of salts: 'Calcium caseinogenate undergoes hydrolysis on heating according to the formula

$$\text{Calcium caseinogenate} + 2H_2O \rightleftarrows Ca(OH)_2 + \text{caseinogen},$$

after a preliminary dissociation of certain calcium caseinogenate molecules:

$$\text{Calcium caseinogenate} \rightleftarrows (\overset{+}{\text{calcium-ion}}) + (\overset{-}{\text{caseinogen ion}})$$

and further a dissociation of a certain number of molecules of water:'

$$H_2O \rightleftarrows (\overset{+}{H}) + (\overset{-}{OH})$$

'By the interaction of the calcium and caseinogen ions with the OH and H-ions of the water, a state of equilibrium is reached in which for each temperature and concentration a fixed number of calcium hydrate and caseinogen molecules will be found. As caseinogen is insoluble hydrolysis will produce turbidity, the acid falling out of solution in excessively fine particles, not visible, however, under the microscope.' That hydrolytic dissociation of colloids is possible, is borne out by Krafft and Stern's observations on palmitic acid [2], for

[1] Osborne: *Journ. of Physiol.* 27, 398 (1901).

[2] Krafft and Stern: *Ber. d. deutsch. chem. Gesellsch.* 27, 1747 (1894).

'palmitic acid will separate out in the form of globules from a boiling solution of sodium palmitate.' Cohnheim's and Krieger's observations on proteids acting both as bases and acids (see p. 27) are also adduced in support of the hydrolytic theory, which seems to be fully proved by the following experiments of Osborne:

1. A 1·25 per cent. solution of calcium caseinogenate was divided into two equal portions; the vessel containing one portion was placed in a thermostat at 40° C., the remaining half of the solution was first warmed to 40° C., then cooled and allowed to remain at room-temperature, 17° C. Both were filtered by Pukall filters connected with the same suction-pump for an equal interval of time. To 50 cc. of each filtrate a few drops of alcoholic phenolphthalein were added —when the filtrate of the calcium caseinogenate solution, made at 17° C., was if anything very faintly acid and required a few drops of $\frac{N}{40}$ NaOH to develop a red tinge, while the filtrate, made at 40° C. and allowed to cool, gave immediately a rich red colour, which disappeared almost completely on adding 1 cc. of $\frac{N}{40}$ H_2SO_4. This proves that at 40° C. the insoluble caseinogen is separated out and that it remains in the filter, while the filtrate obtained at this temperature contains the basic calcium hydrate, $Ca(OH)_2$, which is formed owing to the hydrolysis described above.

2. A salt capable of undergoing hydrolysis does so to a greater extent in a dilute, than in a more concentrated solution, as explained in the chapter on physical chemistry. Söldner[1] having observed that the absolute acidity of milk is decreased by dilution, Osborne has repeated the experiment, using, however, for the titration a Pukall filtrate made with undiluted and diluted milk[2], and has confirmed Söldner's observation.

3. 'If the opalescence at room-temperature and the turbidity at 40° C. be due to dissociation, then they ought to disappear on adding some of the basic hydrate. This is exactly what happens, for the addition of a few drops of lime water to a calcium caseinogenate solution at 40° C. causes the turbidity to disappear in great part.'

4. The electrical conductivity at the temperature when turbidity begins to appear, that is when hydrolysis occurs, shows a small yet distinct rise.

My views on heat-coagulation.

In the light of the facts stated under the heading of heat-coagulation I have arrived at the following conception: Complete coagulation of any proteid takes place only in the presence of electrolytes and if the fluid is slightly acid, that is in the presence of hydrogen ions. Therefore it is necessary to consider under what conditions hydrogen ions can be formed on heating a solution of proteids in the presence of salt.

Even if we grant the view of Starke that different salts have

[1] Söldner: *Die Salze d. Milch*, Inaug. Dissert., Erlangen, 52 (1888).

[2] 'Extraordinary care must of course be taken in the cleaning of these filters.' Osborne.

different dehydrating powers and that they do coagulate in proportion to their water-absorbing power, yet it does not follow that they therefore must coagulate because of this power. Pauli's results, published before Starke's article, make the view of the latter quite untenable, besides which Starke has only taken the kat-ion of the salts into consideration. For the series of salts given on p. 59, Starke showed that the kat-ion radical Mg is not the determining factor, and naturally the question arises, what part do the an-ions play? The feebly coagulating $MgSO_4$ contains the comparatively feeble SO_4 an-ion, while $MgCl_2$ contains the two powerful Cl an-ions, and I believe the latter to be capable of exerting on the proteid-molecule a greater effect than the SO_4 an-ion, especially as they are linked to the feeble kat-ion Mg. Further, as albumin will coagulate even in the presence of such salts as sodium chloride, in which both the kat-ion and the an-ion are strong, it would appear that the presence of an electrolyte, in this case also, serves to induce in the proteid-molecule an intra-molecular change by which hydrogen ions are formed. The possibility of such a change must be admitted on the strength of the work of Spiro and Pemsell, Cohnheim and Krieger, for as to the conversion of pseudo-acids into real acids there can be no doubt.

Any factor which tends to prevent the formation of the hydrogen ions will also prevent coagulation. From this point of view Schadee van der Does' observation (p. 59) can be understood. As silver in the metallic state, especially in fine subdivision, seems to be slightly oxidized under water [1], we may consider its action under the same heading as that of silver oxide Ag_2O. This compound, which is soluble to the extent of 1:3000 parts of water, imparts to the latter an alkaline reaction, which means that silver oxide by uniting with water forms a hydrate which then undergoes hydrolysis, liberating the alkaline hydroxyl ion. We already know that hydroxyl and hydrogen ions unite to form electrically neutral water, and that proteids do not coagulate in an alkaline medium.

By regarding proteids as hydrogen salts (see also p. 58) we may assume that the hydroxyl radical of alkalies prevents coagulation, either by preventing the intra-molecular change which occurs normally when heating neutral or acid proteid solutions, or, if the intra-molecular change does take place, by neutralizing the acid hydrogen-ion which is liberated. At one time I believed that Ag_2O acted by withdrawing from the proteid those hydrogen atoms which on heating seem to be converted into hydrogen ions, a view which for Ag_2O is superfluous, as its watery solution is alkaline; but I still believe a similar explanation to hold good for osmium tetroxide. Bethe and Monckeberg [2] have pointed out that a mixture of equal parts of 2 per cent. OsO_4 and egg-white does not coagulate on heating, and I believe this phenomenon can be accounted for by assuming the hydrogen of the proteid to have a tendency to unite with OsO_4 to form $OsOH_4$.

If the pseudo-basic radical of the proteid is converted into a real acid, it will change the pseudo-basic radical into a real base, which

[1] Skey: *Chem. News*, 35.

[2] See p. 69.

latter by splitting off ammonia could produce the alkaline reaction referred to above on p. 58.

E. *The formation of additive compounds between colloids and non-electrolytes.*

The two most important substances which form additive compounds are osmium tetroxide and formaldehyde. Of these the former is a strong oxidizing and the latter a strong reducing agent.

The effectiveness of an oxidizing solution depends on its concentration and on the ease with which oxygen atoms are separated from it.

How the concentration of reducing and oxidizing substances influences the ultimate result is readily seen in iron compounds. The tendency, for example, of a ferro-ion to become a ferri-ion increases with the number of ferro-ions present and with the absence of ferri-ions.

$Fe^{\cdot\cdot} + Ag^{\cdot} = Fe^{\cdot\cdot\cdot} + Ag$ provided much ferro-ion is present.

$Fe^{\cdot\cdot\cdot} + Ag^{\cdot} = Fe^{\cdot\cdot} + Ag$ provided much ferri-ion is present.

This inverse reaction is also well shown thus: a little iodine in much ferro-salt $= I_2 + 2Fe^{\cdot\cdot} = 2Fe^{\cdot\cdot\cdot} + 2I'$, while few iodine-ions with a large amount of ferri-ion $= 2Fe^{\cdot\cdot\cdot} + 2I' = 2Fe^{\cdot\cdot} + I_2$ [1].

On p. 13 it was pointed out that certain radicals are capable of holding different or multiple charges of electricity; thus the an-ion MnO_4 is divalent in potassium manganate, K_2MnO_4, while monovalent in potassium permanganate, $KMnO_4$. Similarly the tetravalent osmium tetroxide, by giving off two oxygen-atoms, loses two positive loads and becomes reduced to the divalent osmium dioxide; and the divalent ferro-ion $Fe^{\cdot\cdot}$ on becoming converted into the trivalent ferri-ion $Fe^{\cdot\cdot\cdot}$ has transferred to it the positive load of another positive ion. To put it shortly, oxidation of ions means an increase in positive ionic tension (or diminution of negative ionic tension), while reduction means diminution of positive ionic tension (or increase of negative ionic tension).

That oxidation is possible by means of the oxygen in the air is a well-known fact, and is explainable by assuming that the atmospheric oxygen acting on the water forms the negative hydroxyl ion OH, which must be formed to allow the divalent $Fe^{\cdot\cdot}$ ion to assume an extra positive load and thereby to become the ferri$^{\cdot\cdot\cdot}$ ion. The free hydroxyl groups which are required for the conversion of a kat-ion into one of higher valency by means of oxygen, render the fluid in which the kat-ion is dissolved of necessity alkaline; while reversely a fluid must become acid, in which reduction has been brought about through hydrogen ions. When using potassium permanganate as an oxidizer it is very important to know whether this salt is acting in an acid or an alkaline fluid, because in an acid medium a manganosalt MnO and in an alkaline medium a manganese peroxide MnO_2 salt is formed. As the manganese dioxide is more highly oxidized than the manganese monoxide, it follows that to get the maximum oxidizing effect obtainable from potassium permanganate, care must

[1] Ostwald: *Grundlinien der anorganischen Chemie*, 576 (1900).

be taken to have it acting in an acid medium. For this reason it is necessary to know what the ultimate reaction between the interacting substances will be. If we regard the interaction between potassium permanganate and sulphuretted hydrogen from this point of view we obtain:

$$5H_2S + 8KMnO_4 + 2H_2O = K_2SO_4 + MnSO_4 + 7Mn(OH)_2.$$

According to the right half of this equation we are dealing with the neutral salts potassium sulphate and manganous sulphate, while 7 molecules of manganous hydrate contain 14 hydroxyl radicals. To neutralize these it is necessary to add 14 hydrogen ions, which is done most readily by adding 7 divalent sulphuric acid molecules, $7Mn(OH)_2 + 7H_2SO_4 = 7MnSO_4 + 14H_2O$. This explanation is based on Ostwald's view[1] that all oxidizing media in the presence of water may be regarded as hydroxyl (OH) compounds, and all reducing agents as hydrogen (H) compounds.

When discussing the formation of ions (p. 13) attentio was drawn to the fact that oxidizing and reducing agents form a class by themselves and that they are not comparable to salts in their action. This is fully borne out by studying the effect of the non-electrolytes osmium tetroxide, OsO_4, and formaldehyde, OCH_2, on coagulable proteids.

Osmium tetroxide. Hardy, in his investigations on the structure of cell-protoplasm[2], was the first to describe a difference in the appearance of egg-white when fixed by the vapour of osmium tetroxide and when coagulated by such reagents as sublimate. While the latter produces a net-like arrangement, OsO_4 gives rise to the appearance 'of a number of vesicles hollowed out of a continuous solid mass and therefore not communicating with one another. It is, however, very difficult to be certain on this point.'

At the Anatomical Congress in Kiel in 1898, I discussed the oxidizing action of osmium tetroxide, and to this discussion Moncke-berg and Bethe[3] refer in a paper in which they point out that osmium tetroxide is not able to form salts, and that for this reason it does not coagulate as do most other reagents. They state that by acting as an oxidizer, osmium tetroxide becomes reduced to metallic osmium; that white of egg treated with an equal volume of 2 per cent. OsO_4 remains fluid on being boiled; and that it becomes unprecipitable by nitric acid, acetic acid or alcohol, while it is coagulated by a mixture of sublimate and nitric acid. The vapour of OsO_4 acting for 48 hours on white of egg leaves the latter quite uniform, and produces no structure which can be demonstrated by either acid or basic dyes, an observation which bears out my own. These authors also tried the effect of other oxidizing agents, such as peroxide of hydrogen (which tears the tissues), potassium permanganate, and bichromate, which do not act like osmium tetroxide, because their salt-forming is superadded to their oxidizing action.

[1] Ostwald: *Grundlinien der anorganischen Chemie*, 606 (1900).
[2] H. W. Hardy: *Journ. of Physiol.* 24, 170 (May, 1899).
[3] Monckeberg and Bethe: *Arch. f. mikr. Anat.* 54, 135 (1899).

My own investigations on oxidizing agents date back to 1893, when I was engaged on the investigation of changes in nerve cells, and in that year, and more fully in 1894, I published the formula for my osmo-sublimate mixture (see p. 97). Having used OsO_4 both in the form of solutions and vapour I found that it produced a perfectly homogeneous, almost glassy appearance, and I therefore made use of osmium tetroxide to rectify the open structure which results from the use of sublimate[1].

Whenever osmium tetroxide is used in solutions, these should be made isotonic with the tissues by the addition of such neutral salts as sodium chloride or ammonium sulphate (see pp. 80, 83, and 98).

Formaldehyde being a non-electrolyte is incapable of forming salts, but is able to form additive compounds. In 1895 I first commenced adding it to my picro-corrosive fluid with the view of curing shrinkage and the sponge-like appearance of cells. Formaldehyde must be regarded as a reducing agent (see p. 92). In its general effect on tissues it greatly resembles osmium tetroxide, but this does not justify us in considering it therefore as an oxidizer. It has no tendency to cause dissociation of tissues, if used in neutral solutions or as vapour, provided care is taken to use aldehyde free from acid, or to bind the free formic acid by some weak alkali (p. 88). For a general account of formaldehyde see p. 88.

CHAPTER IV.

Fixatives.

Chemistry of Reagents used in Fixation.

In the following account of the chemistry of fixing reagents, the substances have been arranged according to their reaction, for want of a better classification. We should, however, always keep in mind such factors as the amount of electrolytic and hydrolytic dissociation, the nature of their chemical affinities, their oxidizing and reducing powers and their power to form additive compounds.

(1) Acids.

(*a*) **Nitric acid.** Ions, $H^{\cdot} + NO_3'$. It acts as an oxidizer, converting e. g. ferro-ions into ferri-ions, but it is reduced by hydrogen into ammonia $NO_3 + 8\,H = 3\,H_2O + NH_3$. Nitric acid in a 3 per cent. solution is strongly recommended by Altmann, but is not so reliable a fixative as sulphuric acid. It precipitates deutero-albumose in the presence of a high percentage of NaCl. A 50 per cent. solution will precipitate nucleo-albumins permanently.

[1] Still later I combined sublimate with potassium bichromate (see p. 97), and material from Malopterurus, fixed in the double salt of mercury and chromium, served Ballowitz in his investigations on the electric organ. This author has published my formula of the fixing solution (*The Electrical Organ of Malopterurus*, Jena, 1899).

Percentage of HNO_3 and its specific gravity at 15° C.

Percentage.	*Specific gravity.*	*Percentage.*	*Specific gravity.*
1	1·000	40	1·251
2	1·010	50	1·318
4	1·022	60	1·375
8	1·046	70	1·423
10	1·058	80	1·460
20	1·120	90	1·495
30	1·185	100	1·530

After fixing in nitric acid, the tissues have to be placed at once in 70 per cent. alcohol and not in water, and the alcohol must be renewed till all the acid has been washed out (Altmann).

The yellow colour produced by the action of strong acid or the prolonged action of even 5 per cent. acid, is due to the formation of nitro-substitution products in proteids. The organic sulphur becomes under these conditions oxidized, phosphorus and probably nitrogen are removed as well. (See *The Chemistry of Proteids*, by Otto Cohnheim; English translation, Macmillan.)

(*b*) **Sulphuric acid**, H_2SO_4. Its ions are $H^{\cdot}$ + the monovalent HSO_4', and the latter on further dilution becomes $H^{\cdot}$ + the divalent an-ion SO_4''. The specific gravity of the pure liquid acid at 24° C. is 1·834. According to Otto[1], sulphuric acid of different percentages shows the following specific gravities at 15° C.:

Percentage.	*Specific gravity.*	*Percentage.*	*Specific gravity.*
1	1·006	40	1·306
2	1·013	50	1·418
4	1·025	60	1·501
6	1·039	70	1·615
8	1·053	80	1·734
10	1·068	90	1·822
20	1·144	100	1·842
30	1·223		

It has already been stated that sulphuric acid undergoes only about one-half the electrolytic dissociation shown by nitric or hydrochloric acids, and for this reason it is a much better fixative than the other mineral acids. It ought to be used in strengths of 5 to 10 per cent. in water, or still better as 'sulphuric acid-alcohol,' a solution containing ethyl-hydrogen sulphate, and prepared by mixing sulphuric acid 10 ccm. with 90 ccm. of 75 per cent. alcohol. In making up the sulphuric acid-alcohol it is necessary to dilute the acid with the requisite amount of water, and then to add the alcohol to the cooled mixture, as otherwise decomposition may take place.

(*c*) **Sulphurous acid**, $SO_2 + H_2O$, forms the monovalent HSO_3' and the divalent SO_3'' an-ions, which along with the $H^{\cdot}$ kat-ions interact according to the following equation: $2H^{\cdot} + SO_3'' = H_2O + SO_2$.

The SO_2 formed in this way is the anhydride of sulphurous acid and not a true acid, because of the absence of $H^{\cdot}$ kat-ion.

Commercial sulphurous acid contains about 10 per cent. of SO_2, and frequently also sulphuric acid; the latter is readily recognized

[1] *Kalender für Electrochemiker, etc.*, by A. Neuburger, Berlin.

by adding hydrochloric acid and barium chloride. Barium sulphite is soluble in HCl, while barium sulphate is not.

The following are the specific weights and amounts of SO_2 in sulphurous acid at 15·5° C. (Giles, Schearer, and Gerlach):

Percentage of SO_2.	*Specific gravity.*	*Percentage of* SO_2.	*Specific gravity.*
1	1·0051	6	1·0302
2	1·0102	7	1·0352
3	1·0152	8	1·0402
4	1·0202	9	1·0453
5	1·0252	10	1·0504

Sulphurous acid as well as the gaseous anhydride may be used for fixing. It was first employed by Waddington in 1883[1] for the study of paramaecium. He states that it is soluble to the extent of 30 volumes in 1 volume of cold water, whilst Ostwald says the solubility is 50:1. A watery solution by the action of air is quickly converted into sulphuric acid, and therefore Overton recommends that an alcoholic stock solution be made, the SO_2 being much more soluble in alcohol, and being readily given off on diluting the alcohol with water. To saturate 100 grams of alcohol for purposes of fixation, Overton takes ½ gram of anhydrous Na_2SO_3, adds a few ccm. of 80 per cent. sulphuric acid, stoppers the bottle and leads the SO_2 gas into the alcohol. Waddington recommends sulphurous acid also for its powerful bleaching properties, and it is preferable to chlorine, because its action on tissues is less violent. Overton[2] employs sulphurous acid for fixing purposes, and also to convert chromic acid into chromium sulphate, $Cr_2(SO_4)_3$, which latter, by acting similarly to aluminium sulphate, $Al_2(SO_4)_3$, does not interfere with the staining of tissues by haematoxylin. I have tried the method after fixing in Flemming's solution, and find that washing in water is rendered quite unnecessary.

(*d*) **Sulphuretted hydrogen**, H_2S, imparts to water a slight acid reaction, owing to a partial dissociation into $H^{\cdot} + HS'$. Its solubility in 1 ccm. of water is at 0° C. = 4·4, at 10° = 3·7, and at 20° = 3·1 volumes. At ordinary atmospheric pressure and room-temperature 1 molecule (or 1 gram molecular weight) dissolves in 12 litres.

(*e*) **Chromium trioxide**, CrO_3, was first recommended for histological purposes by Hannover (1840). According to Ostwald[3], CrO_3 when in watery solutions forms dichromic acid, $2H^{\cdot} + Cr_2O_7''$. The chromic acid H_2CrO_4 is not formed, because the chromate-ion CrO_4 has a great tendency to undergo condensation into the bichromate ion Cr_2O_7, as is readily proved by heating a chromate solution, when the latter will turn from yellow into the deep-orange colour of the bichromate solution. On cooling the yellow colour is restored.

It is a strong oxidizer, becoming itself reduced by organic bodies to chromic oxide, Cr_2O_3, or even the chromous hydroxide, $Cr(OH)_2$.

[1] *Journ. Roy. Mic. Soc.* 3, part 1, 185 (1883).

[2] Overton: *Zeitsch. f. wiss. Mikr.* 7, 9 (1890).

[3] Ostwald: *Zeitsch. f. physik. Chem.* 2, 78 (1888). A. Fischer, in his book on *Fixirung, etc. d. Protoplasmas*, states that chromium trioxide does not form an acid in a watery solution, a view which cannot be maintained.

It is used in strengths of $\frac{1}{10}$ to $\frac{1}{2}$ per cent. for cell studies; by itself or in combination with acetic acid and osmium tetroxide, as in Flemming's solution, or with corrosive sublimate (see later under Sublimate). All solutions have a considerable solvent action on tissues (p. 139), as is readily shown by using a 10 per cent. solution.

For laboratory purposes it is best to make up a 10 per cent. stock solution.

Tissues fixed in chromic acid or one of its salts have only slight affinity for basic stains, and to overcome this difficulty the following methods have been employed:

(*a*) Edinger, in 1884[1], recommended a 5 per cent. watery solution of nitric acid, with which he treated sections for five minutes. Independently Paul Mayer adopted the same plan. He leaves sections in the ordinary hydrochloric acid-alcohol (1 part of HCl in 1000 parts of 95 per cent. alcohol) till they are almost white, a process which I have found to give very good results. Pieces of tissue, before staining them in bulk, Mayer treats with 5 per cent. sulphuric or 10 per cent. nitric acid. With sulphuric acid I have obtained more intense staining than after nitric acid, and have also found the preservation of tissues to be better.

(*β*) Unna[2] oxidizes the reduced chrome-compounds with peroxide of hydrogen, but great care is needed in employing this method.

(*γ*) Overton's[3] sulphurous acid method depends on the principle that the watery solution of SO_2 unites with chromium trioxide to form chromic sulphate, $Cr_2(SO_4)_3$. This component behaves towards haematoxylin as does the mordant aluminium sulphate, $Al_2(SO_4)_3$, the latter being the essential radical in haematoxylin alum solutions. Gilson[4] uses an alcoholic instead of a watery solution of sulphurous acid for removing chrome-compounds from tissues.

(*f*) **Acetic acid**, $CH_3{\cdot}CO{\cdot}OH$, when anhydrous, is undissociated and therefore neutral to litmus; on being diluted with water the acid kat-ion $H^{\cdot}$ and the monovalent $C_2H_3O_2'$ are formed. It rapidly mixes with water, alcohol, and ether in all proportions, and readily dissolves iodine and sulphur. It has no reducing power and thus differs from formic acid (see p. 74)[5]. With metallic hydroxides it forms acetates.

Anhydrous or glacial acetic acid melts at 16·5° C. At 15° C., according to A. C. Oudemans, different percentage strengths have the following specific gravities. [A complete table is given in Behrens' tables. Bruhn, 16, 1898.]

Percentage of $C_2H_4O_2$.	*Specific gravity.*	*Percentage of* $C_2H_4O_2$.	*Specific gravity.*	*Percentage of* $C_2H_4O_2$.	*Specific gravity.*
1	1·0007	33[6]	1·0447	70	1·0733
5	1·0067	43	1·0552	77–80	1·0748
10	1·0142	50	1·0615	90	1·0713
25	1·0350	60	1·0685	100	1·0553

[1] *Zeitsch. f. wiss. Mikr.* 1, 126 (1884). [2] *Arch. f. mikr. Anat.* 30, 47 (1887).
[3] *Zeitsch. f. wiss. Mikr.* 7, 9 (1890). [4] Lee and Mayer, 32 (2nd ed. 1901).
[5] The interrelation of primary fatty compounds is given in the table on p. 84.
[6] Strength of acid of the *British Pharmacopoeia*.

The specific gravities above 1·0553 correspond to two solutions of different percentages (43 to 78 per cent. and 100 to 78 per cent.). If on the addition of water to an unknown solution the specific gravity is increased, then the solution is stronger than 78 per cent.; if the specific gravity diminishes, then the solution is weaker.

The 77 per cent. solution is a definite hydrate, known as ortho-acetic acid = $CH_3 \cdot C(OH)_3$. Glacial acetic acid behaves as a non-electrolyte and is a good fixative. One per cent. solutions are useful for differentiating between white and yellow fibrous tissue. Acetic acid is mostly employed as an acidifier for osmium tetroxide and potassium bichromate.

(*g*) **Trichloracetic acid** is a substitution product of acetic acid, thus:

$$CH_3 \cdot CO \cdot OH + 3Cl_2 = CCl_3 \cdot COOH + 3HCl.$$

I have used it extensively for fixing purposes, and find it preserves all tissue elements even better than acetic acid, excepting the connective tissue. It also acts as a good decalcifier.

(*h*) **Formic acid**, HCO·OH, on electrolytic dissociation, splits up into HCO_2' and $H^{\cdot}$. It resembles the aldehydes in being a strong reducer (p. 88). The sodium salt (H·COONa) and the potash salt (H·COOK) are deliquescent, while the lead and silver salts are only slightly soluble; hence lead formiate is readily obtained by adding formic acid to lead acetate (see p. 271 for Kronthal's method).

(*i*) **Picric acid** or trinitrophenol, $C_6H_2(NO_2)_3OH$. A saturated solution of this acid in water may mean anything from 0·6 to 1 per cent., according to the temperature of the room, and therefore either a $\frac{1}{2}$ or 1 per cent. solution should be made. The latter does not precipitate crystals at 20° C. It is a stronger coagulant than acetic acid because it precipitates every cell-constituent; it does not alter white fibrous tissue, and after having played its part in fixing the cell may be readily removed by transferring the tissue to a solution of corrosive sublimate. By this means it is extracted more quickly than by alcohol. All the precipitates which it forms have this peculiarity, that they dissolve more or less quickly on diluting the saturated picric acid solution with water, or on warming (which amounts to the same). For this reason tissues which have been fixed in picric acid should never be washed in water, but be transferred at once to 50 or 70 per cent. alcohol.

Its excessive precipitating power is indicated by Experiment 17 on p. 119.

(*k*) **Tannin** (anhydride of gallic acid $[(OH_3) \cdot C_6H_2 \cdot CO_2H]_2 - H_2O = C_{14}H_{10}O_9$). A weak acid which decomposes carbonates; solubility in water = 1 : 1, in alcohol 1 : 2. It was introduced by Carnoy (1885), who recommends the use of $\frac{1}{2}$ per cent. solutions. I used it for many years both for vegetable and animal cells, but have given it up because it acts as a mordant for many coal-tar colours, and therefore makes micro-chemical research more difficult. It further accentuates the precipitating action of sublimate and picric acid to such an extent as to cause over-coagulation. I now no longer use it in com-

bination with my picro-corrosive solution. Good results may be obtained from it, however, with white fibrous tissue.

(2) Acid Salts.

(*a*) **The bichromates.** Chromium combines with oxygen to form a feeble yellow divalent chromate-ion, CrO_4'', and a stronger red bichromate-ion, Cr_2O_7''. The latter, if joined to a strong metallic oxide, as for example K_2O, causes hydrolysis of water, and breaks up, partly, into bichromic acid, $H^{\cdot}_2 + Cr_2O_7''$. The hydrogen ions of the latter are responsible for the acid reaction of bichromate solutions.

Eugen Burchardt (1897)[1] has divided all soluble bichromates into two classes, namely those which fix and those which destroy cell nuclei. He found that all bichromates of the alkalies (potassium, cæsium, rubidium, sodium, and lithium), also those of ammonium, magnesium, strontium, and zinc, preserved the cell body but destroyed nuclei, as the latter were converted into homogeneous bladders with no definite chromatic segments[2]. Barium, calcium, and copper bichromates on the other hand preserve mitoses, with the exception of the achromatic spindle, but they do not preserve the cell body. The nuclei-destroying bichromates penetrate readily, while the nuclei-preserving ones do not; as, further, acetic acid prevents the deleterious action of the nuclei-destroying salts, he uses bichromates belonging to the two different classes in combination with acetic acid. He especially recommends the formula given on p. 100.

Burchardt further found that nuclei become finely granular after treatment with 1 : 1500 or even more dilute chromic acid, while 1 : 1000 potassium bichromate produced a homogeneous and 1 : 1000 calcium bichromate an irregular lumpy appearance.

Strontium bichromate, prepared by E. Merck, does not preserve nuclei, although strontium belongs to the alkaline earths, as do calcium and barium. Zinc bichromate stands about halfway between the nuclei-destroying and the nuclei-preserving bichromates; the latter must be arranged as follows, beginning with the best fixative: barium, calcium, copper, zinc, sodium, potassium, and ammonium.

Tellyesniczky (1898) also arrived at the conclusion that acetic acid prevents the deleterious action of potassium bichromate on cell nuclei (see p. 100).

Potassium bichromate, $K_2Cr_2O_7''$, introduced by Heinrich Müller (1859), has twice the amount of chromium trioxide necessary to satisfy the potassium oxide, for which reason it is ready to part with one chromium trioxide radical whenever it comes into contact with a base. Towards litmus it behaves as an acid body for reasons given above, and will do the same towards inorganic bases and organic potential bases such as albumins. It must be remembered that the whole class of albumins, globulins and their derivatives, possess the power, characteristic of amidogen, of playing the part either of a base or of an acid towards a substance with which they

[1] Burchardt: 'Bichromate und Zellkern,' *La Cellule*, 12, 337 (March, 1897).

[2] All salts were used originally in the same percentage strengths, the tissues were fixed up for twenty-four hours, washed for twenty-four hours in frequent changes of water, and hardened in alcohol.

share chemical affinities, and of being liable to form pseudo-bases and pseudo-acids (see p. 25).

The conversion of the acid bichromate into the alkaline chromate makes it necessary to frequently change the bichromate solution when fixing tissues. To the naked eye the formation of potassium chromate becomes evident by the orange colour of the bichromate solution changing into the yellow colour of the chromate.

Potassium bichromate in a watery solution decomposes hydrolytically into dichromic acid $H^{\cdot}_2 + Cr_2O_7''$ and $K^{\cdot} + OH'$, the acid reaction of the solution depending on the free hydrogen-ions. On adding hydroxyl ions to a solution of potassium bichromate the bichromate ion is converted into the chromate ion, thus $Cr_2O_7'' + 2OH' = 2CrO_4'' + H_2O$. Chromate solutions have an alkaline reaction because the tendency of the chromate ion to become converted into the bichromate ion leads to a hydrolysis of the water; the hydrogen ions are attracted by the chromate ions and partly bound by it, while the free hydroxyl ions cause the alkaline reaction.

When Fischer renders albumins and similar compounds alkaline by the addition of 0·2 per cent. KOH, he no longer studies only the action of $K_2Cr_2O_7$, but, to a certain extent, also that of $KCrO_4$ on these compounds. Notwithstanding this, I have recorded his observations in my chart on p. 102 to impress the importance of that reaction of tissues which depends on inorganic salts.

Potassium bichromate is a slow fixative because of its partial transformation into the chromate.

It is used for fixing large bulks of tissue such as the central nervous system, when the use of chromic acid solutions would lead to a superficial hardening, leaving the centre unacted upon. Till a tissue is hardened with bichromate, there is thus a constant struggle between the potassium chromate and the tissue for the available CrO_3, and in the end the tissue proves the stronger. Potassium chromate has no fixing action whatever, except perhaps on protamin.

The double salt of sublimate and potassium bichromate ($HgCl_2 + K_2Cr_2O_7$) has been used by me since 1895. Dissolve 270·6 grms. of sublimate in 350 ccm. of boiling distilled water; also dissolve 295 grms. of potassium bichromate in another 350 ccm. of boiling distilled water. Mix the two solutions, filter and allow to cool. Red rhombic crystals will separate out on cooling.

It must be borne in mind that all experiments on proteids with chromic trioxide and its salts should be done in complete darkness, because the action of light renders chrome-compounds insoluble.

As first pointed out by Stirling in 1881[1] and subsequently by Virchow[2], tissues to be fixed in alcoholic bichromate mixtures, or tissues which have been fixed in chromic acid or one of its salts and which are then placed into alcohol, have to be kept in the dark, as otherwise an insoluble precipitate of the suboxide of chromium is formed.

To strengthen the fixing action of bichromates, acetic acid may be

[1] Stirling: *Text-book of Practical Histology*, Smith, Elder and Co., 1881.
[2] Virchow: *Arch. f. mikr. Anat.* 42, 117 (1885).

added, as was first done by Burchardt and also by Tellyesniczky (p. 100). Acetic acid leads to a ready dissociation of the bichromate, to an acidification of the proteid compounds, and to a precipitation of the nuclein.

(*b*) **Acid chlorides. Corrosive sublimate**, $HgCl_2$, in water gives an acid reaction because of its hydrolytic dissociation into $HgOH + 2HCl$. Introduced in 1847 by Blanchard [1] for the fixation of planarians, it was used in 1854 by Remak [2] in his researches on the multinucleated cells of embryonic livers. Arnold Lang, in 1878, also working on planarians, was, however, chiefly instrumental in making sublimate popular. Its solubility in water, according to Poggiale, is

at	10°	20°	50°	80°	100°
$HgCl_2$	6·57	7·39	11·34	24·3	53·96

In alcohol it is soluble to about 33 per cent., and in ether to about 25 per cent. It is therefore possible to remove the greater part of the salt from watery solutions by means of ether. With ammonia it forms the mercuriammonium chloride or infusible white precipitate

$$HgCl_2 + 2NH_3 = NHgH_2Cl + NH_4Cl.$$

Analogously to this equation it probably combines with the nitrogenous constituents of the cell. Mercuric oxide + potassium iodide + water forms a strongly basic solution called Nessler's reagent, which is used for detecting traces of ammonia. The interactions by which this solution is made are $HgO + 4KI + H_2O = K_2HgI_4 + 2KOH$, or if written according to the ionic view $4I + HgO + H_2O = HgI_4'' + 2OH'$. Ammonia with mercuric oxide forms the dimercury hydroxide $2HgO + NH_3 = Hg_2N(OH) + H_2O$, which with iodine gives a deep brown colour.

With sodium chloride sublimate forms double salts ($HgCl_2 + 2NaCl$) and ($2HgCl_2 + 2NaCl + 3H_2O$), a fact which must be borne in mind when studying the action of saturated solutions made with $\frac{1}{2}$ or $\frac{3}{4}$ per cent. sodium chloride. A saturated solution of $HgCl_2$ in $\frac{3}{4}$ per cent. NaCl is approximately a 10 per cent. one. These solutions of the double salt of mercury and sodium contain, in addition to the ions of either salt, also such complex an-ions as $HgCl'_3$ and $HgCl''_4$, for it is possible to obtain their alkali salts, for example $KHgCl_3$ and K_2HgCl_4. The corresponding mercurihydrochloric acid $H' + HgCl_3$ is obtained by acting on mercuric chloride with concentrated hydrochloric acid.

Sublimate-precipitates with proteids are soluble in excess of albumin, as well as in salt solutions, and therefore large amounts of the fixative are required. The common salt, when present, plays a double part—it increases the solubility of the sublimate and of the mercury-albuminates, and therefore the penetrating power of sublimate; see Experiments 7 to 12, on pp. 107–114.

[1] Blanchard: *Ann. Sc. N.* (3), 8, 247 (1847).

[2] Remak: *Arch. f. Anat. u. Physiol.* 99 (1854). He also used the following fixatives: 0·2 per cent. acetic and chromic acids, 20 per cent. alcohol and (approximately) 0·5 per cent. potassium iodide, and 0·25 per cent. iodine in water.

Mercuric iodide, HgI_2, formed at low temperatures is yellow, and is in a more unstable state than the red salt. It is only slightly soluble in water (1 : 120), but readily soluble in spirit and in a solution of potassium iodide, because the latter by dissociation liberates iodine-ions, which form with mercury the complex soluble an-ion $HgI_4'' = HgI_2 + 2I'$. The more concentrated the iodine-ions are, the more readily does mercuric iodide dissolve.

The albuminates of mercury are also readily decomposed by iodine-iodide of potash solutions, containing KI_3.

Because of the decomposition of the albuminates of mercury by iodine-potassium iodide, on no condition should this mixture be allowed to act on the tissues till they have been taken through the paraffin process. Sections, for the same reason, should be examined both before and after the application of iodine, to detect the presence of peptones (?) and albumoses. Schaper's [1] objections to the use of iodine after the sections have been made, carry no weight, because the crystals of sublimate, which in his case tore the tissues, are formed during fixing, either owing to a fall of temperature or to evaporation of the fixing fluid. By using large amounts of saturated solutions of sublimate, this salt will dissociate less, and therefore will be less liable to produce in the colloidal proteids those changes which are caused by all electrolytes, but, for most purposes, solutions exceeding 5 per cent. in strength are not to be advocated.

Stannous chloride, $SnCl_2$, gives good results with the nervous system, having a peculiar action on Nissl's substance, rendering it unstainable by toluidin-blue.

(*c*) **The Hydrogen-chlorides.** The so-called chlorides of platinum, namely chloro-platinic acid, H_2PtCl_6, of palladium, H_2PdCl_6, of iridium, H_2IrCl_6, and ruthenium, H_2RuCl_6 (as well as the chloride of vanadium, VCl_2) were repeatedly tried to see whether they had any advantages over mercuric chloride as fixing reagents, but I can only repeat what I stated in 1894[2]: they have not a single advantage as far as general fixation is concerned, although in special cases where general fixation is not wanted 'platinum chloride,' because of the excessive coagulation it produces, may serve to bring out conditions which sublimate would not reveal at that period. I am referring to the longitudinal splitting of chromatin segments, which is seen at a much earlier stage if chloro-platinic acid is used instead of sublimate.

Chloro-platinic acid is a strong dibasic acid, and differs in its constitution completely from mercuric chloride, which latter, as shown above, dissociates electrolytically into hydrochloric acid and the oxide of mercury. Chloro-platinic acid does not liberate any chlorine ions, therefore no hydrochloric acid is formed, and also no precipitate with silver nitrate, there being formed instead, in the latter case, silver platinum chloride, Ag_2PtCl_6. On being electrolysed, H_2PtCl_6 divides in such a manner that the hydrogen-ions $2H^{\cdot}$ go to the kathode, while the $PtCl_6''$ ions go to the anode (see p. 12).

[1] Schaper: *Anat. Anzeiger*, 13, 463 (1897).
[2] *Anat. Anzeiger*, 13, 463-472.

The potash and ammonia salts are only slightly soluble, while the sodium salt is readily soluble in water, crystallizing with $6H_2O$.

The real platinum chloride, $PtCl_4$, is not hygroscopic, as is the chloro-platinic acid, and with water forms H_2PtCl_4O.

Quite apart from fixation, the platinum radical ($PtCl_6$) seems to act as a special mordant for basic dyes, such as safranin and toluidin-blue, because the compounds it forms with proteid contain both platinum and chlorine radicals.

Gold chloride, on being carefully evaporated after having been made, gives aurochloric acid, $HAuCl_4$, which on heating leaves behind gold trichloride or $AuCl_3$. The watery solution of the latter has an acid reaction, and contains the complex an-ion $AuOCl_3''$, for $AuCl_3 + H_2O = H^{\bullet}_2 + AuCl_3O''$. Only a certain amount of the gold chloride undergoes this change, however, while the rest remains as the undissociated $AuCl_3$.

The salts of $HAuCl_4$ are usually called double salts; for example, potassium gold chloride, $KAuCl_4$, and the corresponding sodium salt, $NaAuCl_4 + 2H_2O$.

Pure auro-sodium chloride contains 14·7 per cent. sodium chloride, but the ordinary commercial gold chloride contains frequently much larger quantities. Thus Squire states that 'pure' English gold chloride is the sodium gold chloride containing 50 per cent. of gold, and that the so-called 'sodium gold chloride' is an equal mixture of sodium gold chloride and common salt, reducing the percentage of gold to 25 per cent. According to Apáthy, Merck's aurum chloratum fuscum, $AuCl_3 + 2HO_2$, contains 53 per cent. of pure gold, while aurum chloratum flavum, $AuCl_4H + 4H_2O$, only contains 48 per cent. of gold. The preparations of Grübler and Hollborn contain only hydrochloric acid and water, but no sodium, and are exceedingly hygroscopic (Paul Mayer).

$Au(OH)_3$, formed by the decomposition of the $AuCl_3$ or $HAuCl_4$, is soluble in an excess of the alkali, because the hydroxide has feebly acid properties. Gold chloride solutions with sulphuretted hydrogen form the black amorphous AuS, which is soluble in potassium cyanide, in KCN (becoming colloidal gold on washing), and in yellow, but not in white ammonium sulphide. All gold compounds are readily dissolved in potassium cyanide solutions, forming $Au(Cn)_2'$ and $Au(CN)_4'$.

Gold chloride ($AuCl_3$) for general purposes cannot be recommended as a fixative because of its ready reduction, which seems to depend on the tryptophane radical of certain proteid-molecules (see p. 323). According to Galeotti and Levi[1] gold chloride is a good fixative for cytological work[2].

(*d*) **Mercuric nitrate**, $Hg(NO_3)_2$, is a very deliquescent salt, liable on dilution to pass into the basic salt $2Hg(NO_3)OH + H_2O$ and other basic salts, there remaining ultimately only the oxide of mercury, HgO. The formation of the insoluble basic salt is readily prevented by using as a solvent 1 to 5 per cent. solution of nitric acid, or by taking the requisite amount of mercuric nitrate to make any desired

[1] Galeotti and Levi: *Beiträge z. path. Anat.* 17, 371 (1895).
[2] See also Klemenciewicz in Rollet's *Festschrift* (Jena, 1893).

strength of solution, adding the water, shaking up, and ultimately adding nitric acid drop by drop till the solution becomes quite clear.

(*e*) **The Acid Sulphates.**

Mercuric sulphate ($HgSO_4$), when dry, forms a white opaque mass, which, on the addition of water, turns into the lemon-coloured, insoluble, basic salt Hg_3SO_6. This conversion may be prevented by adding sulphuric acid to the water used for dissolving the salt.

(3) Neutral Salts.

(*a*) **Ammonium sulphate** [$(NH_4)_2SO_4$] dissolves at 0°C. in 71 parts of water, at 20° in 76·3, and at 100° in 97·5 parts. It is very slightly soluble in aqueous alcohol and insoluble in absolute alcohol[1]. The reaction of a saturated solution of the commercial salt is as a rule slightly acid, if tested with litmus solution. Before using it for purposes of fixation, it should be neutralized carefully with ammonia, and complete saturation be ensured by having undissolved crystals on the bottom of the flask. Isotonic solutions of ammonium sulphate, containing 1 per cent. of OsO_4, have been tried repeatedly with good results.

(*b*) **Magnesium sulphate** [$MgSO_4$] has no advantages over ammonium sulphate for histological purposes.

(*c*) **Neutral potash alum** is obtained by carefully adding a 1 per cent. potassium hydrate (KOH) solution to the ordinary potash alum [$Al_2(SO_4)_3 + K_2SO_4 + 24H_2O$], till the precipitate first formed just redissolves.

(*d*) **Sodium chloride** (NaCl) dissolves in aqueous alcohol, and is slightly soluble in absolute alcohol. According to Poggiale, one hundred parts of water dissolve at the stated temperatures the following amounts of the salt :

Temperature :	0°	5°	14°	25°	40°	60°	80°	100°	109·7°
NaCl :	35·52	35·63	35·87	36·13	36·64	37·25	38·22	39·16	40·35

(*e*) **Copper acetate** [$(C_2H_3O_2)_2Cu + H_2O$] is dark-green in colour and is obtained by dissolving cupric oxide in acetic acid. Verdigris is blue in colour, and is a basic acetate $(C_2H_3O_2)_2Cu + Cu(OH)_2$.

(*f*) **Lead acetate**, or 'sugar of lead,' $(C_2H_3O_2)_2Pb + 3H_2O$, is prepared by dissolving lead oxide or litharge in acetic acid. When its solution is boiled with litharge, the soluble basic lead acetate is formed.

(*g*) **Ammonium chromate**, $(NH_4)_2CrO_4$, if correctly prepared is neutral, but possesses as a rule a distinctly acid reaction due to free CrO_3 (see p. 72). Stirling, in his *Text-book of Practical Histology*[2], recommends 50 grms. to be dissolved in 1000 ccm. of water ; to filter and to preserve the filtrate in a stoppered bottle. Fresh tissues are hardened in twenty-four hours ; are washed in water till no more colour is given off, and are then placed in spirit. Small pieces of kidney are fixed for forty-eight hours ; are washed and transferred first into 50 per cent. methylated spirit, and then into pure spirit, which is changed till it becomes quite clear. The whole washing-out operation must be done in the dark.

[1] Roscoe and Schorlemmer : *Treatise on Chemistry.*

[2] Smith, Elder and Co., London, 1881.

(4) Bodies forming additive compounds.

Osmium tetroxide (OsO_4), usually called osmic acid, in a watery solution is neutral to litmus. It is a non-electrolyte. The fact that it is reduced to a varying extent by different tissues was first observed by Franz Schulze, who recommended it to Max Schultze. The latter noticed (1865) that a very dilute solution is reduced by a special group of cells in the glow-organ of Lampyris, if the glow-worm is put living into the solution, while dead tissues did not react in this way. M. Schultze and Rudneff then studied its characteristic reduction by fat-cells, medullated nerves, the outer segment of the retinal rods and leucocytes.

Osmium tetroxide keeps well as a 1 per cent. watery solution made with pure, cold water, but also if dissolved in 1 per cent. sodium chloride or 1 per cent. ammonium sulphate. It readily dissolves in hot water, but seems to become dissociated by heat, because the solution made with cold water is almost colourless, while that made with hot is distinctly yellow, and is apt to become reduced spontaneously in the course of a few days. To make a 2 per cent. solution requires about three days at the ordinary temperature, and if heat is resorted to in dissolving the osmium tetroxide, it must be remembered that we are dealing in all probability not with (OsO_4), but some dissociation product.

If the osmium has become reduced, it is best regenerated by a ten-volume hydrogen peroxide solution (2 to 3 drops), which Bristol (1893) supposes to act according to the formula $OsO_2 + 2H_2O_2 = OsO_4 + 2H_2O$. It is more likely that OsO_4 is reduced to osmium tetrahydroxide, $Os(OH)_4$ (as it is by the action of alcohol), when $Os(OH)_4 + 2H_2O_2 = OsO_4 + 3H_2O$. But osmium tetroxide regenerated in this way is very apt to become precipitated, and, therefore, it is best not to use such a regenerated solution at all.

Osmium tetroxide[1] and ruthenium tetroxide, RuO_4, do not form salts, and in this respect hold a peculiar position. Both act by forming addition-compounds or simply as oxidizers, and are non-electrolytes, for which reason they are of the highest importance to the histologist, as will be shown later (see p. 137).

Osmium may be used for fixing purposes in the form of vapour or in solution. The former method has a twofold advantage: the fixative penetrates more rapidly, and the tissue is not brought into contact with the medium in which the acid has been dissolved. The vapour method is especially to be recommended for thin laminae of tissue or isolated cells, when the action of the reagent on the cell is to be studied. Gilson (1885) uses for better fixation of the nuclei a mixture of osmo-formic acid or osmo-acetic acid vapour. The solution of osmic acid has been used in combination with organic and inorganic acids, salts, and alcohol, and also by Dreyer[2] and myself mixed with formaldehyde (see p. 99).

By referring to the chart on p. 102 it will be seen that 'proteids'

[1] Monckeberg and Bethe : *Arch. f. mikr. Anat.* 54, 139 (1899).

[2] The method employed for the removal of osmium from the tissues will be described later.

in acid solutions are readily precipitated, while in neutral and alkaline solutions, with the exception of amphopeptone and haemoglobin, no precipitate is formed. All proteid compounds containing nucleic acid are not visibly acted upon at all. Flemming (1882) was the first to state that nuclei, after the use of OsO_4, look more life-like than after the action of any other fixative. Loewit (1891), after fixing the blood of crayfish with OsO_4, noticed that by the subsequent use of alcohol a precipitate was formed in the nuclei.

These observations, along with his own investigations, lead A. Fischer to conclude that OsO_4 is 'a very feeble and incomplete precipitant, which will always fail if the cell-contents are alkaline,' and that Flemming is mistaken when he supposes the osmium in his fixative to kill instantaneously, and to precede in its action that of the acetic or chromic acids which are also present.

Fischer reasons further: Because OsO_4 is such a feeble precipitant, all alkaline tissues, which have been subjected to twenty hours' fixation and subsequent washing to remove the acid, will possess their various cell constituents in an unfixed condition, and that proteids are only prevented from leaving the cell walls because of their colloidal nature. By dehydrating such tissues a precipitation in the cells is produced only by the alcohol. We are therefore looking at an alcohol precipitate when we examine a tissue fixed in osmium under the conditions of fixation stated above.

Facts are opposed to these speculations: OsO_4 may and does exert its influence on cells without of necessity causing a precipitation in them. I have injected a rat with 200 cc. of a 1 per cent. OsO_4 solution, left it for twenty-four hours, and then examined the nervous, glandular, muscular, and connective tissues. The cells in every case looked glassy, and quite different from similar cells fixed in picric acid or sublimate. Not a single class of cells, even after days of washing with distilled water, behaved as if its contents were soluble, while glands, muscle, and nerve-cells not fixed in osmium, when placed in aq. dist., rapidly became disintegrated. To meet the possible objection that the rat had become acid due to post-mortem changes, I took a solution of 1 per cent. OsO_4, rendered distinctly alkaline with ammonia, and treated the bladder, mesentery, muscle, pharynx, and retina of a frog with it for one hour[1], and then exposed the tissues to the action of water; in not a single instance did the cells behave as they ought to have done if the fixative had not acted.

It follows that OsO_4 acts in quite a different way from either acids or salts of the heavy metals. It fixes, but it does not precipitate in the same manner as do other fixatives, formaldehyde and perhaps alcohol excepted.

Two other rats were injected with 50 and 90 per cent. spirit, and comparisons of their tissues made with those of the osmium rat. There is not the slightest resemblance either in the fixation of the cells, which is perfect as regards absence of shrinkage in the osmium specimens, or in the staining reactions with aniline dyes. To put

[1] The fixing solution was alkaline at the end of the hour.

it shortly: there is no animal tissue which, when fixed alive, remains unacted upon by neutral 1 per cent. OsO_4.

Osmium solutions may be used in combination with salts, acids, or alcohol, but when we wish to prevent coagulation we may employ one-fifth to a half per cent. OsO_4 in isotonic solutions of such neutral salts as sodium chloride or ammonium sulphate.

It is customary to fix for far too long a time. I find that for complete fixation of a single layer of cells, as in the alimentary canal of the frog, ten to fifteen seconds are quite sufficient. Similarly the retina requires five minutes, the mesentery one minute. The sciatic nerve of the frog, where, for ordinary purposes, not so much fixation as staining of the medullary sheath is required, should be left in a $\frac{1}{2}$ per cent. solution for thirty to sixty minutes. For the study of nerve-cells and axis-cylinders twenty-four hours are required.

To remove the osmium from sections or preparations, either entirely or partially, one of the following methods will be found useful:—(1) P. Mayer (1880) recommends the use of free chlorine (or oxygen), which is most readily obtained by placing some crystals of potassium chlorate in a well-stoppered bottle and then adding two or three drops of HCl (or HNO_3). When the greenish-yellow chlorine gas is being set free, a few cc. of 50 to 70 per cent. alcohol are added. The tissues will be bleached in this mixture in a few minutes. (2) Fol, in 1884, was the first to recommend peroxide of hydrogen. Overton uses a freshly prepared mixture of commercial peroxide of hydrogen, 1 part, and 70 to 80 per cent. alcohol, 10 to 25 parts, when decolourization is produced in a few minutes. I find it best to use only 1 part of H_2O_2 in 50 parts of 80 per cent. alcohol. (3) The following method I have used for over ten years: Treat sections with $\frac{1}{4}$ per cent. potassium permanganate for five minutes. Decolourize the brown sections in a saturated watery solution of sulphurous acid, 1 part, and normal saline (0·75 NaCl), 9 parts [1].

The reduction of osmium tetroxide in the tissues for staining purposes is discussed later.

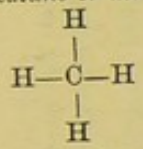
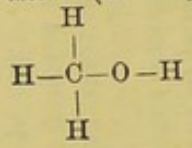
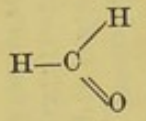
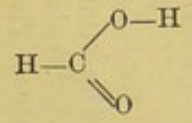
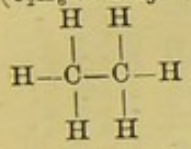
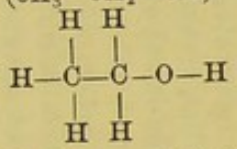
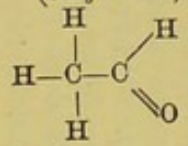
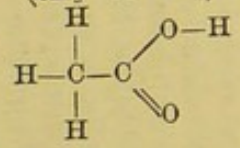
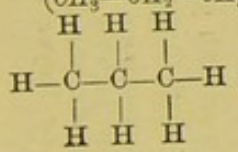
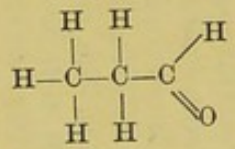
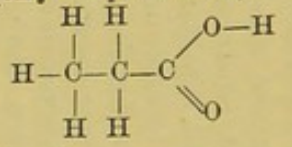
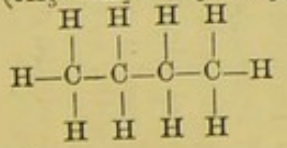
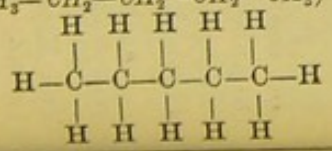
Formaldehyde also belongs to the group of substances forming additive compounds. It will be discussed after the general account of compounds of the fatty series has been given (see p. 88).

(5) Fatty compounds.

Acetic acid, belonging to this group, has already been discussed on p. 73, but it may help the reader to study in a tabular form the inter-relationship of the paraffins, alcohols, aldehydes, and fatty acids (see p. 84). As the formulae are given in a graphic form, it will be easy to understand the differences between lower and higher members of the same series (arranged in vertical rows), and also the changes which methane or marsh gas undergoes on becoming methyl alcohol, formaldehyde, and formic acid (horizontal row), &c. [2]

[1] At first I used Pal's decolourizing fluid (1 gram oxalic acid and 1 gram potassium sulphite, K_2SO_3), but latterly only sulphurous acid.

[2] For a fuller account the reader should consult the *Organic Chemistry* of Perkin and Kipping, edition 1900, or Meyer and Jacobson, *Lehrbuch der organischen Chemie*.

The methane or paraffin series. C_nH_{2n+2}	*The monohydric alcohol series.* $C_nH_{2n+2}O$ or $C_nH_{2n+1}OH$	*The aldehyde series.* $C_nH_{2n}O$ or $C_nH_{2n+1} \cdot CHO$	*The fatty series.* $C_nH_{2n}O_2$ or $C_nH_{2n+1} \cdot COOH$
1. *Methane or marsh gas* $(H \cdot CH_3)$ 	*Methyl alcohol* $(H \cdot CH_2 \cdot OH)$ 	*Formaldehyde* $(H \cdot CHO)$ 	*Formic acid* $(H \cdot CO{-}OH)$ 
2. *Ethane or dimethyl* $(C_2H_6$ *or* $CH_3{-}CH_2 + H)$ 	*Ethyl alcohol* $(CH_3{-}CH_2{-}OH)$ 	*Acetaldehyde* $(CH_3 \cdot CHO)$ 	*Acetic acid* $(CH_3 \cdot CO{-}OH)$ 
3. *Propane or methyl-ethyl* $(CH_3{-}CH_2{-}CH_3)$ 	*Propyl alcohol or normal-ethyl carbinol* $(CH_3 \cdot CH_2 \cdot CH_2 \cdot OH)$ (graphic formula on the above plan.)	*Propaldehyde* $(CH_3 \cdot CH_2 \cdot CHO)$ 	*Propionic acid* $(CH_3 \cdot CH_2 \cdot CO{-}OH)$ 
4. *Butane* $(CH_3{-}CH_2{-}CH_2{-}CH_3)$ 	*Butyl alcohol* $(CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot OH)$ (graphic formula on the above plan.)	*Butaldehyde* $(CH_3 \cdot CH_2 \cdot CH_2 \cdot CHO)$ (graphic formula on the above plan.)	*Butyric acid* $(CH_3 \cdot CH_2 \cdot CH_2 \cdot CO{-}OH)$ (graphic formula on the above plan.)
5. *Pentane* $(CH_3{-}CH_2{-}CH_2{-}CH_2{-}CH_3)$ 	*Amyl alcohol* $(CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot OH)$ (graphic formula on the above plan).	*Valeraldehyde* $(CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CHO)$ (graphic formula on the above plan.)	*Valeric acid* $(CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CO{-}OH)$ (graphic formula on the above plan.)

The monohydric alcohols are mono-substitution products of the paraffins, being formed by the substitution of the monovalent hydroxyl group, OH, for one hydrogen atom in paraffin, thus:

methane $CH_4 + H$ becomes methyl alcohol or $CH_3 + OH$
ethane $CH_3—CH_2 + H$ becomes ethyl alcohol or $CH_3—CH_2 + OH$.

Starting with methyl alcohol or carbinol, $CH_3 \cdot OH$, it is possible to replace one or more of the hydrogen atoms attached to the carbon atom by some other monovalent group, when there are formed mono-, di-, or tri-substitution products[1] of carbinol; thus in the primary alcohols $CH_3 \cdot OH$ becomes converted into $-CH_2 \cdot OH$, while in the secondary alcohols a further reduction takes place into $>CH \cdot OH$.

Primary alcohols on oxidation give rise to aldehydes and fatty acids (see table on p. 84), while secondary alcohols, containing the radical $>CH \cdot OH$, on oxidation become changed into ketones, which are substances possessing the radical $>CO$; for example, acetone, being a ketone, has the formula $\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!CO$.

Ethers are anhydrides of alcohol, formed by the condensation of two hydrocarbon groups into one molecule, with the simultaneous elimination of a molecule of water, thus:

Two molecules of methyl alcohol = one molecule of methyl ether + water.

From the formula it will become apparent that the oxygen is linked directly to the two carbon atoms, and that therefore no hydroxyl group (OH) is present in ether, for which reason ether is such an indifferent substance, not being acted upon by alkalies or weak acids.

Methyl ether ($CH_3—O—CH_3$) is a gas, liquefying at $-23°$; one volume of water dissolving 37 volumes of ether.

Ethyl ether ($CH_3 \cdot CH_2—O—CH_2 \cdot CH_3$) has a specific gravity of 0·736 at 0°, and boils at 35° C. It is soluble in about 10 times its own volume of water.

Esters or ethereal salts are formed by the union of alcohols with acids, analogously to the union of metallic hydroxides and acids, thus:

$$[K \cdot OH + HCl = KCl + H_2O]$$

$$CH_3 \cdot OH + HCl = CH_3Cl + H_2O$$

methyl alcohol + hydrochloric acid = methyl chloride or chloro-methane

Chloroform or trichloro-methane, $CHCl_3$, has a specific gravity of

[1] Substitution means the replacement in a molecule of one atom or a group of atoms by another atom or group of atoms, without the rest of the molecule having its combination of molecules interfered with. For example, C_4H_4, or methane, forms the mono-substitution product CH_3Cl or methyl chloride, the di-substitution product CH_2Cl_2 or methylene chloride, and the tri-substitution product $CHCl_3$ or chloroform.

1·498 at 15°, and boils at 61° C.[1] If chloroform is exposed to the action of light, especially in the presence of air, it becomes oxidized into the very poisonous carbonyl chloride (phosgene gas) and hydrochloric acid [$CHCl_3 + O = COCl_2 + HCl$]. This decomposition is readily detected by adding silver nitrate solution to the chloroform, as the latter, when pure, does not give a precipitate of silver chloride. Chloroform should also not darken when shaken with concentrated sulphuric acid or with strong potash[2].

Dihydric alcohols or glycols are di-substitution products of the paraffins, the two hydroxyl radicals being attached to two carbon atoms, thus:

ethane $CH_3—CH_3$ becomes ethylene glycol or $\begin{matrix} CH_2—OH \\ | \\ CH_2—OH. \end{matrix}$

Ethylene glycol is a colourless viscid fluid, miscible in all proportions with alcohol and water, but only slightly soluble in ether. By oxidation of one of the $CH_2—OH$ groups it is converted into glycolic acid, and by the oxidation of both groups into oxalic acid, thus:

$\begin{matrix} CH_2(OH) \\ | \\ COOH \end{matrix}$ glycollic acid $\qquad$ $\begin{matrix} COOH \\ | \\ COOH \end{matrix}$ oxalic acid[3]

Oxalic acid is a feeble reducing agent, precipitating gold from its solutions. It is dibasic in character, forming soluble alkali salts, e.g. ammonium oxalate:

$$\begin{matrix} COO\ NH_4 \\ | \\ COO\ NH_4 \end{matrix}$$

Trihydric alcohols, for example glycerol or glycerin, are compounds resulting from the substitution of three hydrogen atoms attached to three different carbon atoms by three hydroxyl groups, thus:

propyl alcohol or $CH_3—CH_2—CH_2—OH$ becomes glycerin $CH_2(OH)—CH(OH)—CH_2(OH)$.

Glycerin when pure is crystalline and melts at 17° C., but the commercial glycerin is a fluid having a specific gravity of 1·265 at 15° C. and boils at 290° C. It is insoluble in ether, but mixes readily with alcohol and water. When oxidized it forms acrylic acid $CH_2=CH—COOH$, a mono-carboxylic acid, which, being an unsaturated compound, forms additive compounds, for example:

$$CH_2—CH—COOH + Br_2 = CH_2Br—CHBr—COOH$$[4].

Glycerin is apt to undergo oxidation, especially if it contains traces of salt. By means of dilute nitric acid it is changed into glyceric acid:

[1] Do not heat tissues containing chloroform beyond 58°.

[2] Duncan and Flockart's chloroform prepared from pure ethyl alcohol is the best.

[3] Lactic acid or α-hydroxy-propionic acid ($CH_3—CH(OH)—COOH$) is formed analogously.

[4] See oleic acid, p. 304.

$$CH_2(OH)\cdot CH(OH)\cdot CH_2\cdot OH = CH_2(OH)\cdot CH(OH)\cdot COOH + H_2O$$
glycerine = glyceric acid.

On the addition of acetic or formic acids to glycerin (see p. 249 on mounting picro-carmin sections), mono-, di-, and triacetins or glyceryl acetates will be formed:

$$C_3H_5(OH)_3 + CH_3\cdot COOH = C_3H_5(OH)_2\cdot O\cdot CO\cdot CH_3 + H_2O$$
$$\text{,,} \quad \text{,,} \quad \text{,,} = C_3H_5(O\cdot CO\cdot CH_3)_2\cdot OH + 2H_2O$$
$$\text{,,} \quad \text{,,} \quad \text{,,} = C_3H_5(O\cdot CO\cdot CH_3)_3 + 3H_2O.$$

(6) Alcohols, Aldehydes, and Acetone.

(*a*) **Alcohols**, as shown in the table on p. 84, are derived from hydro-carbons by the substitution of the hydroxyl group, OH, for hydrogen, and have the constitutional formula $(C_nH_{2n+1})OH$. This hydroxyl group may react with other substances in one of two ways, for, on the one hand, the hydrogen, being a comparatively feeble kat-ion, may be replaced by a more powerful kat-ion (see p. 14). Thus methyl alcohol $CH_3\cdot O—H$ can unite with sodium, according to the formula $CH_3\cdot O—H + Na = CH_3\cdot ONa + H$, when it is said to behave as an acid. The new compound, sodium methylate, thus formed, on addition of water again decomposes into alcohol and the alkali. On the other hand, the hydroxyl group OH, being a feeble an-ion, may have its place taken by a stronger an-ion, namely chlorine, thus $CH_3\cdot OH + H\cdot Cl = CH_3Cl + H_2O$, when methyl chloride results. With sulphuric acid it yields either the methyl hydrogen sulphate ($CH_3\cdot OH + H_2SO_4 = CH_3\cdot HSO_4 + H_2O$) or methyl sulphate ($2CH_3\cdot OH + H_2SO_4 = (CH_3)_2SO_4 + 2H_2O$). With nitric acid methyl nitrate, $CH_3\cdot O(NO_2)$, is obtained.

In all these interactions with mineral acids the alcohol is said to act as a base. In the chapter on electro-chemistry it has been pointed out that the terms acid and alkaline ought to be restricted to the hydrogen and hydroxyl ions; and therefore when the methyl alcohol remainder $CH_3\cdot O—$ unites with the kat-ion sodium, it must play the part of an an-ion, and when the methyl alcohol remainder $CH_3—$ links on to the an-ion chlorine, it acts as a kat-ion. On substituting the replaceable OH by ammonia or its salts there result the ammonia bases or amins, e.g. methylamin, $CH_3\cdot NH_2$. It will be evident that alcohol, though neutral to litmus paper, is by no means a chemically inert substance. Towards natural albumins it acts as a coagulator, but freely dissolves both alkali and acid-albumins [1].

If tissues or their constituents be placed for a short time in absolute alcohol they do not lose their solubility, but if left for a long time they become quite insoluble, with the exception of peptones, albumoses, nucleic acid, and protamin. It is generally held that alcohol causes coagulation by the withdrawal of water, but whether it does not exert an effect analogous to the reactions given above is an open question. That nucleo-histones are acted upon by alcohol St.-Hilaire has shown (see p. 320).

[1] Otto Cohnheim: *The Chemistry of Proteids*—English translation, Macmillan and Co.

Ethyl alcohol ($C_2H_5 \cdot OH$) and methylated spirit may be dehydrated by means of calcined copper sulphate sewn up in bags and suspended in the solution. This method is cleaner than pouring the powder into the alcohol, and is also more efficacious; or proceed thus: Thoroughly wash gelatin in alternate changes of 10 per cent. salt solution and distilled water; remove the salt by washing; dry the gelatin; cut it into strips and put these into the alcohol to be rendered absolute[1].

To test alcohol for its purity, P. Mayer[2] adds to it 1 per cent. of the following mixture:—Haematein 1 grm., aluminium chloride 1 grm., and alcohol 100 ccm. With pure alcohol the haematein does not precipitate after 24 hours.

(*b*) **Acetone** ($CH_3—CO—CH_3$) is neutral in reaction. It mixes in all proportions with alcohol and ether; by oxidation with CrO_3 it splits up into acetic and carbonic acids. Towards tissues it probably acts as a reducing agent. I have given it an extensive trial, but it has no special features to recommend it as a fixative. When used pure it causes a greater shrinkage than alcohol (see p. 104). It may be used because of its great diffusibility for purposes of dehydration, and also as a solvent for 'anilin' dyes.

(*c*) **Formaldehyde.** The action of this compound on albumin was first investigated by Trillat in 1892[3], but to F. Blum in 1893 belongs the credit of having introduced it into Histology. Formaldehyde (see table on p. 84) is an oxidation product of methyl alcohol, $CH_3 \cdot OH$. It may be regarded either as methyl aldehyde, $H \cdot CHO$, or as the oxide of the diatomic radical methylene, CH_2, when its formula would be OCH_2. By exposure to light and warmth it is liable to become changed into paraformaldehyde, $C_3H_6O_3$.

Formaldehyde is a gas which is soluble in water to the extent of about 45 per cent. The commercial watery solution contains approximately 40 per cent., and is called formol[4]. The freshly-prepared solution is neutral to litmus, but is very apt to undergo further oxidation into formic acid, $H \cdot COOH$, especially if the solution is freely exposed to light and air. According to Sjöbring (see p. 91) the formol from Höchst is better than the formalin from Berlin. I only know the latter preparation.

When experimenting with it for micro-chemical purposes the free formic acid should be neutralized with magnesium or sodium carbonate and the aldehyde be freshly distilled. Great care must be taken not to use strong alkalies, as they induce aldehydes to polymerize, i.e. the molecules, having no foreign body to unite with, join among themselves. No other organic body is so prone to give such diverse reactions as aldehyde, because of the tendency of the O to pass from its double bond with the carbon $O=CH_2$ into a single one. Owing to this very reaction aldehydes act as reducers. With two

[1] The originator of this method I could not determine.

[2] P. Mayer: *Mittheil. Zool. Stat. Neapel*, 10, 180 (1891).

[3] Trillat: *Compt. Rend. de l'Acad. d. Sc.* 114, 1278 (1892).

[4] Formol is the name used by the firm Meister, Lucius and Brünning, Höchst a/M., while formalin is the term given to the watery solution of formaldehyde by the Chemische Fabrik auf Actien (Schering), Berlin.

molecules of alcohol, aldehydes form acetals, liberating water at the same time, especially readily in the presence of acetic acid (Geuther).

$$2(CH_3{\cdot}CH_2OH) + O{\cdot}CH{\cdot}CH_3 = 2(CH_3{\cdot}CH_2{\cdot}O) - CH{\cdot}CH_3$$

ethyl alcohol + ethylaldehyde = ethyl acetal.

Aldehydes unite, however, not only with hydroxyl groups, but also with ammonia to form ammonium aldehyde:

$$C\begin{matrix}H_2\\O\end{matrix} + NH_3 = C\begin{matrix}H_2\\NH_2\\OH\end{matrix}$$

which then splits up into hexa-methylene tetramine.

Similarly, amido compounds unite with aldehyde. Either one or two molecules of amido groups may join on to one molecule of aldehyde; thus urea behaves as follows:

$$CO\begin{matrix}NH_2\\NH_2\end{matrix} + OCH_2 = CO\begin{matrix}NH\\NH\end{matrix}CH_2 + H_2O$$

urea + aldehyde = methylene – urea + water.

(Blum, 1896.)

Or if two groups of urea unite to one molecule of aldehyde then the following union takes place:

$$CO\begin{matrix}NH_2\\NH_2\end{matrix} \quad CO\begin{matrix}NH_2\\NH\end{matrix}\!\!>\!CH_2 + H_2O.$$
$$+ OCH_2 =$$
$$CO\begin{matrix}NH_2\\NH_2\end{matrix} \quad CO\begin{matrix}NH\\NH_2\end{matrix}$$

(Benedicenti, 1896.)

If we represent a gelatin molecule by the formula (Gel = NH_2), its compound with OCH_2 would be

$$Gel = NH{\cdot}C\begin{matrix}H_2\\OH\end{matrix}$$

or if the gelatin molecule be

$$Gel\begin{matrix}NH_2\\NH_2\end{matrix}$$

its aldehyde compound would be represented by

$$Gel\begin{matrix}NH\\NH\end{matrix}CH_2$$

'Just as aldehyde compounds of ammonia and amido-bodies can be split up into their components, so is it possible to do the same with the aldehydes of egg and serum albumin, of casein and fibrin' (Benedicenti, 1896).

Schwarz[1] states that aldehydes may unite not only with the amido NH_2 group, but also with the imide radical NH or with nitrogen in a tertiary form[2]; further, with the carbon atom CH_2 of the methylene group, if the latter stands in close proximity to a nitrogen atom as it does in hippuric acid —CO·NH·CH_2·CO—. A single NH_2

[1] Leo Schwarz: Über d. Verbindungen d. Eiweisskörper mit Aldehyden, *Zeitsch. f. physiol. Chem.* 31, 460 (1901).

[2] Pal: *Ber. d. deutsch. chem. Gesellsch.* 29, 1086 (1896).

group may fix two or even more aldehyde remainders[1], and as amido-acids of the fatty series are the chief products of dissociation of albumins, such compounds as monomethyl asparagin[2] must be kept in mind; further, as the furfurol reaction given by the tests of Molisch and Liebermann is absent, the carbohydrate radical of proteids may fix the aldehyde, because Tollens and his pupils[3] have shown aldehydes to act on plurivalent alcohols and acids of the sugar group: the oxygen of the aldehyde unites with two hydrogen atoms of the second body to form water, while methylene CH_2 takes the place of the hydrogen. The SH group of the proteid-molecule and the tryptophane radical may also link on to the aldehyde.

As no nitrogen is split off by treating proteid substances with aldehydes, it has been possible to determine that a prolonged action of aldehyde leads to much larger quantities of aldehyde being fixed chemically by the cell. The following table of Schwarz bears out this statement:

	Proteid used.	N : C *in the natural proteid.*	*Aldehyde used.*	*Length of time the aldehyde acted.*	N : C *in the proteid acted upon.*	*Increase of* C *atoms for* 100 N.	*Number of aldehyde molecules which entered for* 100 N.
1	Serum-albumin	100 : 338	formaldehyde	short time	100 : 362	24	24
2	,, ,,	,, ,,	,, ,,	,, ,,	100 : 359	21	21
3	,, ,,	,, ,,	,, ,,	2 months	100 : 381	43	43
4	,, ,,	,, ,,	acetaldehyde	short time	100 : 413	75	37·5
5	,, ,,	,, ,,	,, ,,	2 months	100 : 431	93	46·5
6	,, ,,	,, ,,	benzaldehyde	1 hour	100 : 367	29	4
7	Edestin	100 : 272	formaldehyde	3 days	100 : 291	19	19
8	Hetero-albumose	100 : 307	,, ,,	1 day	100 : 356	49	49
9	Iodized egg-albumin	100 : 336	,, ,,	7 days	100 : 337	1	1

This table also shows how, as the result of maximal action, practically the same number of molecules are absorbed from solutions of formaldehyde and acetaldehyde. Curiously enough, iodized egg-albumin has completely lost its power of taking up formaldehyde, which fact cannot be explained by assuming that the iodine has usurped the place which the methylene group might occupy; because Kurajeff has shown that serum-albumin takes up 5·5 iodine atoms for every 58 nitrogen atoms present in the proteid, while the same amount of nitrogen combines with 24 molecules of aldehyde[4].

It is also interesting to note that trypsin cannot act on albumin which has been treated with form- or acet-aldehyde, but pepsin, probably owing to the presence of hydrochloric acid, does attack both methylated and ethylated albumin.

The visible changes produced in proteids by aldehydes have been

[1] S. Erlenmeyer, jun.: *Ber. d. deutsch. chem. Gesellsch.* 30, 2896 (1897).
[2] H. Schiff: *Chemiker-Zeitung*, 23, 20 (1899).
[3] Tollens: *Ber. d. deutsch. chem. Gesellsch.* 27, 1892 (1894), and 30, 2510 (1897).
[4] Kurajeff has assigned to serum-albumin the formula $C_{223}H_{360}N_{58}S_3O_7$. *Z. f. physiol. Chem.* 26, 462 (1899).

carefully studied by several observers. Trillat, in 1892[1], was the first to state that formaldehyde coagulates albumin, forming a transparent gelatinous mass. Blum, in 1893[2], described how egg and serum-albumin, after having been acted upon by formaldehyde, do not coagulate on heating; how by the addition of acids, alcohol, and acetone, they are precipitated, but on addition of water are rendered soluble again. Benedicenti, in 1896, found gelatin to become hardened and insoluble; blood-serum was converted into a jelly, fibrin and casein no longer swelled up and became, like egg-white, indigestible, and by a stream of steam it was possible to drive off the formaldehyde, and thereby to restore the characteristics of ordinary albumin[3]. Bach, in 1897, noticed that the coagulative power of alcohol after formaldehyde fixation was less than in the case of natural albumin.

Schwarz (p. 89), in 1901, studied not only formaldehyde, but also acet-, propionic, oenanth-, isovaleric and isobutyric aldehydes, and two aromatic compounds, namely, benz- and salicylic aldehydes. His results may be classed into two groups, according as to whether the pure proteids with which he worked contained, or did not contain, salts.

The heat coagulation of egg-albumin, after the addition of formaldehyde, is only prevented in the absence of salts. If the latter be present in abundance, coagulation will occur in the cold. According to Schwarz ordinary egg-white treated directly with formaldehyde behaves as first pointed out by Trillat in 1892; it coagulates into a transparent gelatinous mass. This, according to my observations, never takes place with commercial formaldehyde, because the latter contains free formic acid[4]. A serum-globulin solution containing sufficient ammonium sulphate to just remain in solution, reacts when treated with formaldehyde like a salt-free serum-albumin solution; but if the mixture be kept for some time, a milky opalescence makes its appearance. In such a serum-globulin solution an excess of formaldehyde produces a thick jelly, resembling that of Lieberkühn. Horse-serum with formaldehyde gives primarily a clear solution, non-coagulable by heat; but after twenty-four hours becomes turbid, and after a few days forms a firm jelly.

The presence of salts makes the formaldehyde-serum-albumin coagulable in 96 per cent. spirit; acet- and propionic aldehydes cause a turbidity which increases on further additions of these aldehydes, while benz- and salicylic aldehydes form at once a precipitate on being added to an albumin solution containing ammonium sulphate.

As both hydroxyl groups and amido groups occur in the body, as may be inferred by the decomposition of 'proteids' into oxybenzols and amido bodies (see p. 281), aldehydes act as fixing reagents by forming new chemical compounds with the various cell constituents.

Nils Sjöbring[5] has arrived at the conclusion that formaldehyde

[1] *Compt. Rend. de l'Acad. d. Sc.* 114, 1278 (1892).
[2] Blum: *Zeit. f. wiss. Mikr.* 10, 314 (1893).
[3] Benedicenti: *Arch. f. Physiol.* 219 (1897).
[4] See pp. 106 and 107, Experiments 4 and 5.
[5] Sjöbring: 'Über d. Formol als Fixirungsflüssigkeit,' *Anat. Anz.* 17, 273 (March, 1900).

acts as an oxidizer, because of the similarity in appearance of cells which are fixed in formaldehyde or osmium, or in chrome mixtures containing either osmium or formaldehyde, and, lastly, because of the resemblance between the histological appearances obtainable with methyl alcohol and formaldehyde.

We know that the living organism readily reduces such compounds as methylene-blue; but to suppose a decomposition to take place according to the formula given by Sjöbring, namely, $CHOH + H_2O = CH_3OH + O$, in which formaldehyde is reduced to methyl alcohol and nascent oxygen, seems to me very unlikely, besides which methyl alcohol produces quite different appearances histologically from those seen after aldehyde fixation.

Formol, when not used in full strength, should always be diluted with normal salt solution (0·75 NaCl), and never with water, because watery solutions cause such tissues as blood-corpuscles and the central nervous system to swell up in whatever strength formol may be used. In most cases it will also be advisable to neutralize the free formic acid by the means indicated above. In fixing whole eyes, however, either an incision must be made into the sclerotic coat or formol solutions be used which contain no salt, because otherwise the bulb will collapse owing to exosmosis taking place.

(7) Soaps and alkalies.

(*a*) **Lysol** was introduced by Reinke in 1893. It is a solution of creosol in neutral soap. Creosol is the mono-methyl ether of homo-pyrocatechin $C_6H_3 \cdot CH_3 \cdot OH \cdot OH. = 1 : 3 : 4$, and possesses the formula $C_6H_3CH_3(OH)(O \cdot CH_3)$. Cresol $= C_6H_4(CH_3)OH$.

Lysol is a good macerating agent for the keratinized cells of hair and epidermis; although it does not precipitate (Fischer) it is used by Reinke for the demonstration of his oedematin granules in nuclei.

I have not given this method as yet a fair trial, and therefore refrain from any other remarks, but call to mind that precipitation and fixation are not necessarily one and the same, as pointed out in connexion with osmium on p. 82.

(*b*) **Caustic potash**, especially in 33 per cent. solutions, may be regarded as a fixative for muscle cells and glands, although, generally, it is classed amongst the macerating agents.

(*c*) **Ammonia** acts as a precipitant for histone, and on this account is perhaps not so objectionable in picro-carmin as is believed by Paul Mayer, especially as this stain should only be used for fresh tissues.

(8) Other substances.

Iodine dissolved in potassium iodide forms probably the compound KI_3. Iodine thus plays the part of an acid radical in the potassium iodide compound; but if the latter, chemically pure and quite free of carbonates, be placed on litmus paper or on turmeric, a distinctly alkaline reaction is obtained on adding water, which suggests that KI breaks up hydrolytically into $KOH + HI$.

This curious behaviour explains why a mixture of iodine-iodide

of potash in some respects behaves as a feeble alkali, extracting, for example, that radical of Nissl's bodies in nerve-cells, which has an affinity for basic dyes. The first to use iodine for fixing purposes was Kent[1] in 1881 (see formula on p. 95).

Hofmeister[2] has shown that the yellow colour of a watery iodine solution and the blue colour of iodide of starch solution disappear on the addition of crystallized pure egg-albumin. Further additions of iodine to neutral egg-albumin produce an acid reaction, and terminate in the formation of a yellow coagulum. Iodized albumin contains nearly 9 per cent. of iodine, which latter replaces the hydroxyl group in the tyrosin radical of albumin, and thereby prevents the Millon reaction (see p. 321). Because of this alteration in the aromatic nucleus the Adamkiewicz test (see p. 323) also fails. Iodine and iodic acid further act on the unoxidized sulphur in albumin crystals, changing it from a mercaptan or sulphide form into sulphonic acid.

Potassium permanganate, $KMnO_4$, may be used in $\frac{1}{10}$ to 1 per cent. solutions for objects not thicker than 1 mm. As regards histological appearance it fixes somewhat like osmium tetroxide, but is inferior to the latter because it acts as an electrolyte. The tissues also become very brittle when transferred to alcohol.

CHAPTER V.

Fixing Fluids.

Formulae of the more important Fixing Fluids, Arranged in chronological order.

Instead of using the reagents which have been mentioned so far, singly, it has been found that combinations of two or more of them give better results, because of the different nature of the cell contents which have to be acted on. These compound mixtures are usually termed fluids or solutions, and are called after their originators.

Heinrich Müller's Fluid, 1859 (*Verhandl. d. phys.-med. Gesellsch.*, Würzburg).

Potassium bichromate	2·5	grm.
Sodium sulphate	1	,,
Water	100	cc.

Fix tissues in the dark for about three weeks. A human spinal cord requires for good fixation fully three months, a human brain six to ten months. Change the fluid during the first week every day. During the second week every other day. Twice in the third week, and after that weekly up to six weeks. Henceforth every other week. The great advantage possessed by it is that it fixes and hardens uniformly. The chief disadvantage is bad fixation of the nuclear structures, and hence arises the necessity of combining it for general work with acetic acid as recommended by Burchardt and Tellyesniczky (p. 100). For

[1] Kent: *Manual of the Infusoria*, 1881, p. 114; *Jour. R. Micr. Soc. London* (2), 3, 730 (1883).

[2] Hofmeister: 'Über jodirtes Eieralbumin,' *Zeitsch. f. physiol. Chemie*, 24, 159–172 (1897).

special work on the central nervous system it has been combined with osmic acid (p. 274), sublimate (p. 272), and formaldehyde (p. 275). After the fixation has been completed the tissues are rinsed in water for one minute and then transferred to 70 per cent. spirit, which must be kept in the dark (Stirling, *Text-book of Practical Histology*, Smith, Elder and Co., 1881, and Virchow, *A. f. mikr. Anat.* 24, 117 (1885)). Care must be taken to ensure ready access of the spirit from all sides, for which purpose the tissue should be either laid on absorbent cotton wool or be suspended by means of a thread, which is prevented from injuring the tissue by wrapping the latter in some absorbent cotton wool.

The 70 per cent. spirit should be changed four times in the course of as many days. It may be found to be still coloured yellow, which indicates that the whole of the free chromic salts have not been removed. A complete removal of the fixative is, however, unnecessary because the last traces are readily got rid of by Paul Mayer's method (Lee and Mayer, *Mikroscopische Technik*, p. 29), who treats sections which are stained yellow by either chromic acid or any of its salts, with Flemming's acid alcohol, containing 1 part of HCl in 1000 parts of absolute alcohol. [16 drops of 25 per cent. HCl = 1 cc.] To allow of ready staining in bulk he treats tissues for some hours in a mixture of sulphuric acid 5 parts and water 95 parts; they will turn a greyish-green colour, and should be very carefully washed in running water to remove all traces of acid if carmin solutions are to be employed.

Erlicky's (1877) and **Kultschitzky's** (1877) **Fluids** (Erlicky: *Warschauer Med. Zeit.* 22, Nos. 15 and 18, 1877. Kultschitzky: *Z. f. wiss. Mikrosc.* 4, 348, 1887).

Erlicky's fluid (potassium bichromate $2\frac{1}{2}$, copper sulphate $\frac{1}{2}$, water 100) I have discarded altogether, for although it fixes quicker than Müller's solution, I prefer the latter. A modification of this solution, namely that of Kultschitzky, is however still one of the best for the central nervous system. To make the solution, proceed as follows:— Dissolve in 100 cc. of a hot 20 per cent. potassium bichromate solution, ten grams of copper sulphate; allow this mixture to cool; in the photographic dark room add to the cooled mixture an equal quantity of absolute ethyl alcohol, or methylated spirit rendered absolute, so as to have a saturated solution of bichromate and copper sulphate in 50 per cent. alcohol. Kultschitzky saturates 50 per cent. spirit in the dark ad lib. with finely powdered bichromate and copper sulphate. Before use add 5 to 6 drops of glacial acetic acid to every 100 cc. of the fixing solution. Fix tissues not thicker than 5 to 10 mm. in this solution for forty-eight hours, taking care to prevent all access of light. Then transfer them, still in the dark, to two changes of 75 per cent. spirit, and one change of 90 per cent. spirit. The further manipulations necessary for the celloidin or paraffin process may now be done in the light.

Kleinenberg's Fluid, 1876 (Foster and Balfour).

To a saturated picric acid solution 100 cc. add one cc. of sulphuric acid. Shake vigorously. Filter off the precipitated picric acid and dilute the filtrate with twice its bulk in distilled water.

Fix tissues not thicker than $\frac{1}{2}$ cm. or small embryos for three hours, and then transfer them directly into 70 per cent. spirit. Change this three times in the course of the first day. Now transfer to two changes of 80 per cent. spirit and 90 per cent. spirit during the second and third day. Do not trouble to remove all traces of the picric acid, because it is readily discharged from the sections with 75 per cent. spirit. On no account use alkalies in any form for extracting the yellow colour.

Paul Mayer's Fluid, 1880 (*Mitth. Zool. Stat. Neapel*, 2, 5).

Saturated picric acid . .	100 cc.	} Filter and use
Nitric acid . . .	2 „	} undiluted [1].

This fluid, in addition to fixing purposes, may be used for decalcifying bones which have been fixed in formol or sublimate solutions; I only use it for the latter purpose.

Kent, in 1881 (*Manual of the Infusoria*, p. 114), introduced iodine + potassium iodide as a fixative. He saturated with iodine a saturated solution of potassium iodide in water, and diluted this stock solution for use with distilled water till it was of a light-brown colour. For Remak's method of 1854, see footnote, p. 77.

Overton, 1890 (*Z. f. wiss. Mikr.* 7, 14) has used the vapour of iodine for fixing purposes by proceeding in this way: A few crystals of iodine are placed in a test-tube and heated till the violet iodine gas is formed. By inverting the test-tube the heavy gas is made to stream over the tissue to be fixed. To remove the iodine the tissues are next warmed for two to three minutes up to 40° C., and are then mounted. (See p. 72 for Overton's sulphurous acid method.)

Betz, in 1893 (*Arch. f. mikr. Anat.* 9, 101), used iodized 75–80 per cent. alcohol for hardening the spinal cord, adding new iodine as the brown colour disappeared. After the cord, without the dura, had remained for three days in this alcohol the pia mater was removed and the cord allowed to stay another three days. It was then removed to 3 per cent. potassium bichromate till a brown precipitate showed on its surface and then kept in 0·5 to 1 per cent. potassium bichromate; the cerebrum halved sagitally is hardened ultimately in 4 per cent. and the cerebellum in 5 per cent. bichromate solution. I have used, especially for investigating the structure of nerve cells, a 5 per cent. solution of potassium iodide containing 2·5 per cent. of iodine. After fixing for twenty-four hours the tissues are placed directly in 70 per cent. spirit.

Waddington, 1883 (*Journ. R. Micr. Soc. London* (2), 3, 185), recommends sulphurous acid as a substitute for osmic acid. See p. 72 for Overton's method of readily preparing an alcoholic solution of SO_2.

Overton uses also SO_2 dissolved in a watery solution of picric acid, or in a solution of picric acid in 30 to 50 per cent. alcohol.

Sulphurous acid can be recommended both for nuclei and the cell body. I use preferably the gas (SO_2), or where this is inadmissible, the gas dissolved in 70 per cent. methyl alcohol.

Walther Flemming's Fluids: weak fixative, 1882 (*Zellsubstanz, Kern und Zelltheilung*, Leipzig, p. 381), and strong fixative, 1884 (*Z. f. wiss. Mikr.* 1, 349).

	Weak solution.	*Strong solution.*
1 per cent. osmium tetroxide . .	10 cc.	40 cc.
20 per cent. glacial acetic acid .	0·5 „	25 „
10 per cent. chromic acid . .	2·5 „	7·5 „
Water	87 „	27·5 „
	100	100

The solutions should be freshly prepared and be used in the dark.

The strong solution is to be preferred to the weaker one, and is especially applicable for the study of nuclei and the testis. I cannot recommend it for

[1] Paul Mayer states in Lee and Mayer, p. 48, that he takes water 100 parts, nitric acid (25 per cent. N_2O_5) 5 parts, and then saturates this mixture with picric acid.

tissues containing much white fibrous tissue, because the latter is swelled by the acetic acid, and requires to remain at least a month in the fixative to allow the chromic acid to render it resistant to the washing-out process, while in the meantime the other constituents macerate.

Tissues to be fixed in this solution should not exceed 2·5–3 mm. in thickness, and must remain in the solution at least twenty-four and not longer than forty-eight hours. They are then to be washed in running water at least twenty-four hours, or to be treated with sulphurous acid (see p. 73).

The disadvantage of this fluid, as that of all mixtures containing osmic acid, is the 'overfixation' of the outermost layers of the tissue, which therefore have a glassy homogeneous appearance and do not stain well. A great improvement may be achieved by decomposing the osmium with oxidizing agents as indicated on p. 83.

F. Hermann's Fluid, 1889 (*A. f. mikr. Anat.* 34, 58).

1 per cent. platinum chloride . .	75 cc.
2 per cent. osmium tetroxide . . .	4 (or 2)
Glacial acetic acid	1

This solution is a modification of those of Flemming and Podwyssozki. The latter had added corrosive sublimate to Flemming's solution to increase the penetrating power of the osmium, and thus to get better fixation of gland-cells (Podwyssozki, *Ziegler's Beiträge*, 1, 1886).

Comparisons between a solution made up according to the above formula and one made up with the same amount of corrosive sublimate instead of the 'platinum chloride' have convinced me that the sublimate mixture is at least equal, if not superior, to Hermann's solution as regards the power of delicate fixation, and has the great advantage of cheapness. It must be remembered, however, that platinum salts form with safranin especially stable compounds.

Tissues not thicker than 2–3 mm. are fixed for forty-eight hours, and subsequently are treated as stated for Flemming's solution.

Mann's Fluids, 1890–1900[1].

(*a*) 1890–3 Formulae (*Transact. and Proceed. Bot. Soc. Edinb.* 18, 429, 443, Session 1889–90. Also 1893, *Anat. Anz.* 8, 441).

Saturated watery and alcoholic solutions of corrosive sublimate were saturated with picric acid.

(*b*) 1894 (May) *Journ. of Anat. and Physiol.* 101, and *Z. f. wiss. Mikr.* (November), 480.

Saturated solution of sublimate in 0·75 salt solution	100 cc.
Picric acid	1 grm.
With or without tannin	1 „

The solutions (*a*) and (*b*) I no longer use. (See p. 119.)

(*c*) 1895.

Saturated $HgCl_2$ in $\frac{3}{4}$ per cent. NaCl = 1
Saturated water sol. of picric acid = 3

This solution has a specific gravity of 1020.

(*d*) From 1895 up to the present time I have used two solutions.

The one without formaldehyde (A) is used for micro-chemical investigations, while the solution B, containing formaldehyde, is

[1] I have thought it advisable to place all my methods together to facilitate references.

used to obtain the best fixation possible with the least subsequent shrinkage during the paraffin process:

Sol. A. (Sol. B.)

Boiling water . .	100 cc.
Sublimate . . .	2·5 grm.
When dissolved add—	
Picric acid . .	1 grm.
Allow to cool, and immediately before use add—	
Formol . .	10 to 25 cc.

Preferably inject the fluid, and where this is inadmissible, fix tissues not exceeding 7–10 mm. in thickness according to the times specified herewith: 1 mm. for 20 minutes, 2 mm. for 1 hour, 3 mm. for 4 hours, 4 mm. for 8 hours, and for every additional mm. allow also four hours. Thus a tissue measuring one cm. in thickness requires twenty-seven hours twenty minutes, or in round figures thirty hours. Transfer directly to 50 per cent. spirit and change this in the course of the first day twice, then take through 60 per cent., 70 per cent., 80 per cent., 90 per cent. and absolute alcohol. The precautions to be taken are detailed below under 'General Principles' on p. 144.

(*e*) In 1894 (*Z. f. wiss. Mikr.* 9, 481), I recommended osmo-sublimate for nerve-cells, but have also used this mixture extensively for gland-cells and other tissues:

Saturated sublimate in $\frac{3}{4}$ per cent. salt sodium	50 cc.
1 per cent. osmium tetroxide . . .	50 ,,

Fix for forty-eight hours in the dark. Transfer to 50 per cent. spirit in the dark, and treat subsequently as stated for the picro-corrosive solutions.

A modification of this solution is that published by Cox, 1896, who fixes spinal ganglia in: Saturated sublimate solution = 30 parts; 1 per cent. osmic acid = 10 parts, and glacial acetic acid = 5 parts.

The first to employ osmic acid in combination with sublimate was Braun, 1886 (*Zool. Anz.* 458), who fixed Alcyonium, Sympodium, Gorgonia, &c., with a boiling, saturated mixture of sublimate in sea-water (20–25 cc.), to which 4–5 drops of 1 per cent. osmic acid had been added. He fixed for five minutes and then transferred the animals to 30 per cent. spirit. As already stated above under 'Hermann's Fluid,' Podwyssozki used a combination of sublimate and Flemming's solution.

(*f*) A 5 per cent. solution of the double salt of corrosive sublimate and potassium bichromate ($HgCl_2 + K_2Cr_2O_7$) has given good results with electric organs, tissues rich in connective tissues and gland-cells, and has been in use since 1895 (see p. 76). (First published in Ballowitz's paper on the electrical organ of Malopterurus (Jena, 1899).)

(*g*) The chromo-corrosive solution for the central nervous system (1896) contains:

Corrosive sublimate	5 grm.
Chromic acid	1 ,,
Water	100 cc.

(*h*) The formol-sublimate mixture (a modification of Brause's Fluid, see p. 100):

Sublimate, 2·5 per cent.	50 cc.
Formol	20 ,,
Water	30 ,,

This solution is much weaker than Brause's solution as regards the sublimate mixture (see experiments on pp. 117–121, and deductions to be drawn from them).

(*i*) The formol-osmium mixture is given on p. 99.

(*k*) The sulphurous acid methyl-alcohol mixture is mentioned on p. 95, and sulphuric acid-alcohol on p. 71.

Experiments have been made, during the last five years, with sulphurous acid as vapour and in solution, with sulphuric, phosphoric, phosphomolybdic, phosphotungstic, trichloracetic, nucleic, and other acids, all in strength of 1–10 per cent. solutions, and with cyanogen compounds. Particulars of these experiments will be published later. Attention was drawn (p. 78) to the fact that of all the chlorides of the heavy metals sublimate is the best. Experiments, not quite completed, with mercuric nitrate and mercuric sulphate lead me to suppose that they are more delicate fixatives than sublimate.

(*l*) Sodium chloride and ammonium sulphate, used either by themselves or in combination with osmium tetroxide, and iodine potassium iodide solutions were used in connexion with the examination of fresh tissues.

F. Blum, 1893. Formaldehyde[1] Methods. This exceedingly important fixative (see p. 88) was advocated by F. Blum in 1893 because of its great penetrating power, the good preservation of the tissue elements, and the readiness with which cells are stained. By many observers the use of formaldehyde is neglected, probably because of bad results which were obtained owing to a faulty method of using it. Formol (= 40 per cent. solution of formaldehyde) should not be used diluted with water, but with some solution which is isotonic with the juices of the organism to be fixed. In general practice a 0·75 sodium chloride solution answers this purpose. Secondly, if formol is not to be followed by some other fixative, such as salts of the heavy metals or chrome-compounds, tissues should be transferred from the formol fixative directly into at least 90 per cent. spirit.

Blum's Fluid, 1893 (*Zeitsch. f. wiss. Mikros.* 10, 314).

Dilute the commercial 40 per cent. solution of formaldehyde with ten times its volume of water. In 1896 Blum fixed tissues for six or eight hours, and then transferred them directly to alcohol.

Hoyer, junr., 1894 (*Verh. Anat. Gesellsch.* 9. Vers., 236) fixes in pure formol, and then transfers directly to alcohol.

Lavdowsky, 1894 (*Anat. Hefte*, Abth. 1, 4, 355), was the first to use a mixture of alcohol and formaline, advocating the following mixture:

Formol.	95 *p.c. Alcohol.*	*Water.*	*Glacial acetic acid.*
6	20	40	1

Mann, 1895. I found on treating blood with 10 per cent. watery

[1] It is called formalin by Schering and Co., or formol by Meister, Lucius, and Brüning, or formalose by an American firm; according to Lee and Mayer: *Grundzüge d. mikr. Technik.* 1901, p. 62.

formol (=4 per cent. formaldehyde), that red blood-corpuscles swelled up and behaved as if they had been placed in water. Since that time formol has been always firstly neutralized (see p. 88), and then diluted with normal saline (0·75 per cent. NaCl). R. Muir tells me that he has also worked on this principle of using normal saline when working with blood.

Parker and Floyd, in 1895 (*Anat. Anz.* 11, 156), attained the same end by using an alcoholic solution of formol for hardening brains. They found a 2 per cent. watery solution of formol, two parts, mixed with 95 per cent. alcohol, three parts, to answer best.

Sjöbring, in 1900 (*Anat. Anz.* 17, 273–304), insists also on using isotonic solutions and hardening thoroughly with alcohol. Mammalian tissue is best fixed for forty-eight hours or longer in 20 per cent. formol (= 8 per cent. formaldehyde) and then transferred directly, for at least two days, to 95 per cent. alcohol. Paraffin sections, fixed to slides, may be mordanted with 1 per cent. chromic acid or 5 per cent. potassium bichromate.

Dreyer and Mann, 1900, have used combinations of formol with osmium tetroxide:

	Formol.	OsO_4.	*Water.*	1 *p.c.* NaCl.
Dreyer .	10 cc.	1 grm.	90 cc.	——
Mann .	20 ,,	0·5 ,,	——	80 cc.

Dreyer uses the mixture for blood and Mann for fresh tissues generally, in particular ciliated cells, peri-oesophageal membrane of the frog, and nerves.

Formol has been most extensively used for the preservation of the central and peripheral nervous system.

Weigert (1895) fixes for his neuroglia method (see p. 236) pieces of the central nervous system not thicker than 5 mm. for four days in 10 per cent. formol. Lachi uses 20 per cent. formol (*Zeitsch. f. wiss. Mikr.* 12, 32, 1895); van Gieson 4, 6 and 10 per cent. formol (*Anat. Anz.* 10, 494, 1895) for medullated nerves. Vassale and Donaggio substituted, in 1895, formol for osmium tetroxide in Golgi's impregnation method (*Monitore Z. Ital. Anno* 6, 82, 1895), a procedure also advocated by Strong (*Anat. Anz.* 10, 494, 1895); Durig (*Anat. Anz.* 10, 659, 1895); Fish (*Trans. Amer. Micr. Soc.* 17, 319, 1896); and Kopsch (*Anat. Anz.* 11, 727, 1896).

The impregnation methods are given on p. 264.

C. Rabl, 1894 (*Z. f. wiss. Mikr.* 9, 165), quite independently of me, discovered the good qualities of picro-corrosive solutions. His formula is:

Saturated watery solution of sublimate . .	1 cc.
Saturated watery solution of picric acid . .	1 ,,
Water	2 ,,

R. Altmann's Fluids, 1894 (*Die Elementarorganismen*, &c., Leipzig):

(*a*) Equal parts of 5 per cent. potassium bichromate.
2 per cent. osmium tetroxide.

Fix fresh tissues for twenty-four hours; wash for some hours in running water; transfer in succession to 75, 90, and 100 per cent. alcohol.

(*b*)

Saturated solution of mercuric nitrate . .	1 cc.
Water	3 ,,
Formic acid (1·12 sp. gr.)	1 ,,

Prepare this solution immediately before use, and fix small pieces of tissue for several hours.

K. Zenker's Fluid, 1894 (*Münchener med. Wochenschr.* Jahrg. 41, 532–534):

Müller's solution	100 cc.
Sublimate	5 grm.
Glacial acetic acid	5 cc.

The glacial acetic acid must be added immediately before use.

This solution is one of the very best for all purposes, and I find it often difficult to say whether it or the picro-corrosive formaldehyde gives the best results as regards fixation, although the presence of the chrome modifies the primary staining reactions considerably. This latter point, however, can be overcome by removing the chromic acid by Mayer's method given under 'Müller's Fluid.'

I usually take instead of Müller's solution simply a 2·5 per cent. bichromate solution, omitting the 1 per cent. sodium sulphate, without having noticed any difference in the fixing reaction; see, however, p. 131, on the difference of Müller's solution and a 2·5 per cent. bichromate solution.

O. vom Rath, 1895 (*Anat. Anz.* 11, 286), has given several formulae, of which the following is the best:

Saturated watery picric acid . . .	100 cc.
Saturated sublimate solution . . .	100 ,,
2 per cent. osmic acid	20 ,,
Glacial acetic acid (optional) . . .	2 ,,

After-treatment with crude pyroligneous acid or tannin for staining purposes and with iodine potassium iodide to remove the sublimate.

Weigert's 1895 fixing methods are given on p. 236.

Brause, 1896 (*Denksch. med.-naturw. Gesellsch.*, Jena, 309, 1896):

Saturated (7·5 per cent.) watery sublimate .	75 cc.
40 per cent. Formaldehyde (Formol) . .	25 ,,

Burchardt, 1897 (*La Cellule*, 12, 337):

Barium bichromate, 4 per cent. watery solution	60 cc.
Potassium bichromate, 5 per cent. watery solution	30 ,,
Glacial acetic acid	5 ,,

The barium bichromate fixes the nuclei, the potassium salt the cell, while the acetic acid prevents the deleterious action of the potash salt on the nuclei. See also the next fixative.

K. Tellyesniczky's Fluid, 1898 (*Arch. f. mikr. Anat.* 52, 202):

Potassium bichromate	3 grm.
Glacial acetic acid	5 cc.
Water	100 ,,

This is a thoroughly rational formula, according to the author's own showing, as the cytoplasm is preserved by the bichromate, while the nuclear elements are fixed by the glacial acetic acid (and also by the chromic acid which will be liberated by mass action). I have used this solution both for injecting animals and also for the fixation of pieces of tissue. Its penetrating power is as great as that of Müller's fluid, and it is thoroughly to be recommended for embryos of all ages, for ovaries and testes and glandular structures. The cells in the central nervous system are well preserved by it, and those who believe, which I do not, that the thorns of the protoplasmic or dendritic processes represent a normal

appearance, will find them brought out by this solution as by no other, especially after staining in Ehrlich's acid haematein.

Alcohol is perhaps the oldest of all fixatives, having been used by Leeuwenhoek. In modern times it is used very considerably because it is especially suitable for micro-chemical investigations; but when used pure, it has the great tendency, although to a lesser extent than acetone, of causing the nuclear contents to be driven against that side of the nuclear membrane furthest away from the surface of the tissue. There are produced in this way crescentic or rather hemispherical conglomerations in the nuclei (Tellyesniczky). This phenomenon is not observed if animals are injected with 90 per cent. alcohol.

The following alcoholic fixatives are all to be relied on for general histological purposes:

Squire in 1892[1] advocated the placing of tissues for fixing purposes in gradually increased strengths of alcohol, commencing with 50 per cent. alcohol for 1 to 2 days; the same length of time in 70 per cent. and to complete fixing and hardening in 90 per cent. strength; after fixation is achieved tissues should be preserved in 70 per cent. alcohol.

Carnoy[2] recommends two formulae, namely glacial acetic acid 1 part, absolute alcohol 3 parts, and secondly this mixture: glacial acetic acid 1 part, absolute alcohol 6 parts, chloroform 3 parts. The chloroform is said to hasten the action of the other constituents, but it also causes coagulation of albumin.

After fixation I carefully wash out the acetic acid with pure absolute alcohol.

Van Beneden and Neyt[3] used for their researches on the eggs of Ascaris a mixture of absolute alcohol and glacial acetic acid in equal parts. It should be made up immediately before use.

It must be remembered that glacial acetic acid, either by itself or in combination with absolute alcohol, is a non-electrolyte, and that for this reason a truer histological fixation is attained by it than with solutions containing electrolytes such as the following, which has been devised for the study of centrosomes in the non-striped muscle of the cat's intestine.

Lenhossék, 1899 (*Anat. Anz.* 16, Nos. 13, 14, pp. 334–342), has found the following alcoholic mixture best;

Lenhossék's Fluid:

Apáthy's mixture	50 per cent. alcohol	100	75 cc.
	Sodium chloride	5	
	Sublimate	4	
	Absolute alcohol		25 ,,
	Glacial acetic acid		5 ,,

Fix for six hours. Transfer to 90, 96, and 100 per cent. alcohol for twenty-four hours each.

[1] P. W. Squire: *Methods and formulae used in the preparation of animal and vegetable tissues*, &c., London, 1892.

[2] Carnoy: *La Cellule*, 3, 6 and 276 (1887).

[3] V. Beneden and Neyt: *Bull. Acad. Belg.* 14, 218 (1887).

CHART SHOWING THE MACROSCOPICAL EFFECTS PRODUCED BY FIXATIVES ON DIFFERENT CELL-CONSTITUENTS.

%	Fixative.	*Amphopeptone.*	*Deuteroalbumose.*	*Protalbumose.*	*Serumalbumin.*	*Globulin.*	*Haemoglobin.*	*Nucleoalbumin.*	*Nuclein.*	*Nucleic acid.*	*Protamin.*	*Gelatin.*
		r | *b*	*r* | *b*	*r* | *b*	*r* | *b*	*r* | *b*	*r* | *b*	*r* | *b*	*r* | *b*	*r* | *b*	*r* | *b*	*r* | *b*
3	Nitric acid	O	O	♁	♁	♁	+	X	+	+	O	O
1	Chromic acid	O	+	+	+	+	+	+	+	+	+	+
10	Acetic acid	O	O	♁	♁	♁	♁	+	+	+	O	+ O
Sat.	Picric acid	X	X	X	X?	X?	X?	X?	X	X		X
2	Tannic acid	X?	X?	X?	+	+	+	+	+	?	+	+
1	Osmium tetroxide . .	+	+ | O	+ | O	+ | O	+ | O	+	O	O	O	—	O ?
2·5	Potassium bichromate .	O	+ | O	+ | O	+ | O	+ | O	+	+ | O	O	O	+	+
2·5	Corrosive sublimate . .	+	+	+	+	+	+	+	+	+	+	+
1	Platinum chloride . .	+	+	+	+	+	+	+	+	+	+	+
100	Alcohol	X	X	X	+	+	+	+	+	X	X	+
100	Acetone	X	X	+	+	+	+	+	+	X	X	+
10	Formaldehyde. . . .	O	+ | O	+ | O	+ | O	+ | O	+	?	?	O	O	O
10	Lysol	O	O	O	O	O	O	O		O		

Meaning of the signs employed:—
r = alkaline *b* = acid. + = Precipitate insoluble in water. X? = Precipitate very slightly soluble in water.
X = Precipitate soluble in water. ♁ = Precipitate soluble in an excess of the fixative. O = No precipitate.

This chart has been constructed partly from A. Fischer's account, and partly from my own observations. The behaviour of protalbumose and conglutin I have not verified.

Mucin is not represented because different natural mucins vary greatly in their behaviour, and pure mucin I have not yet prepared. Special reference should be made to osmium tetroxide (pp. 81–83) and potassium bichromate (p. 75).

CHAPTER VI.

The Macroscopical Effects of Fixing Reagents.

The following test-tube experiments will show along what lines I have been working ever since 1888. I started originally with the idea that the most vigorous coagulating agents ought to give the best histological results, on the supposition that by their rapid action post-mortem changes would be prevented. I now know that such treatment will not allow the ordinary changes occurring at death to take place, but also that the finer details in many cells are completely destroyed by such exceedingly vigorous precipitants as sublimate, picric acid, and tannin, especially if they be used in combination as I advocated ten years ago. The experiments detailed below ought to be studied in the light of physical chemistry (Chapter II), and reference should also be made to the views on coagulation set forth in Chapter III. If not expressly stated otherwise the freshly prepared egg-white of newly-laid winter eggs was taken. Fresh summer eggs contain more globulin and more reducing substances than winter eggs.

Experiment 1.

To determine the 'coagulating' power of ethyl-alcohol on egg-white:

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*	*L*
Absolute alcohol . .	—	1	2	3	4	5	6	7	8	9	10
Distilled water . .	10	9	8	7	6	5	4	3	2	1	—
Egg-white . . .	1	1	1	1	1	1	1	1	1	1	1

The alcohol was mixed with the distilled water in *B* to *K*. After the addition of the egg-white each tube was closed by putting the thumb over its mouth, and the tube was turned sharply over ten times so as to mix the albumin with the alcohol. No shaking was resorted to.

Tubes *A* to *D* showed mechanical coagula (see p. 50) in decreasing amounts. A slight turbidity was seen in *A*, containing no alcohol, due to a precipitation of globulin and ovomucoid. This tube after twenty-four hours had set into a thin jelly. The opacity increases slightly from *B* to *D*, very small flocculi (mechanical coagula) are seen in *D*. *E* in twenty-four hours forms a perfectly homogeneous, thin jelly with no mechanical coagula. *E* is more transparent than *D* because of its greater homogeneity. In *F* the jelly is sufficiently firm to allow of the test-tube being held horizontally, and *G* may be inverted for several seconds without the jelly, enclosing air-bubbles, moving in any way. There is in *G* a suspicion of fine flocculi. *H* contains well-marked fine flocculi in a very thin jelly; the tube cannot be held horizontally without disturbing the jelly. *I* shows coarser flocculi in an opalescent mother liquor, the whole mass being so fluid that the tube cannot be held horizontally. The flocculi have partly settled, leaving a milky supernatant fluid.

K is composed of very coarse membranous and filamentous flocculi; the mother liquor is almost clear, the slight turbidity being due to minute flocculi. The coagulum after 10 minutes has settled sufficiently to show a narrow superficial clear layer. *L* contains the coarsest flocculi, partly membranous, intermixed with numerous small flocculi which also rapidly subside. After 20 minutes the upper third of the fluid in the test-tube is perfectly clear.

The general conclusions to be drawn from the above experiment are that 40 to 50 per cent. ethyl-alcohol is a very efficient coagulant if it be allowed to act for 24 hours. Used in weaker solutions coagulation does not take place uniformly, and if used beyond 60 per cent. strength coarse flocculi are apt to result because of the dehydrating action. It is therefore best to fix in 50 per cent. ethyl-alcohol for 24 hours and then gradually to substitute the higher percentages, after the coagulable material has acquired a certain resistance. The tendency of weak alcohols to prevent the formation of mechanical coagula is very interesting, and is probably due to a double action, namely, partial dehydration of the proteid-molecules, and also to an alteration in the surface tension of the fluid.

Experiment 2.

To determine the relative coagulative power of methyl-alcohol, ethyl-alcohol, and acetone. The reagents were obtained from E. Merck, and were chemically absolutely pure. The egg-white of newly-laid summer eggs freshly prepared was used. The coagulants were mixed with the water and allowed to stand till all bubbles had disappeared. After adding the egg-white to the alcohol and acetone mixtures, the test-tubes were quickly closed with the thumb and then were turned ten times upside down in the course of 20 seconds. No shaking was resorted to.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*	*L*
Methyl-alcohol (Merck's)	—	1	2	3	4	5	6	7	8	9	10
Ethyl-alcohol . .	—	1	2	3	4	5	6	7	8	9	10
Acetone . . .	—	1	2	3	4	5	6	7	8	9	10
Distilled water . .	10	9	8	7	6	5	4	3	2	1	—
Egg-white . . .	1	1	1	1	1	1	1	1	1	1	1

One hour after having completed this experiment the following appearances were seen:—In *A* all three tubes showed a marked foam-formation on the surface of the fluid, and mechanical coagula were also noticeable in all three tubes, most markedly with methyl-alcohol and least with acetone. In *B* considerably less foam than in *A*, again more with methyl than with ethyl-alcohol. The mechanical coagula were finest with methyl-alcohol, and many of them were entangled in the foam. In *C* the mechanical coagula with ethyl-alcohol had sunk to the bottom of the tube, while they floated in the methyl and acetone solutions. A distinct opalescence was seen in all three tubes, most in the acetone and least in the methyl mixture. In *D* the ethyl-alcohol had produced a clear jelly, enclosing fine air-bubbles, methyl-alcohol had produced no jelly, and flocculi were seen floating near the surface. The acetone had formed an opaque jelly

much thinner than in the ethyl-alcohol mixture, with flocculi near the surface. In *E* ethyl-alcohol showed a thicker and more opaque jelly than *D*. The methyl-alcohol had given rise to large flocculi mixed with air-bubbles, and the jelly was much more opaque than in the ethyl-alcohol mixture. Acetone formed an opaque jelly, less firm than the ethyl-alcohol jelly, and containing numerous small flocculi. In *F* the ethyl-alcohol jelly was somewhat less resistant than in *E*; numerous air-bubbles and minute flocculi could be made out. The flocculi were apparently formed first, and were then fixed in their position by the remainder of the mixture setting into a jelly. The ethyl-alcohol jelly was firmer than the methyl-alcohol one. The acetone mixture had formed no jelly, and flocculi were floating in an opaque mother liquor. In *G* to *K* in the ethyl-alcohol series membranous flocculi were noticed gradually increasing in size; the opacity of the mother liquor, still well marked in *H*, was much less in *I*, and had completely disappeared in *K*, owing to a complete primary precipitation in the form of large membranous coagula. The flocculi adhered to one another, most in the ethyl-alcohol tubes, less in the methyl-alcohol series, and not at all in the acetone tubes. In the latter the coagulum was settling rapidly.

After two days *D* in the ethyl-alcohol tube had formed a firm jelly, including air-bubbles, while the corresponding methyl-alcohol was liquid. *E* in the ethyl-alcohol tube was a very firm jelly, while *F* showed a slight exudation of clear fluid on the top, and a not very firm jelly. Acetone had not caused a jelly in any of the tubes.

General conclusions.—Methyl-alcohol with a molecular weight of 32, if used in the same percentage strengths as ethyl-alcohol with a molecular weight of 46, is much less efficient in producing a homogeneous coagulation with normal alkaline egg-white. Acetone has no 'binding' power as compared with the alcohols; it seems to act on the proteid-molecules individually, causing them to contract, and thereby leads to a disruption of any arrangement which may exist. These test-tube experiments are fully borne out by histological results, for although there is little to choose between ethyl and methyl alcohols, the former being slightly better, acetone causes such bad fixation as to justify its disuse.

Experiment 3.

To determine the 'coagulating' power of acetic acid. 1 ccm. of albumin was added to 10 ccm. of 25, 50, and 100 per cent. of glacial acetic acid. The 25 per cent. solution showed a few ill-defined flocculi, while in the 50 per cent. acid a large number of minute membranous and transparent flocculi floated in a jelly-like mother liquor, which latter exhibited a slight opalescence. Glacial acetic acid throws down large membranes of a white, slightly transparent colour; the albumin being completely precipitated, no opalescence is seen in the mother liquor. These white membranes do not change in their appearance even if kept for months.

General conclusions: Pure glacial acetic acid acts thus chiefly as a dehydrating agent. It would appear that for fixing purposes this

acid should be at least 50 per cent. strong, as otherwise soluble acid albumins will be formed.

Experiment 4.

To determine the action of formaldehyde on egg-white.
Newly bought formol was used in the following proportions:

	A	*B*	*C*	*D*
Formol	2·5	5	7·5	10
Water	7·5	5	2·5	—
Egg-white	1	1	1	1

Perfectly fresh egg-white of summer eggs was used. The reaction of the white of egg was strongly alkaline, while that of the formol was slightly acid. From *A* to *D* a gradually increasing opacity was seen, but no coagulation after ten minutes. *D*, to begin with, was milky and white, and showed after one hour large flocculi in a fluid resembling watered milk. On acidifying the tubes *A*, *B*, and *C* with glacial acetic acid, a marked neutralization precipitate was obtained, which rapidly disappeared on a further addition of acetic acid.

On adding to 10 ccm. of formol 2 drops of glacial acetic acid, mixing these two and then adding 1 ccm. of egg-white, a coarse membranous precipitate resulted, the coagula floating in a clear fluid.

On rendering 10 ccm. of formol exactly neutral by the addition of some formol which had stood over magnesium carbonate and which was alkaline in its reaction, and then adding 1 ccm. of egg-white, an apparently perfectly clear solution resulted, but on holding the test-tube in a certain way, a perfectly transparent coagulum, in the form of shreds floating in a clear mother liquor, was seen. This tube after 24 hours had set into a firm jelly which, although slightly opalescent, still showed the clear strands of coagulated materials.

General conclusions: The most homogeneous fixing effect is produced by acting on the normally alkaline albumin with carefully neutralized formol. For this reason I recommend the use of neutral formol (p. 88).

Experiment 5.

To determine more fully the action of weaker watery solutions of Formol (= 40 per cent. Formaldehyde) on white of egg.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*
Formol . . .	10	9	8	7	6	5	4	3	2	1
Water . . .	—	1	2	3	4	5	6	7	8	9
Egg-white . .	1	1	1	1	1	1	1	1	1	1
Proportion of CH_2O to egg-white	4 : 1	3·6 : 1	3·2 : 1	2·8 : 1	2·4 : 1	2 : 1	1·6 : 1	1·2 : 1	0·8 : 1	0·4 : 1

The immediate results were:—In *A* within 5 seconds a milky appearance, settling quickly to a white jelly. *B* was very similar to *A*, but about 15 seconds were required to form the milky appearance similar to *A*. *C* could be best compared to watered milk, while *D* was milky opalescent with a slight tendency to the formation of a sediment. *E* was opalescent, while *F* and *G* resembled in turbidity a 5 per cent. gelatine jelly. *H*, *I*, and *K* were very similar to *G*, but

a gradual diminution in the turbidity could distinctly be traced from *H* to *K*.

After 24 hours very coarse secondary flocculi had developed in *A*, *B*, and *C*. They were least marked in *C*. In *A* about four drops of supernatant clear fluid were seen, about two drops in *B*, and just a trace in *C*. *D* was milky in appearance, but no secondary coagula had formed. It resembled tube *C* as the latter appeared after adding the albumin. In *E* the opalescence had slightly increased, but no further changes were seen in *F* to *K*.

General conclusions: Three distinct factors come into play in this experiment, namely, the concentration of the formaldehyde in the solution; the total amount of the aldehyde in relation to the amount of egg-white; and, lastly, time (see p. 106). The action of formaldehyde differs from that of sublimate in being more protracted, therefore to get full fixation by this reagent it is necessary to fix for at least three days, because in the above experiment after three days the opalescence had increased in *H* and *K*[1]. From this experiment it would appear also as if solutions should not be stronger than *D*, as otherwise secondary coagula may be formed; but I have obtained good results histologically with pure formol acting for one week.

Experiment 6.

To determine the action of 1 per cent. osmium tetroxide on egg-white. These substances were mixed in all proportions. With newly-laid winter eggs a slight deepening in colour of the mixture and a considerable increase in viscosity were noticed, while with the egg-white of newly-laid summer eggs the osmium tetroxide was reduced to a deep brown colour approaching black, but no deposit was seen even after exposing the mixture to direct sunlight. The viscosity in summer eggs seemed to me greater, as far as I could recollect, than that produced in egg-white of winter eggs.

Conclusion: Osmium tetroxide does not produce a visible coagulation, but forms more or less intimate additive compounds.

Experiment 7.

To determine the action of different strengths of sublimate on the same amount of egg-white. The sublimate was a 5 per cent. solution in distilled water. The albumin was to the fixing solution in the proportion of 1 : 10. (See table on p. 108.)

Ten minutes after having mixed the albumin with the $HgCl_2$ mixtures the following results were obtained:—*A* showed a milky opalescence with some sediment. In *B* the opalescence was denser, with less sediment than in *A*. *B* to *D* showed a gradually increasing diminution of the opalescence in the upper layers, owing to the albumin having been precipitated in a somewhat coarse form. The sediment was most bulky in *B* and least bulky in *D*, because in the latter it had settled to a much greater extent. This tendency of

[1] It was omitted to make tests as to the amount of acid reaction in the test-tubes at the commencement of the experiment and after the lapse of several days, for it is quite possible that the turbidity may have been due to the formation of formic acid from the excess of formaldehyde present.

the coarser flocculi to settle into a firm sediment was even more evident in *E*, but the upper layers in *E* are more opaque than in *D* because of the greater formation of fine flocculi which had not as yet settled. From *F* to *N* the main bulk of the precipitated albumin was forming coarser and coarser masses, while the remainder of the albumin in *F* formed a more, and in *G* a less milky supernatant layer than in *E*. From *H* to *N* the whole of the albumin was precipitated as a sediment, the bulk of which, owing to firmer settling, gradually diminished up to *N*. Because of the complete precipitation of the albumin, the upper layers were perfectly clear.

After 24 hours the following changes had occurred:—In *A* the sediment formed about one-fifth the total bulk of the fluid, the middle three-fifths were opalescent, while the upper fifth commenced to become clear. In *B* slightly more sediment had been formed than in *A*, and the upper fifth was less clear. In *C* the sediment

	5 *per cent. sublimate solution.*	*Water.*	*Percentage strength of the sublimate solution.*	*Actual amount of sublimate by weight acting on 1 ccm. of egg-white.*
A . .	0·1	9·9	0·05	0·005
B . .	0·25	9·75	0·125	0·0125
C . .	0·5	9·5	0·25	0·025
D . .	1	9	0·5	0·05
E . .	2	8	1	0·1
F . .	3	7	1·5	0·15
G . .	4	6	2	0·2
H . .	5	5	2·5	0·25
I . .	6	4	3	0·3
K . .	7	3	3·5	0·35
L . .	8	2	4	0·4
M . .	9	1	4·5	0·45
N . .	10	—	5	0·5

resembled that in *B*, the middle layer was more milky than *B*, and the upper tenth clear. The upper layer in *D* and *E* appeared opalescent, most so in the latter. Commencing with *F* and increasing to *M* is a gradual clearing up of the superficial layers till in *M* and *N* the upper part of the fluid is perfectly clear, owing to a complete settling of the precipitated albumin. From *I* to *N* the amount of sediment in the test-tubes diminished in bulk because of the firmer aggregation of the precipitated albumin.

General conclusions: Solutions of sublimate containing 0·05 up to 1 per cent. of this salt, and used in amounts equal to 10 times the bulk of the tissue, will produce a more even coagulation than stronger solutions, as these are apt to form with increasing strengths coarser and coarser precipitates. The opalescence seen in *A* to *E*, on the other hand, is a sign of incomplete coagulation, and tissues left in these weak solutions for several days might show, in addition to the primary effect of coagulation, a deposit of secondary coagula on the primary ones. *H*, which represents a 2·5 per cent. sublimate solution, is the lowest percentage which gives a complete coagulation, and I have therefore adopted this strength in my mixture (p. 97).

Experiment 8.

Knowing that a saturated 35 per cent. solution of sodium chloride readily causes a sublimate precipitate of albumin to dissolve, three distinct sets of experiments were made to determine what proportions of salt will cause a distinct visible change in such a precipitate, and what amounts will prevent the formation of the precipitate.

Experiment 8 a.

In the first experiment 5 per cent. watery solutions of sublimate and of common salt were used in the amounts stated in the table, 1 ccm. of normal egg albumin being added to 10 ccm. of the sublimate-salt mixture. After the addition of the albumin the test-tubes were each shaken three times, a shake consisting of a single sharp movement.

	$HgCl_2$ 5 *per cent.*	NaCl 5 *per cent.*	*Albumin.*	*Proportion in gram weights of* $HgCl_2$: NaCl.
A . .	10	—	1	10 : 0
B . .	9	1	1	9 : 1
C . .	8	2	1	4 : 1
D . .	7	3	1	7 : 3
E . .	6	4	1	3 : 2
F . .	5	5	1	1 : 1
G . .	4	6	1	2 : 3
H . .	3	7	1	3 : 7
I . .	2	8	1	1 : 4
K . .	1	9	1	1 : 9
L . .	0·5	9·5	1	0·5 : 9·5
M . .	0·25	9·75	1	0·25 : 9·75

The immediate results are these:—In *A* a very coarse membranous precipitate is formed, which after 5 minutes commences to settle, leaving a faintly opalescent supernatant layer. *B* resembles *A*, but the precipitate is less coarse. *C* shows a finely floccular precipitate in a bluish opalescent mother liquor. Soon the flocculi aggregate into larger flocculi like cumuli, and these commence to settle quickly. *D* resembles *C*, but the flocculi are smaller and settle less quickly. The mother liquor is more opalescent than in *C*. The specific gravity of the egg-white lies between that of the solutions *D* and *E*, nearer *D*. The tube *D* shows a uniformly opalescent fluid with no flocculi. From *F* to *M* a gradually diminishing opalescence is seen, which in *M* is just perceptible. Comparing *M* with *B* of Experiment 7, shows that the sodium chloride has prevented the formation of a sediment and diminished the opalescence.

After 24 hours in *A* a thick curdy precipitate occupies the lower quarter of the mixture, while the supernatant fluid is perfectly clear. *B* shows a thick curdy precipitate in the lower third, the middle third being distinctly opalescent and sharply marked off from the upper third, which exhibits the faintest trace of opalescence.—*C* to *M* form a definite series in which the amount of the precipitate

and the opalescence slowly decrease till only a few very light flocculi are seen in *H*, which thus forms the most homogeneous tube. In *I* to *M* the opalescence gradually diminishes till it is just visible in *M*, but there is no precipitate. Thus after 24 hours H shows the same condition which was seen in *E* immediately after mixing the egg-white with the sublimate-salt mixture.

General conclusions are given on p. 113.

Experiment 8 B.

In Experiment 8 A the only constant was the egg-white, namely, 1 ccm., while both the sublimate and the salt solutions were variables. Following the suggestion of G. J. Burch I repeated the experiment having two constants, namely, the sublimate and the egg-white, and two variables, the salt and the water, as seen in Experiment 8 B, while in Experiment 8 C the albumin and the salt were constants, the sublimate and water being the variables. (The differences in the electrolytic dissociation of NaCl and the hydrolytic dissociation of $HgCl_2$ were not taken into consideration.)

The molecular weight of sodium chloride is 58·4, and a 'normal' solution is made by dissolving 58·4 grms. of this salt in 1,000 ccm. of water, sodium being monovalent. The molecular weight of mercuric chloride (sublimate) is 270·6, and, as mercury is divalent, to obtain a normal solution of sublimate equivalent to that of a monovalent substance such as sodium chloride, one-half the molecular weight in grams, namely 135·3 grams, would have to be dissolved in 1,000 ccm. of water. The solutions used in this experiment were a two-fifth normal sublimate solution, i. e. 54·1 grams in 1,000 ccm. of water, and a double-normal salt-solution, i. e. 116·8 grams in 1,000 ccm. of water. The reason for choosing a two-fifth sublimate solution was because the insolubility of sublimate prevents a 'normal' watery solution being made, its solubility being only 70 instead of 135·3 grams in 1,000 ccm. of water. The sodium chloride was used in double its normal strength to bring the action of this salt quickly into play.

Care was taken that the total amount of fluid amounted in each case to 10 ccm. and that the sublimate, salt, and water were well mixed before adding the albumin.

25 ccm. of sublimate solution contain 1·3525 grams of $HgCl_2$, while 5 ccm. of salt solution contain 0·584 gram of NaCl (Table on p. 111).

The immediate results were:—*A* showed no change, while in *B* a dense flocculent coagulum appeared which commenced to settle after 10 minutes. The coagulum was so dense that ordinary letterpress could not be seen through the test-tube. *C* contained finer flocculi than *B*; letterpress at first was just visible, but the writing became more distinct after 10 minutes because of the settlement of the flocculi. *D* resembled *C*, but the flocculi were still finer and hence the print plainer than in *C*; after 10 minutes individual letters could be recognized, the sediment was finer and less abundant than in *C*.

E to *L* exhibit a gradually descending series of opalescence. The egg-white floated on the solutions from *A* to *G* and had to be mixed with the fixing solution by shaking. At *H* the specific gravity was approximately the same ; the egg-white sent downwards fingerlike processes resembling the tentacles of a medusa. *I* at first sight might be taken to be almost clear, and *L* compared with *A* showed the merest trace of opalescence. In *M* within 2½ minutes after adding the albumin and shaking vigorously, a thick curdy precipitate settled down. In *N* the salt-solution did not quite prevent a slight opalescence, indicating that some albumin was still combined with sublimate. *O* showed a very faint opalescence due to incipient globulin precipitation.

	$HgCl_2$ *in ccm.*	NaCl *in ccm.*	*Water in ccm.*	*Albumin in ccm.*	*Proportion in grms. of* HgClr : NaCl.
A . .	—	2·5	7	·5	·0 : 2·92
B . .	2·5	—	7	·5	1·3525 : 0
C . .	2·5	0·5	6·5	·5	1·3525 : 0·584
D . .	2·5	1	6	·5	1·3525 : 1·168
E . .	2·5	2	5	·5	1·3525 : 2·336
F . .	2·5	2·5	4·5	·5	1·3525 : 2·920
G . .	2·5	3	4	·5	1·3525 : 3·504
H . .	2·5	4	3	·5	1·3525 : 4·672
I . .	2·5	5	2	·5	1·3525 : 5·840
K . .	2·5	6	1	·5	1·3525 : 7·008
L . .	2·5	7	—	·5	1·3525 : 8·176
M . .	10	—	—	·5	5·41 : 0
N . .	1	8·5	—	·5	0·541 : 9·625
O . .	—	8·5	1	·5	0 : 9·625

After 24 hours :—In *A* the solution was absolutely clear. In *B* a heavy white curdy precipitate occupied the lower one-third, while the upper two-thirds showed a just perceptible opalescence. *C* to *G* showed a slight precipitate, most marked in *C* and gradually diminishing till just visible in *G* ; the supernatant fluid showed a marked bluish opalescence, most marked in *C* and gradually diminishing till *G*. In *H* to *L* no precipitate was visible, and the solutions gradually became less opalescent, till in *L* the opalescence was just visible.

After 48 hours traces of a fine floccular precipitate were seen in all the tubes from *H* to *L*, being least marked in *L*.

M contained a dense precipitate in the lower one-fifth of the tube, the upper four-fifths being perfectly clear. With transmitted sunlight the flocculi in the precipitate were seen to be much coarser than in *B*. In *N* the opalescence was still visible, while *O* was perfectly clear, with the merest trace of a sediment.

Experiment 8 c.

The sublimate and salt solutions were of the same strength as in Experiment 8 b, the constants in this case being the salt and the variables the sublimate and water.

	NaCl *in ccm.*	$HgCl_2$ *in ccm.*	H_2O *in ccm.*	*Albumin in ccm.*	*Proportion in grams of* $HgCl_2$: NaCl.
A . .	10	1	84	5	0·0541 : 1·168
B . .	10	2·5	82·5	5	0·13525 : 1·168
C . .	10	5	80	5	0·2705 : 1·168
D . .	10	10	75	5	0·5410 : 1·168
E . .	10	20	65	5	1·082 : 1·168
F . .	10	25	60	5	1·3525 : 1·168
G . .	10	30	55	5	1·623 : 1·168
H . .	10	40	45	5	2·164 : 1·168
I . .	10	50	35	5	2·705 : 1·168
K . .	10	60	25	5	3·25 : 1·168
L . .	10	70	15	5	3·787 : 1·168
M . .	10	80	5	5	4·328 : 1·168

The immediate results :—All solutions from *A* to *M* are opalescent ; *D* to *H* show a flocculent precipitate after the first three shakes ; on continuing to shake this precipitate disappears, but reforms after 5 minutes, though to a much lesser extent than at first. The specific gravity of egg-white lies between *I* and *K*, nearer to *I*. *I* after 10 minutes shows traces of a flocculent precipitate, similar to the primary one seen in *D* and *H*. *K*, *L* and *M* are milky opalescent with no trace of flocculi 10 minutes after the experiment.

After 48 hours all the tubes from *A* to *M* are markedly opalescent, the opalescence and the amount of precipitate in the bottom of the test-tube increasing gradually from *A* to *M*. In *M* the upper one-tenth of the solution is somewhat clearer than the subjacent portion, showing that a second crop of flocculi is about to settle down. *K* appears more milky than either *I* or *L*. *M* in general character strongly resembles *C* of Experiment 8 A.

General conclusions are given at end of Experiment 9.

Experiment 9.

A saturated solution of sublimate in 0·5 per cent. sodium chloride (Gaule's solution), and this solution diluted with an equal bulk of water, were compared with a saturated solution of sublimate in distilled water and also with this solution diluted with an equal bulk of distilled water.

Result : The saturated sublimate-salt solution formed a sediment consisting of coarser flocculi than the saturated watery sublimate solution, and therefore did not settle down as firmly as the former. The sediments formed by the half-saturated solutions only settle down to one-half the extent of those formed by the saturated ones.

There was no difference noticeable in the sediments of the two half-saturated solutions, while the supernatant fluid in that test-tube containing the half-saturated salt-sublimate mixture was opalescent, because the salt leads to the formation of some very fine coagula, or partly dissolves the mercury precipitate. After shaking up the two half-saturated solutions, the one containing salt settles more quickly.

General conclusions: Sodium chloride, if present in even minute traces, has a distinct solving action on the sublimate precipitate, and if it be to the sublimate in the proportion of 3:7 gram weight (Exp. 8A, *D*) the formation of a solid coagulum is prevented altogether, —the proteid-molecules being fixed separately give rise to a fine opalescent emulsion. Still further additions of salt, especially if the amount by gram weight of sodium chloride to the sublimate is as 5·84:1·35, prevent coagulation altogether (Exp. 8B, *I*), because the sodium chloride, by its ready dissociation into sodium ions and chlorine ions, saturates the watery solution with the latter, and thereby prevents any more chlorine ions being formed. Sublimate, which would normally break up hydrolytically according to the formula $HgOH + 2HCl$ (p. 21), and its hydrochloric acid into the hydrogen ion with an acid reaction (p. 15) and the chlorine ion, cannot do so now, as the formation of the chlorine ions in a solution already saturated by them is impossible.

When sublimate, therefore, is used in strong saline solutions, it cannot dissociate hydrolytically, and no hydrogen ions being formed the reaction of the sublimate and salt solution remains neutral, as has already been noticed by Lee and Mayer[1].

When a proteid, coagulated by sublimate, is treated with a solution of sodium chloride it becomes soluble, because we are dealing with the following changes: From the table of electro-affinities (p. 14), it will be seen that sodium is a much stronger kat-ion than mercury, and hence sodium will displace the mercury in the albuminate, a sodium instead of a mercury albuminate being formed; the mercury ions, turned out of their union with the albumin, link on to the chlorine an-ions to form sublimate, and as the solution is already saturated with an excess of chlorine ions, the $HgCl_2$ cannot dissociate, and we arrive at the same result as if we had added a solution of sublimate in a strong salt solution to the proteid. The sodium albuminate does not coagulate, because all neutral salts, such as sodium chloride, fail to produce coagulation.

These experiments make it clear that it is by no means immaterial whether tissues are fixed in sublimate or in sublimate-sodium chloride solutions. Some years ago M. Heidenhain was good enough to inform me by letter that his object in using sublimate dissolved in salt solution was to increase the solubility of the former. At that time I had made these experiments, but refrained from arriving at any rash conclusion because I did not know then, nor do I now, to what extent the electrolytic dissociation of sublimate is prevented by the addition of salt, particularly as the new factor of increased solubility of the mercury salt comes in[2].

When staining sections the instability of the mercury albuminate must also be borne in mind.

[1] Lee and Mayer: *Grundzüge d. mikr. Technik*, 1901, p. 43.

[2] Experiments have been going on for some time to settle experimentally the precise effect produced on tissues by adding sodium chloride to sublimate solutions in different proportions.

Experiment 10.

To determine the coagulating power of the double salt $HgCl_2 + NaCl$ when used in different strengths. The double salt was made up as a 5 per cent. solution.

	A	*B*	*C*	*D*	*E*	*F*
5 per cent. double salt solution	10	8	6	4	2	1
Water	—	2	4	6	8	9
Albumin	1	1	1	1	1	1
Double salt by weight	0·5	0·4	0·3	0·2	0·1	0·05

After twenty-four hours the amount of sediment diminished from *A* to *F*, showing that to get a complete precipitation, as far as this is possible in the presence of sodium chloride, the fixative to the object to be fixed must be in the proportion of 10 to 1. That precipitation was not complete was determined by adding some of the 5 per cent. double salt solution to the tubes *D*, *E*, and *F*, when an increase in the amount of opalescence was observed.

Experiment 11.

To determine the effect of a 10 per cent. watery solution of the double salt $HgCl_2 + 2NaCl$.

	A	*B*	*C*	*D*	*E*
10 per cent. double salt	10	8	6	4	2
Water	—	2	4	6	8
Albumin	1	1	1	1	1

After twenty-four hours all test-tubes had a milky opalescent appearance, the tube *C* being the most milky because the specific gravity of this fixing solution was primarily equal to that of the albumin solution, and for this reason also no sediment was formed. In the remaining four tubes the sediment diminished slightly in this order, *E*, *D*, *B*, *A*.

Experiment 12.

To determine the effect of the double salt $HgCl_2 + K_2Cr_2O_7$ in a 5 per cent. watery solution.

	A	*B*	*C*	*D*	*E*
5 per cent. double salt	5	4	3	2	1
Water	—	1	2	3	4
Albumin	0·5	0·5	0·5	0·5	0·5

The immediate results: The specific gravity of egg-white lies between *A* and *B*, but nearer *B*. Both *A* and *B* formed, on shaking with the egg-white, coarse coagula; in *C* the coagulum was intermediate. After 1½ hours a clear supernatant layer was seen in *A*, *B*, and *C* in the proportion of 3:2:1. *D* shows a fine coagulum, and is also the best, inasmuch as no supernatant clear layer and no precipitate had formed after 1½ hours. *E* shows a uniform precipitate, which, after 1½ hours, had divided into an upper milky layer and a lower flocculent sediment.

After 24 hours: *A*, *B*, and *C* show a supernatant clear layer, diminishing from *A* to *C*, with perhaps a trace of opalescence in *C*.

The sediment had settled down most in *A*, and least in *C*. *D* with a faintly opalescent upper layer and a sediment which had not settled down so much as in *C*. *E* with no further change except that the sediment had settled a little more.

Experiment 13A.

To study the effect of adding acetic acid to sublimate solutions. The test-tubes were turned upside down sixteen times, after having their mouths closed with the thumb.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Glacial acetic acid . . .	1	1	1	1	1	1	1	—
5 per cent. sublimate . .	—	1	2	3	4	5	9	10
Water	9	8	7	6	5	4	—	—
Egg-white	1	1	1	1	1	1	1	1

The effect after 10 minutes: In *A* the coagulum first noticed quickly disappeared. In *B* small flocculi buoyed up by air-bubbles were seen at the top, small flocculi occupied the main middle portion, while a considerable precipitate covered the bottom of the tube. *C* resembled *B*, but the flocculi in the middle portion of the tube were smaller. *D* and *E* resembled *B*, but the opacity in the middle layer was gradually increasing, and the flocculi were so small as to be hardly visible. *F* had the same specific gravity as the egg-white. The middle layer was milky, and contained about twenty times the amount of flocculi seen in *B* to *E*. In *F* and *G* the flocculi were membranous, owing to the test-tubes having been moved about gently at the time of coagulating the egg-white. In *F* the sediment was about five times that of *D*. The middle layer was very milky. In *G* all the egg-white was precipitated, and formed a sediment with the exception of a few fine flocculi which were floating in the supernatant clear mother liquor.

The effect after 60 minutes: *B* to *E*, on being shaken up, showed that a gradual increase in the amount of coagulum had been produced, but in *B* and *C*, as compared with the effect noticed 10 minutes after the experiment, a partial solution of the coagulum was seen to have taken place.

The effect after 12 hours: In *A*, as stated above, the primary precipitate seemed to have disappeared, but now a very scanty flocculent precipitate was seen, the mother liquor being very slightly opalescent. The same held good, in increasing amounts, from *B* to *D*. In *E* the mother liquor was strongly opalescent; in *F* milky opalescent; in *G* milky, with a slight tendency towards a complete clearing in the uppermost layers. The sediment increased regularly from *A* to *E*, the most marked increase being in *F*, and four times that of *F* in *G*. The amount in *G* and *H* was approximately the same, but while the mother liquor had a faint opalescence in *G* it was perfectly clear in *H*.

Experiment 13B.

Similar to the last, but with stronger percentage solutions of sublimate and glacial acetic acid. In every case, after mixing the 5 per

cent. sublimate and the glacial acetic acid in the proportions stated, only 10 ccm. of the mixture was used and 1 ccm. of egg-white added.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Glacial acetic acid . . .	1	2	3	4	5	10	10	10
5 per cent. sublimate . .	10	10	10	10	10	10	5	—

In *A* the immediate result was the formation of a milky opalescent fluid, in which comparatively fine flocculi were floating, resembling the appearance seen in the test-tube *G* of Experiment 13 A. After 30 minutes three distinct layers could be distinguished—an upper opalescent one, a middle layer of very fine coagula which were settling, and at the bottom a third stratum composed of coarse coagula. After 12 hours the uppermost layer, owing to a continued settling of the fine flocculi, was perfectly clear; the middle stratum had now the appearance which the upper stratum had after completing the experiment. The flocculi in *A* are coarser than in *B*, and for this reason the mother liquor appears more transparent in *A* than in *B*. From *B* to *F* the mother liquor gradually becomes more and more opalescent and less milky. The amount of the coagulum from *C* to *F* seemed increased, as the formation of larger and larger membranous precipitates prevented a settling into so small a bulk as in *B*. *G* contained in a perfectly clear mother liquor exceedingly large membranous flocculi resembling those in *H*, except that they were more transparent, owing to the presence of the glacial acetic acid.

After 12 hours, from *B* to *G* a decreasing amount of opalescence was seen, which was milky in *B*, milky opalescent in *C*, and different degrees of opalescence up to *G*, in which it was very slight. The amount of sediment was diminished in *A* to *C* because of the dissolving action of the acetic acid, but from *D* to *G* it increased because of the formation of membranous coagula due to the combined action of strong acetic acid (see Experiment 3) and of the sublimate.

General conclusion: On comparing the effect of acetic acid with that of sodium chloride (Experiment 8 A) it will become evident that in the weaker solutions the acetic acid (Experiment 13 A, *B*, *C*) increases the coagulating effect of sublimate, and that in stronger solutions (Exp. 13 B) the action of acetic acid peculiar to itself (Exp. 3) comes into play.

Experiment 14.

To determine the effect of adding a 1 per cent. osmium tetroxide solution to a 5 per cent. corrosive sublimate solution.

	OsO_4.	$HgCl_2$.	*Water.*	*Albumin.*	*Proportion of* OsO_4 *to* $HgCl_2$.
A . .	1	5	4	1	0·1 : 2·5
B . .	2	5	3	1	0·2 : 2·5
C . .	3	5	2	1	0·3 : 2·5
D . .	4	5	1	1	0·4 : 2·5
E . .	5	5	0	1	0·5 : 2·5
F . .	—	5	5	1	

As regards the amount of settling no difference could be seen after 24 hours in *A* to *F*, but while the precipitate in *F* containing no OsO_4 was exceedingly mobile, the cohesion in the precipitates was noticed to increase from *A* to *E*, being most marked in *E*, which latter, corresponding to my osmium-sublimate solution, contains the osmium tetroxide to the sublimate in the proportion of 1 in 5.

Experiment 15.

To determine the extent to which formol modifies the action of sublimate. Compare this experiment with Experiments 4, 5, and 7. The sublimate solution used was a 5 per cent. watery solution. The formol is the commercial 40 per cent. formaldehyde containing traces of formic acid.

	5 *per cent.* $HgCl_2$.	*Pure formol.*	*Albumin.*	*Proportion of sublimate to formaldehyde in percentages.*
A . . .	9	1	1	0·45 : 0·4
B . . .	8	2	1	0·40 : 0·8
C . . .	7	3	1	0·35 : 1·2
D . . .	6	4	1	0·30 : 1·6
E . . .	5	5	1	0·25 : 2
F . . .	4	6	1	0·20 : 2·4
G . . .	3	7	1	0·15 : 2·8
H . . .	2	8	1	0·10 : 3·2
I . . .	1	9	1	0·05 : 3·6
K . . .	—	10	1	— : 4
L . . .	10	—	1	4 : 0

Immediate effect: After the primary three shakings the coarseness of the coagulum decreased in regular order from *A* to *I*. After 10 minutes, on trying to break up the primary precipitate, a resistance was noticed which became especially marked at *E* and then gradually increased till it reached its maximum at *I*. *K* offered no resistance when shaken up. After three very vigorous shakings the test-tubes were left standing vertically, when after 5 minutes the coagulated egg-white was seen to be moving upwards in *E* and *F*, leaving a small clear area in the bottom of the test-tube. An explanation of the peculiar behaviour of the clot is difficult to give, but it would appear that the air-bubbles entangled in the coagulum by the shaking process cause a certain amount of it to float, and that this portion forms a nucleus to which the rest of the coagulated egg-white attaches itself.

After 24 hours: In *A* the greater part of the coagulum forms a flocculent sediment, but about one-tenth floats on the surface of the liquid. *B* to *F* show a dense white coagulum in the upper part of the test-tube, leaving the lower portion of the fluid perfectly clear. The amount of clear fluid in the bottom of the test-tubes *B* to *E* is in the proportion of 2 : 3 : 4 : 6 : 7, owing to a gradually increasing amount of contraction of the floating clot. The most uniform and homogeneous coagulum is that in *D*. In all tubes from *B* to *E* the

lower surface of the coagulum forms a convex sharply-defined surface. In *G* the floating clot has a coarse flocculent appearance, its lower convex surface being quite ragged, and some flocculi, having become detached, form a very slight sediment in the bottom of the tube. *H* is similar to *G*, but the flocculi are aggregated into coarse masses in the floating clot, the lower surface of which is only slightly convex and beset with loose flocculi, a number of which, having dropped off, form a slight sediment. *I* contains a floating clot similar to *G*, but appearing more open and being quite irregular on its lower surface. Flocculi having detached themselves form a sediment. *K* is similar to *H*, but the floating clot is much less abundant. In all tubes from *G* to *K*, the fluid in the bottom is no longer clear, but slightly turbid owing to the formaldehyde overcoming the action of the sublimate. In *L* two-thirds of the coagulum form a dense white sediment, one-third forms a floating mass from which minute flocculi are constantly detaching themselves and falling to the bottom. The fluid in the middle of the tube is perfectly clear.

General conclusions: Judging by this experiment perhaps *E* is the best fixative, but all solutions containing formol and sublimate can be relied on provided they contain at least 10 per cent. formol; see the next experiment.

Experiment 16.

It was desired to obtain a 2·5 per cent. sublimate solution containing respectively 2·5 per cent., 5 per cent., 10 per cent., and 20 per cent. of formaldehyde. Therefore 5 per cent. watery sublimate and 40 per cent. formaldehyde (= formol) and water were taken in the following proportions:

	$HgCl_2$.	*Formol.*	*Water.*	*Proportion of sublimate to formaldehyde.*
A . . .	50	6·25	43·75	2·5 : 2·5
B . . .	50	12·5	37·5	2·5 : 5
C . . .	50	25·0	25	2·5 : 10
D . . .	50	50·0	—	2·5 : 20

Immediate results: All tubes gave a primary milky, curdy precipitate, the finest coagulum being in *B*; then came in this order *C*, *A*, and *D*. After again shaking up secondary flocculi form in the order *A*, *B*, *C*, *D*, being coarsest in *D*.

After 24 hours: In *A* the sediment had settled most, and least in *D*; *B* and *C* were intermediate. The bulk of the sediment was to the supernatant clear fluid in *A* in the proportion of 4 : 1, showing that this precipitate has not the same tendency to settle as has a pure sublimate precipitate. From *A* to *D* the cohesion of the sediment increases regularly with the increase in the amount of formaldehyde. *B* showed more cohesion than *A* and less coarseness in the formation of secondary flocculi.

Experiment 17.

To test the relative precipitating power of 2·5 per cent. watery sublimate; of 2·5 per cent. sublimate in 1 per cent. picric acid; of 1 per cent. picric acid (at 20° C.), and of a saturated ammonium-picrate solution. 10 cc. of each fixing solution and 0·5 cc. of albumin were taken.

The immediate result was a very coarse precipitate in the picro-corrosive mixture, a less coarse coagulum in the sublimate. The picric acid produced a coarse membranous precipitate, which on again being shaken up commenced to settle within two minutes; ammonium picrate produced only a faint turbidity.

After 24 hours: Sublimate showed a fine precipitate occupying twice the bulk of that produced by picro-corrosive. The sediment in the latter was more bulky than that in the picric acid tube (which as mentioned had received a second shaking). The supernatant fluid in the picro-corrosive tube was very slightly turbid, more so in the picric acid, when a settling of the very finely distributed precipitate manifested itself by the uppermost layer of fluid being perfectly clear. No change was visible with the ammonium-picrate solution excepting that the opalescence had increased slightly, probably owing to an escape of ammonia.

General conclusions: This series shows firstly, that picric acid has an enormous precipitating power used by itself, and secondly, that it increases the coagulating power of sublimate, because the picro-corrosive sediment occupied less space than did the sublimate one.

If it is therefore essential to coagulate everything present at the expense of fine structure, picro-corrosive sublimate is the best reagent. To preserve minute histological detail it is necessary to add formaldehyde, as shown by the next experiment.

Experiment 18 a.

To study the effect of adding formol to my weak picro-corrosive solution the two following experiments were made. (The weak picro-corrosive solution consists of a 2·5 per cent. sublimate in 1 per cent. picric acid.)

	Picro-corrosive solution.	*Formol.*	*Albumin.*	*Proportion of* $HgCl_2$ *to* OCH_2.
A . . .	10	0·5	1	2·5 : 2
B . . .	10	1	1	2·5 : 4
C . . .	10	2	1	2·5 : 8
D . . .	10	2·5	1	2·5 : 10
E . . .	10	3	1	2·5 : 12
F . . .	10	4	1	2·5 : 16
G . . .	10	5	1	2·5 : 20
H . . .	10	10	1	

The immediate result: In *A* very coarse membranous flocculi were formed, which after being broken up by three additional shakings

settled quickly. In *B* the membranous flocculi were distinctly smaller than in *A* and settled less quickly after being broken up. *C* compared with *B* as did *B* with *A*. From *D* to *G* the precipitate gradually became finer and finer till in *G* it almost resembled the milky appearance shown by *H*.

H contained the finest coagulum and therefore seemed to reflect more white light, quite apart from the formalin-albumin coagulum being white itself (see Exp. 4, p. 106).

D corresponds to my picro-corrosive formaldehyde mixture, and the retarding influence exerted by formol as regards overcoagulation is proved by the sediment in *D* occupying twice the bulk of that seen in *A*.

After three days the picric acid seems to overcome the formaldehyde effect, if the latter, by becoming partly converted into formic acid, itself does not lead to a disintegration of the coagulum which was formed primarily. To me the coagulum in *F*, *G*, *H*, seemed to have become more transparent and less bulky, quite apart from the settling.

It is best therefore not to fix for more than 48 hours in the picro-corrosive formaldehyde mixture.

Experiment 18 b.

Instead of keeping, in the next experiment, the picro-corrosive mixture a constant factor, the picro-corrosive and the formol were mixed in inverse proportions.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*	*L*
Picro-corrosive .	10	9	8	7	6	5	4	3	2	1	—
Formol . . .	—	1	2	3	4	5	6	7	8	9	10
Albumin . .	1	1	1	1	1	1	1	1	1	1	1

In this experiment only a single vigorous shake was resorted to, by moving the arm once rapidly up and down. The specific gravity of albumin lies between *C* and *D*. In *A* to *F* after two hours the following changes were seen: If the amount of sediment in *A* as regards bulk is expressed by 2, then in *B* and *C* it was 3, in *D* it was 4, in *E* it was 5, while *F* showed only a trace of a clear supernatant fluid.

In *G* after the first shake a cohesiveness of the coagulum made itself felt and after two hours the precipitate commenced to settle. In *H* after the first shake an increase in the cohesiveness as compared to *G* manifested itself. The coagulum was milky and homogeneous. After two hours the precipitate had not commenced settling, and the test-tube could be inverted without the coagulum moving, but a few drops of a yellow fluid oozed out. *I* showed still greater increase in the cohesiveness, as the test-tube after two hours could be inverted without any contents flowing out. *K* exhibited after the first shake the same milky appearance as *H*, but was whiter, and the test-tube could be inverted without the contents flowing out. In *L* within $2\frac{1}{2}$ seconds after the first shake a milky jelly was formed,

but of a greater consistency than milk. After two hours the test-tube could be inverted without the contents flowing out.

After 24 hours: In the tubes *A* to *D* a clear supernatant layer was seen, and the precipitated albumin, which occupied the least bulk in *A* and the largest in *D*, showed how the addition of formol prevents the precipitation of albumin in a dense form. *E* as compared to *D* showed in the upper clear layer several large floccular masses which had not settled. In *F* a narrow but distinct clear layer was seen, while on inverting *G* two clear drops and from *H* only a single drop exuded. Even after three days not a single drop was obtained from *I* on inverting the test-tube, care having been taken, of course, to prevent evaporation. *K* and *L* resembled *I* in forming a firm jelly; the small amount of the picro-corrosive in *K* did not visibly interfere with the formol.

Experiment 19.

To determine differences produced by an increase in the concentration of precipitable material.

	A	*B*
Picro-corrosive . . .	10	10
Formol	2	2
Albumin	1	2

Within half an hour the supernatant turbid part in *A* as compared with *B* was as 12 to 1, showing that the rate at which a sediment settles with the different fixatives is determined by the amount of coagulable material present to begin with, or, to put it differently, that the same fixative will cause a much greater apparent shrinkage in cells which are poor in proteids. This necessitates diluting the fixing fluid according to the needs of the special tissue to be investigated.

After three days the amount of sediment in *A* was about one-third the bulk of that seen in *B*.

To make a microscopical investigation of the coagula and precipitates formed in these experiments I considered useless, as everything depends on the ease and quickness with which the fixative and the coagulable material are mixed. Definite experiments bearing on the microscopical effect produced by fixatives are given in the following chapter.

CHAPTER VII.

The Microscopical Phenomena of Physical Coagulation and Chemical Precipitation.

A. Physical Coagulation.

Solutions and suspensions of such bodies as silicic acid, cupric hydrate, agar, gelatin, and white of egg, have the power of passing under certain conditions into the firm state of jellies, which on theoretical grounds the physicist van Bemmelen (see p. 35) believes

to be arranged in the form of a framework holding the water in its meshes, a view shown to be correct by the microscopical investigations of Bütschli and Hardy.

Bütschli in his last paper[1] expresses an opinion on the structure of protoplasm and on some of his critics. He complains, and rightly, that many histologists are not in any way affected by the simplest physical considerations, for how could they otherwise mention, in support of the view that protoplasm is not a fluid, the fact that protoplasm can 'stream up a mountain,' which no water does (Flemming)? Bütschli as the result of careful observations of both living and dead cells and other colloids maintains the existence of a foamlike arrangement. Hardy's careful work[2] is given in the following pages.

Whenever a colloid separates out, an insoluble network with nodal swellings or a honeycombed structure is formed, the meshes or cavities of which contain the original solvent of the colloid in a fluid state (Hardy).

To demonstrate physical coagulation microscopically Hardy gives the following directions:

1. Render egg-white insoluble by William Roberts' method (p. 38), which consists in diluting white of egg with eight times its bulk of water, filtering the mixture and boiling it, and subsequently dialysing it, when a mixture will be obtained in which the proteid is in a fine state of suspension, called desolution by Minchen.

2. Soak a piece of thin silk thread in 2 per cent. calcium chloride solution; lay the thread on a slide and place over it a cover-glass.

3. Allow some of the boiled egg-solution to fill the space under the cover-glass, and watch the effect under a magnification of 500 diameters.

A fine cloud will be seen surrounding the silk thread, and soon the particles in it grow to 0·75 or 1 μ in size. Subsequently these aggregate into threads, which join to form an open network with regular polygonal meshes, the diagonals of which measure about 6 μ. This stage is one of unequal equilibrium, because the net soon shrinks, the granules shift their position, and thus the typical network with nodal points is formed.

Solid particles in colloidal solutions, such as various grades of finely divided carmin in egg-solution, are found after fixation to occupy the nodal points of the framework, and, if they are large, also to considerably increase the size of the mesh. Such insoluble granules are comparable to those of the pancreas and the coarsely granular leucocytes of the frog, and they do not belong to that class of granules found, for example, in mucous glands which swell up on coming in contact with water.

Cold acts on soluble jellies by producing the structural features of an 'insoluble' jelly at temperatures which vary with the nature of the colloid. Thus a 1½ per cent. watery gelatin jelly shows no change until the temperature has been lowered to 1° C., when minute droplets

[1] Bütschli: *Arch. f. Entwickelungsmechanik d. Organismen*, 11, 499 (1901).
[2] Hardy: *Journ. of Physiol.* 24, 172 (1899).

measuring 0·5μ in diameter are formed, which at $-1°$ C. enlarge till a honeycomb or spongelike appearance is given to the jelly. Lowering the temperature still more causes the water in the spaces to crystallize out. The critical temperature for 1½ per cent. gelatin is thus 1 to $-1°$C., while for 2 per cent. agar it is $-5·5°$C. Rapid cooling, produced by placing a drop of 1·5 per cent. of gelatin, previously heated to 90° C., on a slide cooled to $-15°$ C., eliminates the droplet-stage altogether, there being formed at once a trabecular arrangement, which starting at the periphery of the drop projects into the still fluid central portion.

Stress and Shear Effects.

To study the changes produced in colloids by the action of stress and shear, Hardy proceeds thus :—Films of egg-albumin are stretched across a ring of cork ; then a tiny drop of mercury is rolled on the film and the latter is fixed in sublimate ;—or colloids are poured over a drop of mercury on the slide and fixed at once ;—or a colloidal solution is drawn along the slide with a pointed instrument and then quickly coagulated (l. c. 187, 188).

In the last case, the effect produced by the shear is an internal heterogeneity which lasts for some time because the viscous nature of the colloid prevents a quick recovery towards a stable equilibrium. As long as such an internal heterogeneity persists, singly refractile substances become doubly refractile, owing to a rearrangement of the molecules in a definite linear pattern, as first shown by Bütschli [1].

The bearing of this on histological research is important, because an originally homogeneous plasm, when it is subjected to stress of any kind, may give rise to apparently two distinct substances, although there exist only one. It follows, if it were not for other evidence, that the spindle of a karyokinetic figure might be simply 'a coarse diagram of a dynamic phase of the cell history.'

Homogeneity of Thin Films.

The last point referred to by Hardy is the optical homogeneity of very thin films after fixation, a phenomenon 'probably related to the fact that when colloidal mixtures separate to form conjugates a thin layer next the surface remains homogeneous.' If one corner of two cover-glasses, held in a pair of forceps, is dipped into a solution of white of egg, then the latter by capillary attraction is sucked in between the cover-glasses, and if these be held, at once, for a moment in a steam jet, the albumin will be coagulated while still moving. Such a preparation stained in diverse ways and examined in media with different refractive indices will always appear at its edge homogeneous and unstained, but where the film is thicker a network with nodal swellings and stained deeply is to be made out. Histologically this again is important, because of its bearing on the question whether a hyaline exoplasm, as distinguished from a granular endoplasm, really exists in leucocytes and amoebae.

In connexion with the study of effects produced by reagents we

[1] Bütschli : *Unters. ü. mikrosk. Schäume u. d. Protoplasma*, Leipzig, 1892.

must refer to a macro-chemical method, destined before long to play an important part in micro-chemistry, namely, the method of preparing 'proteids' in crystalline form.

Method of preparing Pure Albumin Crystals.

Hofmeister (1891) was the first to obtain egg-albumin in a crystalline form, but his original method is not so simple as its modification by Hopkins and Pincus, and therefore I give the latter according to the most recent account by Hopkins (*Journ. of Physiol.* 25, 306 (1900)).

Procure newly-laid eggs and collect the egg-white. Measure it carefully, and add exactly the same amount of a saturated ammonium sulphate solution. Beat the two together till the whole mass forms a stiff froth, and let it stand overnight.

Filter off the precipitated globulins and mucoids, and to the clear filtrate add very gradually, under constant stirring, a solution of 10 per cent. glacial acetic acid till a slightly milky permanent precipitate is formed. To litmus paper the mixture will by this time be slightly acid. Now add to each 100 cc. of this milky mixture 1 cc. of the 10 per cent. glacial acetic acid, when a bulky amorphous precipitate is formed which in the course of five hours will become crystalline. To obtain the full yield of crystals (at least 60 grms. per litre), let the mixture stand till the next day.

Pure crystals are got thus: Filter off the precipitate and wash it in three changes of a half-saturated solution of ammonium sulphate containing 1 per 1000 of glacial acetic acid. Dissolve the crystals in a minimal quantity of water; add very slowly, stirring gently all the while, a saturated solution of ammonium sulphate till a distinct precipitate is formed. Then add, in addition, for each litre of the solution 2 cc. of the saturated ammonium sulphate solution. As a rule in twenty-four hours the albumin will have recrystallized. Should the crystals, however, not form readily, agitate the vessel containing the solution gently, but do not shake violently, as mechanical coagulation (see p. 50) is apt to occur.

To remove the ammonium sulphate wash the crystals repeatedly with a completely saturated solution of pure sodium chloride containing 1 per cent. acetic acid.

For histological purposes the crystals may be obtained in a permanent form by one of the two following methods I devised: (1) While the crystals are in the half-saturated ammonium sulphate solution they may be pipetted off and allowed to fall into a saturated watery solution of corrosive sublimate. They will thus be converted into the insoluble egg-albuminate of mercury. After fixation they may be freely washed in alcohol, or ether and water, or water, to remove the free sublimate. (2) The other method consists in allowing the half-saturated ammonium sulphate solution to become quite saturated by spontaneous evaporation, or by substituting for it a saturated solution. Then boil the crystals in this solution for five minutes. The rise of temperature will render the ammonium sulphate unsaturated, and the available water will coagulate the crystals without distorting their shape. The crystals, coagulated in this manner,

are then freed from the salt by boiling in at least ten changes of distilled water. They may be kept indefinitely in absolute alcohol.

According to Arthur Wichmann[1], however, Hofmeister was the first to render the crystals insoluble by coagulating them with alcohol, and Gürber previously to myself had employed the method of coagulating the crystals by heating them in the mother liquor[2].

B. Chemical Precipitation.

To study the effects produced by different fixatives on solutions of chemically pure substances, Alfred Fischer employs two methods. The first consists in mixing the fixing solution and the 'proteid' in little flasks or Petri-capsules, as used by bacteriologists; allowing the precipitate to settle during twenty to twenty-four hours; removing the fixative by washing, and finally making cover-glass preparations of the precipitate for subsequent staining.

His second method is to impregnate small pieces of elder-pith under the air-pump with the 'proteid,' and then to treat the pith as if it were a tissue. In repeating Fischer's experiments I used a slide and a test-tube method.

The Slide Method.

For examining the behaviour of fixatives towards 'proteids,' I originally mounted proteid solutions under the cover-glass, then placed a drop of fixative on one side of the cover-glass and connected the fixing solution and the 'proteid,' but now I use threads soaked in the fixative according to Hardy's method, who uses silk threads. I proceed as follows:—Pieces of cotton-thread about five inches long are laid across a watch-glass, into which the fixative is poured, and the centre of each thread is thoroughly soaked in the reagent. After ten minutes, one thread is chosen and placed along the centre of a slide, great care being taken not to let the fixative come in contact with any part of the slide except along the line of the thread. A thin mica cover-glass (2 × 3 cm.) is laid on the thread; the latter is brought into focus under a magnification of 500 diameters, and now the 'proteid' to be tested is placed by means of a glass pipette on the right side of the cover-glass close to one corner, when by diffusion it will spread along the thread. A mica instead of a glass cover is chosen because the former, being more pliable and also lighter, offers less resistance to the inflow of the viscous 'proteid' solutions.

The Test-tube Method.

The other method depends on the use of narrow test-tubes for mixing the fixative with the 'proteid.' This has the advantage of allowing the two to act slowly or quickly on one another by either pouring the fixing solution into the proteid or vice versa, according to their respective specific gravities, and also by either shaking the

[1] Wichmann: 'Über d. Krystallformen d. Albumine,' *Zeitsch. f. physiol. Chem.* 27, 575 (1899).

[2] Wichmann, employing these crystals for experiments in staining reactions, arrived at the conclusion that all staining is intermolecular, but this question is fully discussed later in the chapter on the theory of staining.

test-tube or keeping it quiet. It is easy in this manner to produce membranous precipitates, to which reference will be made later on.

Fischer classifies the precipitates formed by reagents under two headings. He considers that substances such as albumins, globulins, and nucleo-albumins, because of their more colloidal and therefore less crystalline nature, are thrown down in the form of exceedingly minute particles, which are all of the same size and which he terms 'globulites.' These elements are grouped together to form either solid masses or membranes or trabeculae.

On the other hand, the less colloidal, more crystalline and therefore more diffusible bodies, namely the peptones, albumoses, haemoglobin and nucleic acid, are precipitated as isolated sphaero-crystals or granules. These may differ in size, and may remain single or become arranged in pairs, in short chains, or even appear like growing yeast (Fischer).

For purposes of description, I propose to distinguish these two varieties as 'grumous[1]' and 'granular.'

This division into two groups made by Fischer is, however, a purely artificial one, for the grumous and granular characters are not primarily inherent in the different classes of 'proteid,' but are only induced by the fixing reagents. Whether a grumous or a granular precipitate is formed, depends primarily on the nature and strength of the fixative, and only secondarily on the nature, reaction, and concentration of the 'proteid.'

We have seen how easy it is to obtain albumin crystals (p. 124), and Fischer may be right when he speaks of granules as sphaero-crystals, although their crystalline nature is not revealed by the microscope.

Experiments with the Slide Method.

As examples of various 'proteids,' the following have been chosen: amphopeptone, prepared by Kühne's method; pure deutero-albumose and Witte's peptone, which latter consists mainly of albumoses; prot-albumose, haemoglobin, nucleic acid, prepared especially for me by Dr. Grübler; white of egg diluted with three times its bulk of water, and egg-albumin crystals (prepared by Hopkins' method, free from ammonium sulphate (p. 124)) dissolved in a minimum of water, and this stock solution diluted so as to form a 10 per cent. solution.

1. Peptone is thrown down most efficiently by 7 per cent. sublimate, in the form of a fine grumous precipitate with a few scattered larger granules. The double salt of sublimate and potassium bichromate (p. 76) produces glassy homogeneous membranes and minute granules only slightly aggregated. Sublimate and osmium tetroxide, in combination, produce a less copious precipitate than does sublimate in a saturated solution, the peptone being thrown down as a fine grumous structure with some minute and small granules interspersed. Picro-corrosive and picro-corrosive-formaldehyde form small granules, with a few larger ones interspersed and with but little tendency to aggregate. Picro-corrosive containing 5 per cent. formic acid effects a decided aggregation of the isolated granules. Alt-

[1] Grumous is an old English term for clotted.

mann's mixture shows small, gnarled, granular fragments intermixed with large and small well-formed granules. The latter, however, form the chief bulk of the precipitate obtained with platinum chloride, Hermann's solution, and osmium. Flemming's solution not only does not precipitate, but actually dissolves solid peptone. If a solution of the latter has been precipitated by either picric acid or sublimate, the addition of a sufficient quantity of Flemming's solution redissolves the precipitate.

2. Deutero-albumose (Witte's peptone) is of all the 'proteids' the most difficult to fix. As it is important for micro-physiological research to know how it behaves towards different reagents, I give a somewhat full account of the phenomena to be observed on bringing a filtered 10 per cent. solution of albumose into contact with the ordinary fixatives.

(*a*) Picric acid in a saturated watery solution. The immediate effect close to the cotton thread consists in the precipitation of the albumose in very minute granules, which are carried a little distance away from the thread as the fixative diffuses into the solution. Each granule at first is separate and shows considerable Brownian movement. On shifting the slide till the granular precipitate is just visible (2–3 mm. distance from the thread), careful examination of the still clear albumose solution reveals the sudden appearance of isolated granules, the distance between individual granules being twenty to thirty times their own diameter. As the solution was vigorously boiled and filtered before use, it is difficult to understand why the granules are not formed close together, except on the theory that certain albumose molecules have a greater affinity for the picric acid, and that they form the nucleus round which other molecules aggregate—or that an aggregation of ions is brought about by electrical charges (see p. 53).

After two to three minutes, the originally small granules, close to the thread, have grown into three or four times their first diameter, and many are seen to be joined in pairs. These granules are either of the same size, or one is much bigger than the other.

After five to ten minutes, the picric acid solution will have become much weakened by diffusion, and now the granules coalesce or fuse together till large homogeneous masses result, containing vacuoles in their interior. Thus the jelly stage is reached, which is a preliminary to complete solution. To see the large aggregations dissolve readily, a little albumose solution may be added to weaken the action of the fixative still more.

(*b*) Picro-corrosive. Close to the thread a fine grumous appearance is seen, but the individual granules soon aggregate into a meshwork. In the next zone, isolated granules with Brownian movement soon grow, and these may coalesce into structures like diplococci or short diplobacilli. The further away from the thread the larger do the granules become, till they reach about ten times the diameter of those close to the thread. At the periphery gnarled masses are formed by the coalescence of the large granules; it is, however, rare to see vacuoles in their interior. The difference between the appear-

ance close to the thread and at the periphery is to be accounted for by the quicker diffusion of the picric acid component and by its simultaneous dilution.

(*c*) Picro-corrosive-formaldehyde (see p. 97). The immediate effect is a grumous precipitate, which rapidly becomes arranged to form a coarse net of granules, the individual particles being slightly larger than those obtained with picro-corrosive. Quite at the periphery, the granules, which have been gradually increasing in size, fuse to form large lobed masses, enclosing vacuoles of all sizes. This effect is produced by the formaldehyde diffusing more rapidly than the other ingredients and inducing a primary jelly-like coagulation, which later is partly fixed by the advent of the picric acid and sublimate. After ten minutes the granules close to the thread have fused into homogeneous masses because the fixative has become weakened by diffusion.

(*d*) Formaldehyde (commercial). At first minute granules are seen, with distinct Brownian movement and with no tendency to unite; but these grow, and sooner or later unite with their neighbours to form long chains, which in their turn by fusion produce a coarse spongework; but, curiously enough, the individual granules still possess slight Brownian movement.

(*e*) Corrosive sublimate (saturated watery solution). The primary effect is a grumous deposit, with a slight tendency towards membrane-formation. Then sharply defined granules appear. Further away from the thread the granules become larger and larger, till there is reached a zone, occupied by a distinct jelly with vacuoles. This is again the preliminary stage towards solution, for it must be remembered that all albumins, albumoses and peptones precipitated by sublimate are soluble in an excess of the 'proteid.'

When the meshwork close to the thread is being acted upon by the surrounding albumose solution, the finer meshes coalesce and gradually a trabecular arrangement results, which has a great resemblance to the pith of the bullrush. The originally grumous aspect has disappeared, each trabecula being a perfectly transparent strand of jelly.

(*f*) Mercuric nitrate, 10 per cent., is of all the reagents the most energetic in its action. The initial granules in the grumous precipitate are exceedingly small. The albumose solution, passing by diffusion under the mica cover, is fixed at once, and thus membranes are formed similar to those seen in test-tube experiments, although not so perfect. Such a membrane shows the granules arranged so closely that an aperture is nowhere to be seen. Halfway between the centre and the periphery of the preparation the individual granules become larger and are aggregated into clusters and trabeculae. Quite at the periphery a homogeneous jelly, with vacuoles, is formed as in the sublimate experiments, and for the same reason.

(*g*) Osmium tetroxide, 2 per cent. The immediate effect is the formation of granules, as minute as those obtained with mercuric nitrate, but they have only a slight tendency to unite. Brownian movement is more marked than with any other reagent. After two

to three minutes some of the granules start joining into threads, and after five minutes a coarse reticulum is formed close to the thread, while at the periphery the minute granules continue their Brownian movement. This aggregation of albumose molecules into larger particles is no criterion as to the action of osmium tetroxide on albumin molecules, as the latter are arranged in a definite manner in the cell, and as they cohere to one another, owing to their viscosity being greater than that of albumose molecules.

(*h*) Flemming's strong solution. The immediate effect is the production of very minute granules, with lively Brownian movement. After five minutes the granules have increased to twice their original diameter, and form chains. These unite in places to form a very loose spongy framework. Even after thirty minutes no large granules similar to those in the test-tube experiments can be made out, because the fixative and the albumose are used in relatively small amounts.

Experiments with the Test-tube Method.

1. **Albumose.** Test-tube experiments made with 10 per cent. Witte's peptone (2·5 cm.) and fixatives (7·5 cm.) agreed on the whole with the slide experiments. In all test-tubes after twenty-four hours, in addition to the precipitate which had sunk to the bottom or which was adhering to the sides, some albumose was seen in suspension. A granular deposit was obtained with 1 per cent. solutions of chromic acid, platinum chloride, osmium tetroxide, the fluids of Flemming and Hermann, picro-corrosive and picro-corrosive-formaldehyde. With the four last fluids the average smallest granules are formed with picro-corrosive-formaldehyde, while the largest result from Flemming's solution. All these solutions allow some of the granules to fuse together into large homogeneous aggregates.

To overcome this tendency, experiments were made by adding absolute alcohol to the picro-corrosive, in such quantities as to give 25, 50 and 75 per cent. alcoholic solutions, and also by using a 25 per cent. solution of sublimate in absolute alcohol, which was then saturated with picric acid. In one set of experiments the albumose solution was poured into the fixative, in the other this order was reversed. The result was the formation of a few granules, while the main bulk of the albumose appeared as large spheres, strands, and irregular masses, which are best likened to coke. The masses were full of cavities, varying in size from pin-points to that of a human red corpuscle when both were magnified 500 times. Afterthought told me that I might have expected this on the ground of the following observation, which I had made previously.

If albumose is precipitated by alcohol, and the precipitate with some alcohol is placed on a slide, covered and examined under a magnification of 500 diameters, fine granules are seen clustered together. While observing, allow some water to diffuse under the cover-glass. The precipitate will suddenly appear to boil, small cavities run together to form bigger ones, and there results the formation of a jelly enclosing vacuoles, or, if the action be quicker, a fleeting foam-

like arrangement. Both these stages are soon followed by a complete dissolution.

What happens on adding an alcoholic solution of a fixative to a solution of albumose, is shortly this: the alcohol, diffusing more quickly than the other reagents, temporarily precipitates the albumose, but then, by further diffusion, becomes weakened. The albumose will therefore commence to pass through the phases of solution, until by the advent of the picro-corrosive it becomes fixed.

The granular precipitate of albumose which is formed by the addition of Flemming's solution or picric acid, if the latter be not in excess, is dissolved on heating, but reappears on cooling, while the slight precipitate caused by 1 per cent. osmium tetroxide in a neutral solution is greatly increased in amount on heating.

Granular-grumous precipitates are formed in test-tubes by osmo-sublimate, bichromate-sublimate, sublimate by itself, tannin and picric acid. Solutions of albumose, if alkaline, give with neutral formaldehyde a grumous, if acid, a granular yeast-like deposit.

How the effect produced by some of these different reagents varies with the same solution is well seen in Fischer's table :

Fixative.	10 *per cent. slight'y acid deutero-albumose.*	
(1) Altmann's solution	1 -3	Size of granules in micromillimetres (μ).
(2) Platinum chloride, 1 per cent. .	0·7–1	
(3) Hermann's solution	0·7	
(4) Potassium bichromate, 2½ per cent.	0·5–0·7	
(5) Corrosive sublimate, 7 per cent. .	0·4-1	
(6) Chromic acid, ½ per cent. . .	0·4–0·5	

The fact that the same reagent, for example Altmann's fluid, will give different results, according to the percentage of albumose present, is seen in another table of the same observer. (Two cc. of albumose to one cc. of undiluted Altmann's fluid were taken.)

Percentage of albumose.	*Size of granules.*	
10	1·3	μ.
3	0·7–1·5	
1	0·5–0·7	
0·5	0·5–0·7	
0·1	0·4	

This table may serve to estimate, approximately, the amount of albumose in a cell, if its presence has been established by other reagents.

2. **Prot-albumose**, according to Fischer, is thrown down, in addition to the reagents enumerated under deutero-albumose and peptone, by potassium ferrocyanide and acetic acid mixtures. The granules formed by the last reagent give iron reactions (p. 290). Sublimate, tannin, formaldehyde and osmo-acetic acid mixtures give a granular-grumous precipitate ; thus, for example, with a 0·5 per cent. sublimate solution the granular, and with a 7 per cent. solution the grumous character predominates.

3. **Haemoglobin** reactions I also cite from Fischer's account, because of the occurrence of haemoglobin, apart from the red corpuscles, in extravasations and in blood-destroying organs, such as the liver and spleen.

Exceedingly fine granules (almost grumous) are seen if a 2 per cent. watery or normal saline solution be treated with osmium picric, osmo-acetic and chromic acids, Flemming's solution, sublimate, platinum chloride, and formaldehyde. Coarser granules are obtained with nitric acid and nitric acid alcohol, while very large granules result from 96 and 100 per cent. alcohol and 2·5 per cent. potassium bichromate.

It is important to notice that Müller's solution (p. 93) and $2\frac{1}{2}$ per cent. potassium bichromate do not act similarly. This bears out Burchardt's researches (p. 100) and my own experience with regard to the nervous system, for which I prefer Müller's solution.

4. **Nucleic acid** is precipitated as granules or gnarled fragments by chromic acid, Flemming's solution, 1 per cent. chloro-platinous acid (platinum chloride) and Hermann's mixture. With Flemming's fixative exceedingly minute granules, very slightly elongated and joined after the manner of diplobacilli, are seen. The granules soon become arranged into masses, which in their turn aggregate so as to form a coarse spongework or larger granules.

A more or less grumous appearance is produced by the following reagents: (1) osmo-sublimate forms absolutely homogeneous glassy membranes along with a grumous precipitate; (2) sublimate in saturated watery solutions resembles the previous reagent, but the general arrangement, in the aggregate, is coarser, while the membranes, in places, have a ground-glass appearance; (3) picro-corrosive-formaldehyde coagulates the acid into a spongy and membranous precipitate, the constituent granules of which are all of the same diameter and much finer than the smallest produced by Flemming's solution; (4) picro-corrosive, formaldehyde, iodine, alcohol and 50 per cent. nitric acid.

5. **Serum-albumin** is a typical representative of the class of bodies giving a grumous precipitate, which is not altered by the nature of the fixative, the reaction of the medium in which the albumin is dissolved, or by its concentration. A 2 per cent. acid solution of serum-albumin, precipitated by Altmann's solution (p. 99), has the same grumous trabecular arrangement as a neutral 2 per cent. normal saline solution precipitated by 1 per cent. chloro-platinous acid (platinum chloride).

Egg-albumin with picro-corrosive-formaldehyde gives a more evenly arranged coagulum than with picro-corrosive. The tendency towards membrane-formation is also greater with the latter.

6. **Mixtures of deutero-albumose and serum-globulin** are thrown down, with each constituent in that form which is characteristic of the action of chloro-platinous acid and of the solutions of Hermann and Flemming. Sublimate does not answer so well for reasons given under deutero-albumose and peptone (p. 126). Those fixatives giving large granules, on coming in contact with a mixture of proteids are supposed by Fischer to precipitate in the first instance the albumins and to affect the albumoses only secondarily; these, therefore, must aggregate in the meshes of the albumin precipitate, and then, becoming more and more concentrated, crystallize out after

the manner of sphaero-crystals. As it is, however, not possible to see the crystals in these artificial albumose granules, we may be simply dealing with an agglutination of the viscid particles.

If one compares the strongly acid Flemming's solution with the feebly acid mixture of Altmann, it is easy to understand that the more acid solution will produce a precipitation of granules in an alkaline solution of albumose, where Altmann's fixative will fail altogether (see chart, p. 102).

For the same reason a neutral or feebly acid fixative acting on a living tissue with neutral reaction may fail to show the presence of granules, while a tissue in which post-mortem acidity has developed will show very distinct granulation (Fischer).

The minimum percentage of albumose in a solution of albumin which will give a distinctly recognizable granular precipitate may be taken as 1 per cent. (Fischer).

Hardy, who has studied the coagulation of colloids from the physical point of view (p. 37), states that gelatin in the form of jelly examined at the ordinary temperature does not reveal any structure till it has been treated by one of the common fixing reagents such as alcohol, sublimate, or formaldehyde. The latter, for example, acting for about 16 hours on a 15 per cent. gelatin jelly, produces a typical network with nodal swellings, while sublimate gives this appearance only with solutions containing less than five grams of gelatin in 100 cc. of water, as stronger solutions become honeycombed.

Jellies behave towards a rise of temperature in one of two ways: they either become soluble, by reverting to the fluid state, when they are said to be reversible, or they do not, and then they are known as irreversible or fixed (Hardy).

The reversion to the fluid state is accompanied by a loss of the netlike or honeycomb arrangement of the colloid material, which latter, now, forms a homogeneous mixture or solution with its solvent. A typical example of a reversible jelly is that of pure gelatin and water.

In irreversible jellies (such as gelatin jelly which has been chemically fixed with formaldehyde or a silicic acid jelly) heating causes the colloid framework to shrink, by producing an aggregation or synaeresis (Graham) of the insoluble colloid particles. Because of this shrinkage of the solid constituents or 'clot,' the liquid part is forced out to form the 'serum' (Graham).

Hardy also experimented with egg-albumin in this manner:

White of egg, beaten up, centrifugalized and found to contain about 13 grams of solid in 100 cc., was suspended in loops of silk and exposed to the action of various fixatives; after fixation it was washed in distilled water for about six hours, dehydrated by changes of 15, 30, 40, 50, &c. per cent. spirit, imbedded in paraffin from cedar-wood oil and cut into sections. How the size of the meshes varied with the different fixatives may be gathered from his table:

Fixative, and its time of action.	*Size of mesh.*
Osmium vapour, 12 hours	0·5–0·7 μ.
Steam, a moment	1 μ.
Potassium sulpho-cyanate, ?	1·0 μ.
Potassium bichromate, 21 days	1·3 μ.
Corrosive sublimate, 20 hours	1·7 μ.

With very low percentages of a colloid, instead of a netlike arrangement, a granular precipitate was obtained.

Hardy's results with osmium tetroxide are only to be obtained by proceeding exactly as stated above, for after fixing pure egg-white for two days in the vapour of 2 per cent. osmium, and then transferring the albumin directly to 40, 50, &c., per cent. changes of pure ethyl alcohol, no honeycombed or reticular appearance will be seen.

The alteration produced by the action of electrolytes in the arrangement of the colloid molecules, judging by Hardy's results, leads to the liberation of fluid which, as a rule, can be forced out of the colloidal framework comparatively easily. For example, a 13 per cent. watery pure gelatin jelly withstands, at the ordinary temperature, a pressure of 400 lb. to the square inch without parting with its water; but after having been treated with formaldehyde (containing free formic acid), it can have its water removed by hand pressure.

CHAPTER VIII.

Guide to the Selection of a Fixative.

We should be led in the selection of a fixative in the first instance by general considerations, and secondly by special ones. Proceeding according to this rule the most important question is that of the reaction of the fixative.

When dealing with the theory of staining I shall discuss how albumin after alcohol fixation shows no affinity for acid stains, while albumin will stain readily with acid dyes, if some acetic acid is added to the alcohol used for fixing; or how, if an alkali be added to a basic stain, proteids fixed in neutral alcohol will readily combine with the coloured base. The explanation offered is that living proteid-matter exists in a pseudo-acid pseudo-basic state (p. 28).

Proteids must be regarded, therefore, as neutral compounds. This view has received an unexpected confirmation by Hans Friedenthal[1], who has collected a great deal of evidence to prove that all living tissues, both vegetable and animal, have a neutral reaction. When blood is said to be alkaline, it is simply on the strength of the observation that red litmus paper turns blue on coming in contact with fresh blood or blood plasma. The reason for this reaction is that the comparatively strong, acid litmus turns out the much feebler carbonic acid from its combination with sodium bicarbonate, $NaHCO_3$, which in a chemical sense is an acid substance, but neutral in its reaction to such indicators as are capable of showing the

[1] Friedenthal: *Verworn's Zeitsch. f. allgemeine Physiologie*, 1, 56 (1901).

presence of carbonic acid, such indicators being, for example, phenolphthalein, Porrier's blue, and α-napthol-benzene.

$NaHCO_3$ + litmus = Na litmus + H_2CO_3. Na litmus = NaOH + litmus.

The hydroxyl OH acting on the red litmus converts it into blue litmus. The bicarbonates of the alkali metals are therefore basic to litmus, but neutral to phenolphthalein, because the latter, being a much feebler acid substance, does not turn out the CO_2 from its combination with the alkali. Carbonic acid led into a red alkaline solution of phenolphthalein turns it colourless.

The conception that organic matter is for the greater part in a chemically inert colloidal state, is the only one which makes life possible; life being the expression of a struggle between the proteid colloids with a tendency to become more colloidal, and the electrolytes striving either to break up the already existing colloidal particles, or tending to convert them into still larger masses.

We have, however, to remember (p. 45) that no absolute difference exists between an electrolyte and a colloid, the latter being either an $H^{\cdot}$ or OH' compound, carrying positive or negative charges, but not able to exert any influence on its environment because the $H^{\cdot}$ or OH' ions, with their charges, are joined to an unwieldy mass of aggregated colloidal particles.

It has also been shown, how, by increasing the $H^{\cdot}$ or OH' ions in the fluid holding the colloidal aggregates in suspension, provided the ions which we add are the same as those already joined to the larger aggregates, the latter are broken up into smaller ones, the number of these smaller masses being directly proportional to the number of ions added. An $H^{\cdot}$ colloid by the addition of acids, that is of more $H^{\cdot}$ ions, passes into solution, and similarly an OH' colloid by adding alkalies, that is more OH' ions, also passes into solution.

It follows that this alteration in the state of aggregation must bring about a change in that structural arrangement of the colloidal particles which exists during life.

Hardy is quite right in his statement that the addition of electrolytes capable of producing a coagulation must of necessity produce morphological changes; but it must also be remembered that only those electrolytes are capable of changing a colloid which have a potent negative or positive ion; which, in other words, are either acid or basic in nature.

Neutral salts do not give rise to coagulation, but only to a physical precipitation by the withdrawal of water, provided they be used in sufficient concentration (p. 52).

Again, non-electrolytes, such as osmium tetroxide and formaldehyde, having neither negative nor positive charges, cannot induce morphological changes in a colloid. They act by forming additive compounds.

To sum up: The truest histological fixation of tissues is obtained by non-electrolytes capable of forming additive compounds, and would also be obtained by neutral salts, if the latter did not cause, by the withdrawal of water, a collapse of the cells.

Altmann seems to have been the first who realized the importance of using neutral fixatives, and his mixture (p. 99) is still one of the best we possess; for although the bichromate is slightly acid (p. 75), this acidity for histological purposes is an advantage, as it makes the recognition of detail easier, and its excessive action is prevented by the presence of the osmium tetroxide in this mixture.

As regards the special considerations which must guide us we have to consider what special line of research we are pursuing, whether it is morphological, cytological (see below), or micro-chemical (p. 139).

1. **For morphological purposes**, as distinct from cytological work, an average fixation of all the elements is of greater value than one which is only suited for a particular tissue.

The four methods specially to be recommended are (*a*) sublimate and acetic acid[1]; (*b*) bichromate and acetic acid (see p. 100); (*c*) formol diluted with isotonic salt solutions, or in special cases, such as the entire eye, diluted with water; (*d*) formol and sublimate (see p. 97).

2. **For cytological work** we may divide fixing solutions into those specially suited for nuclei, and those best adapted for the cell-plasm. Generally speaking, the salts of the heavy metals are good fixatives for nucleic acid compounds (the so-called 'nuclear chromatin'); but they cannot be relied upon either for other nuclear elements, such as nucleoli and nuclear sap, or for cytoplasmic structures, except perhaps the centrosomes. These, after sublimate fixation, become readily demonstrable by certain staining methods, simply because of the open structure which has been induced in the rest of the cytoplasm by the action of the fixative.

All salts of the heavy metals have a tendency to produce comparatively coarse coagula, because they become electrolysed themselves, lead to a hydrolysis of the water, and also call forth the electrolysis of proteids. The greater the valency of the metal the more marked does shrinkage in the tissues become, for platinum chloride (H_2PtCl_6) shows the longitudinal splitting of chromatin segments, during mitosis, much more markedly than does mercuric chloride ($HgCl_2$), although the radical $PtCl_6$ is divalent.

Certain investigators use almost exclusively corrosive sublimate either in saturated watery solutions or saturated in normal saline, as does, for example, Martin Heidenhain; others, as Henneguy, speak of the brutal action of sublimate, and state that it produces the same appearance in all cells. A. Fischer seems to object to the use of sublimate because it does not produce such beautiful crops of granules out of a homogeneous mixture of albumose and albumin, as do the solutions of Altmann, Flemming, and Hermann.

Sublimate does precipitate proteid matter in a comparatively coarse net, and the homogeneous appearance seen either after osmium tetroxide or formaldehyde fixation is absent; cells fixed by it do have a certain superficial resemblance to one another, as have cells fixed by any other reagent such as Flemming's or Hermann's solutions: but any one who knows by personal experience, and not

[1] Sublimate, 2·5 grms.; glacial acetic acid, 1 cc.; water, 100 cc.

only by contemplating other people's figures, the appearances produced by sublimate in different classes of cells, will agree with me that Henneguy's charge is greatly exaggerated.

Tellyesniczky has pointed out that sublimate is not a good fixative for testicles, and in this I agree; for the large amount of inorganic salts normally present in this organ dissolves the originally formed coagulum, on the principle of the sublimate-sodium chloride experiments given on pp. 109–113.

Sublimate, further, cannot be relied on for fixing the delicate connective tissue of mucous membranes, and in particular that of the villi, as its use leads to a contraction of the core of the villus and to a separation of the epithelial cells. Probably the reason is the same as that just stated when describing its action on the testicle.

These disadvantages may be overcome to a great extent by the addition of formaldehyde, osmium tetroxide, or potassium bichromate according to the formulae given on p. 97, because such additive compounds are less affected by electrolytes (see Experiments, pp. 116–119).

Assuming that we have at our disposal a given amount of a fixative which is an electrolyte, the exceedingly difficult question arises, How far shall we dilute it for use? Keeping in mind the fact that only ions produce coagulation (p. 68) the two following alternatives have to be considered: A large quantity of a dilute solution will contain the electrolyte more or less fully dissociated (p. 11), but the ions will be distributed over a large space, while a small quantity of a strong solution may possess only a few ions, which for this reason will also be widely separated (see Kohlrausch's table, p. 20). On bringing these solutions together with a coagulable substance, the diluted fixative will commence to coagulate, but inasmuch as its ions are far apart the effect will be a very gradual one, and in consequence the coagulum exceedingly loose; and further, provided the solution is not moved, ions will have to travel considerable distances (pp. 10 and 54).

The concentrated solution, on the other hand, contains in addition to its ions a store of non-dissociated molecules, which latter will commence to dissociate at a gradually increasing rate, for this reason: whenever the ions which were present to begin with are rendered inert either by their chemical union with the coagulable material or by loss of their electrical load, new ions must be formed out of the undissociated molecules to keep the solution saturated with ions (p. 16). The rendering inert of ions means, therefore, diminishing the total number of molecules, or, what amounts to the same, the percentage strength of the fixative, and we know that the smaller the number of molecules in a solution, the greater is the dissociation of the electrolyte.

Therefore to use a concentrated solution of an electrolyte means to employ a solution in which the number of ions is gradually increasing and, what is more, increasing in close proximity to the coagulable material, with the result that an exceedingly coarse coagulum is formed (Experiment 7, p. 107).

Generally speaking, it follows that dilute solutions of electrolytes, for example of sublimate, will tend to fix more uniformly and evenly; there is, however, always the risk of the primary coagulum dissolving in an excess of the coagulable material (p. 77); while strong solutions, on the other hand, ensure complete coagulation, but in so vigorous a manner as to lead to the destruction of fine histological detail.

The time factor is also of very great importance, for, given an albumin which forms insoluble monochlorides and soluble dichlorides, it becomes necessary to interrupt the process of fixation at a period when approximately the whole of the albumin is in the insoluble state. For this reason, fixing with sublimate should not be a prolonged operation, as, owing to hydrolysis of the mercury salt, free HCl is always present, which may in time lead to the solution of the primary precipitate, for albumin precipitated by mercury is readily soluble in strong hydrochloric acid.

For the cell-plasm the best fixatives are osmium tetroxide; formaldehyde; mixtures of osmium tetroxide and formaldehyde; glacial acetic acid by itself or in combination with absolute alcohol; the vapour of sulphurous acid, or 75 to 80 per cent. alcohol saturated with sulphurous acid.

Osmium tetroxide, unable to form salts with other metals, holds amongst inorganic fixatives a place similar to that held amongst organic ones by formaldehyde. Both are good fixatives because they are non-electrolytes (see p. 68), and because by directly combining with the tissues they form additive compounds which do not become electrolysed. They leave, however, the tissues on which they have acted in such a state, that subsequent hardening by means of alcohol or sublimate mixtures becomes imperative. It is omission of this precaution which has brought formaldehyde into discredit, as has justly been pointed out by Sjöbring.

While experimenting with various oxidizing agents, such as osmium tetroxide and chromium trioxide, I was soon struck by the dissimilarity between their action and that of the salts of the heavy metals; and, believing at first that osmium owed its peculiar action essentially to its oxidizing power, was led to combine the oxides of osmium and chromium with sublimate (see p. 97). Subsequently potassium bichromate was substituted for the chromium trioxide to prevent undue hardening. While sublimate, taken by itself, causes excessive coagulation of the cell-plasm, and while potassium bichromate does not fix the nuclei, the double salt $HgCl_2 + K_2Cr_2O_7$ produces not only good fixation of the nuclei, but also prevents, very considerably, the overcoagulation of the cell-plasm.

In this respect osmium tetroxide resembles potassium bichromate; and formaldehyde, used in combination with sublimate, can also completely prevent excessive coagulation (see test-tube experiments on pp. 117 and 118).

It follows that to obtain the best histological preservation one of the following methods has to be adopted: namely, either to fix whatever differentiation exists in a tissue by non-electrolytes, and then to still further harden the tissue for subsequent manipulations

by salts of the heavy metals, alcohol and similar substances,—or to combine the non-electrolytes with such electrolytes as sublimate.

By using osmium tetroxide or formaldehyde, free from formic acid, in the form of vapours, the risk of electrolytic dissociation of the tissue-constituents is reduced to a minimum; but if osmium tetroxide and neutral formaldehyde have to be used in solution, then let them be dissolved in isotonic solutions of neutral salts, or mix neutral formaldehyde with 50 to 70 per cent. neutral alcohol.

Glacial acetic acid is neutral to litmus, and is mentioned here in connexion with the non-electrolytes proper. I have found it a very good fixative, if used either by itself or mixed with an equal quantity of absolute alcohol, as in the mixture of van Beneden and Neyt (p. 101). After fixation is completed the tissue should be suspended in absolute alcohol to remove the specifically heavier 'acid.' M. Geiger[1] has shown experimentally that formic, acetic and chloracetic acids are not dissociated in methyl-alcohol solutions, while hydrochloric and nitric acids do dissociate.

When fixing tissues by employing non-electrolytes in combination with electrolytes we may assume that osmium, if it has free access, is quicker in its action than sublimate, and that formaldehyde, because of its great penetrating power, is always certain to act on the cells before the sublimate can do so. Therefore my method of combining osmium tetroxide or formaldehyde with sublimate is theoretically correct and in practice gives good results.

Alcohol must also be regarded as one of our best fixatives provided it is used in the proper strength. From my experiments in the test-tube (see p. 103) and also on tissues, it appears that 50 per cent. absolute alcohol is a delicate fixative, and that good results may be obtained by gradually increasing at short intervals the strengths to 60, 70, 80, 90, 95 and 100 per cent. If alcohols of 90 per cent. or stronger be used the nuclei are greatly distorted, an appearance with which I was acquainted in 1890, but which Tellyesniczky first recorded[2].

In choosing fixatives, we must further bear in mind whether in the resulting precipitate the individual molecules have their cohesiveness preserved, or whether they lose their viscosity and are made to separate from one another. Thus picric acid and sublimate make the proteid particles separate, while formaldehyde, osmium tetroxide, and weak alcohols make them cohere to one another, as is seen from the experiments on pp. 103–121.

Theoretically the amount of fixing solution should be proportional to the amount of proteid to be precipitated, but the principle generally adopted, and on the whole rightly, is to use an excess of the fixing solution.

Further, we should not expose the tissues to the action of coagulants longer than is absolutely necessary, but I believe all

[1] Geiger: *Gazetta*, 30, 225 (1900).

[2] Tellyesniczky: *Arch. f. mikr. Anat.* 52, 202. With some tissues, such as seminal tubules, even 70 per cent. methyl and ethyl alcohols are too strong.

histologists sin in this respect. Heidenhain fixes for too long a time in sublimate ; and till recently, after having injected animals with fixing solutions, I used to fix the excised tissues for another twenty-four hours in the same fixing solution, but now I proceed at once to 75 per cent. alcohol. Tissues must not remain in solutions of electrolytes longer than is absolutely necessary, especially if we are dealing with chromic acid, picric acid, or with sublimate. Flemming's solution commences to macerate in many instances after twenty-four hours owing to the continued action of chromic acid. M. Heidenhain has drawn attention to this fact, and I fully agree with him for the following reasons. Egg-white coagulated with 5 to 10 per cent. chromic acid will pass mostly or entirely into solution within twenty-four hours ; nerve-cells fixed for prolonged periods in chromic acid or its salts lose so much of their contents as to collapse ; but perhaps the following instance is the most striking. Cole tells me that flatfish fixed in Flemming's strong solution, on being kept in this fluid, invariably open their mouths on the fifth day, owing to shrinkage, and that fish in this state are macerated.

When dealing, however, with either Müller's fluid or with solutions of the bichromates of potassium, sodium (and ammonium), very prolonged action becomes necessary, especially in hardening the central nervous system. Whatever advantages quickly hardening reagents such as Weigert's new fixatives may have, I personally prefer to use Müller's solution, for then axis-cylinders can be stained by Stroebe's method (see p. 222), as after no other fixative. The cord of a cat, for example, should be fixed for at least three months at the ordinary room-temperature.

3. **Micro-chemical research** should be carried on according to the following principles:

1. The tissues at the time of fixation must be absolutely normal.
2. Fixation must be brought about by physical reagents, which neither produce a change in the organic molecules, nor induce a redistribution of the inorganic constituents.
3. Chemical fixation must allow of the reconversion of the newly formed compounds into the original state.
4. Unknown chemical changes induced by certain fixatives must allow us, by means of definite colour reactions, to recognize specific tissue elements, such as elastic or white fibrous tissues.

Following the suggestions just made, it is theoretically best to employ the dehydration method of Altmann (see p. 143) as a means of fixing tissues, but this necessitates very special apparatus. To procure rapid fixation I have mixed solid carbonic acid (CO_2—snow) with alcohol and ether, which gives a temperature of about -80° C. ; have cooled down absolute alcohol to the temperature just stated, and then have used this alcohol for freezing tissues. A whole frog is frozen through and through in a few seconds. At this temperature alcohol is of an oily consistence and does not act as alcohol, as legs of frogs after remaining for hours in the alcohol are still

contractile after thawing. Tissues fixed in this manner were divided or rather broken into small pieces not thicker than 1·5 mm. and dehydrated over sulphuric acid at a temperature of −30° C.

On the assumption that alcohol acts primarily by dehydrating tissues I inject animals with 50 per cent. ethyl alcohol to keep the blood-vessels patent (about half a litre in a cat), then follow up without diminishing the pressure with 75 per cent. alcohol (about one litre) and 90 per cent. alcohol (two to three litres), or, having dilated the blood-vessels with 0·9 per cent. sodium chloride tap-water solution, follow up immediately with absolute alcohol. The animal is quickly dissected and its tissues are placed at once into absolute alcohol. Large quantities of alcohol are required to throw down all free salts in an insoluble form and thus to prevent their union with 'proteid' substances. It should, however, be remembered that prolonged action of alcohol denaturalizes most proteids, and that it is necessary to quickly pass the sections through pure benzene into solid paraffin if they are to be examined immediately, or into pure liquid paraffin if they are to be preserved for some time for future use. It is always necessary to avoid the use of xylol or toluol, as they contain acids (p. 377), and of chloroform, because of the action of the latter on proteids in the presence of salts. It may be argued that chloroform only acts on watery solutions of albumin, but it is exceedingly difficult to absolutely dehydrate tissues.

For some years I have also used the salting-out method employed in physiological chemistry for the study of micro-chemical reactions. Tolmatscheff in 1867 was the first to employ common salt[1], while Heynsius in 1884[2] introduced ammonium sulphate. The advantages of salting out are that the proteid substances do not become denaturalized, provided the salt solutions are perfectly neutral.

It is best to inject a carefully neutralized solution of ammonium sulphate which has been saturated for warm-blooded animals at 39° C., and for cold-blooded animals at the room-temperature.

Tissues are then excised after fixation and left in a further quantity of the saturated ammonium sulphate solution for 48 hours (because of the difficulty with which the salt penetrates), and are finally transferred directly to absolute alcohol, and taken through the celloidin method. (The razors suffer, however, very badly.)

To use heat as a fixing agent I do not consider right, because of the great chemical changes produced by heat coagulation (see p. 58); I also avoid, whenever possible, taking tissues through the paraffin process, as celloidin is preferable.

Amongst chemical fixatives osmium tetroxide and sublimate are the best, because the former can be removed completely (see p. 83) and the latter at least in part (see p. 78), for a modified Millon's reaction is seen in white fibrous tissue after iodine-potassium iodide treatment of picro-corrosive sections.

Theoretically fixing solutions should be isotonic with the tissues

[1] Tolmatscheff: 'Zur Analyse d. Milch,' *Hoppe-Seyler's med.-chem. Untersuch.* 272 (1867).

[2] Heynsius: *Pflüger's Arch.* 34, 330 (1884).

they come in contact with, which means that there should be neither exosmosis nor endosmosis, as the former will tend to cause collapse of the cell and the latter a swelling up. Histologists remember this, as a rule, only when they are dealing with blood, and forget that all tissues ought to be treated on the same principle. It is for this reason also, quite apart from the prevention of electrolytic decomposition, that formol and osmium tetroxide should be combined with normal saline ; that I no longer use sublimate in saturated solutions ; that I have reduced my original picro-corrosive solution to one quarter its strength, the dilute fixing solution having a specific gravity of 1,020. (Unsaturated solutions of sublimate are further also less liable to form crystals in the tissues.)

When dealing, however, with such soluble substances as peptone and albumose it is advisable to use saturated solutions of sublimate in water or in alcohol, but even then difficulties arise (see p. 129).

The disadvantages of using dilute solutions, on the other hand, become evident on studying my slide experiments (see p. 126), which show that the precipitates first formed are liable to redissolve in an excess of the proteid. An entirely different disadvantage is, that with greater dilution hydrolysis also becomes more marked (see p. 21), and that hereby the tendency to overcoagulation is increased.

In preparing organs for histological research we should always remember that there are, in addition to cells, various structures which have been derived from them, e. g. white fibrous tissue. Most observers are in the habit of neglecting the connective tissue, as is shown by the readiness with which they employ dilute acetic acid to ensure better penetration of the object.

Such a procedure is quite legitimate if by no other means a proper fixation of the rest of the organ or animal can be ensured, but inasmuch as we possess in potassium bichromate an agent which penetrates readily we should use it by preference. Picric acid also penetrates with great readiness, and, according to Flemming's and my own experiments, preserves nuclei and cells better than dilute acetic acid, but it is apt to cause a greater amount of overcoagulation in cells than almost any other fixative (see p. 119).

If we suspect that certain appearances in cells are due to physical causes coming into play during the act of fixation, we should vary the mode and direction of access of the fixative. It is easy for example to slit open the alimentary canal of Oniscus, and then to allow fixation to take place from the sides of the large cells or from their inner or outer surfaces.

Another method of determining the pre-existence of certain structures is to study cells in different phases of functional activity. Thus L. Huie has been able to show that eight chromatin segments occur during certain phases of glandular secretion[1], and also during mitotic division[2]. Hermann and Meves have further established the connexion between the cell centrosomes and the middle piece in spermatozoa.

[1] Miss L. Huie : *Quarterly Journal*, 39, 387 (1896-7), and 42, 203 (1899).
[2] Unpublished observation.

Are we justified in using such fixatives as Altmann's, knowing that they precipitate from an intimate mixture of albumose and albumin, the former in the shape of large granules, and the latter in a grumous manner (see p. 126)?

I believe we are for a double reason: (1) granted that we have albumose in solution in the cell, we may use this method for quantitative estimations of the amount of albumose present, that is to say for micro-physiological purposes (see p. 130); and (2) because the fact that Altmann's solution precipitates albumose in a granular form outside the cell does neither signify that granules seen in the cell are albumose nor that they have been produced artificially. To put it shortly, Fischer's observation has been applied recently in the most senseless manner to deprecate the appearances seen in cells. I hold with Michaelis[1] that Altmann's mixture is still one of the very best which we possess.

The last point which should be remembered, is not to undo the fixation we took great care to obtain by thoughtless after-treatment of the tissues, as, e.g., washing material fixed in picric-acid solutions in water; or by treating sublimate-tissue with iodine solutions before the tissue has passed through the paraffin process; or by staining material fixed in alcohol only in watery stains, for Hardy and Wesbrook[2] have found that the granules of certain large basophil cells after alcohol fixation disappear on being brought in contact with watery stains; and Weski[3] confirmed the observation of Dreysel and Oppler[4] that dyes help to fix eleidin granules in sections, for palmar and plantar skin fixed for five to six hours in saturated sublimate solution, dehydrated in increasing strengths of alcohol to which iodine was added, and taken through the paraffin process, behaved as follows: The eleidin granules were unaltered by alcohol, ether, and xylol, but were extracted by an even momentary immersion in water, and therefore it was impossible to use the ordinary method of floating sections on warm water, before mounting them on slides[5].

CHAPTER IX.

Application of Fixatives to Tissues.

I. **The dehydration method.** As far as I have been able to ascertain, the process of drying tissues for microscopical research is one of the oldest of all methods. Leeuwenhoek (1720) describes

[1] L. Michaelis: 'Die vitale Färbung, eine Darstellungsmethode d. Zellgranula,' *Arch. f. mikr. Anat.* 55, 558–575 (1900).

[2] Hardy and Wesbrook: *Journ. of Physiol.* 490–524 (1895). They rediscovered the 'mantle layer' of mast-cells described first by Ehrlich in 1877 (*Arch. f. mikr. Anat.*, 13, 263 (1877).

[3] Oskar Weski: *Anat. Hefte*, 17, 199 (1901).

[4] Dreysel and Oppler: *Arch. f. Derm. u. Syph.* 30.

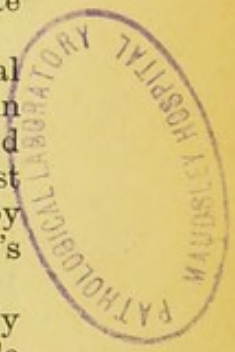
[5] To fix the eleidin granules use water-blue (methyl-blue), which was introduced by Frickenhaus (*Monatssch. f. prakt. Dermat.* 23). See also Franz Weidenreich: *Arch. f. mikr. Anat.* 57, 583 (1900).

how he gave orders to the captain of a whaler to procure a piece of whale muscle, on the supposition that big animals have big tissues. He carried such a piece of dried muscle about with him, and whenever he wanted a little pleasant relaxation, slices cut off with a knife were examined under the microscope. His method is still practised at the present day with some tissues such as tendons.

A more refined proceeding, depending, however, on the same principle, is Altmann's method:—Small portions of an organ are frozen and kept at a temperature of at least -20° C. over sulphuric acid in vacuo. After some days the tissue, having become quite dry, is placed in melted paraffin in the vacuum chamber, and is readily impregnated. As far as my experience goes the method ought to be practised more frequently, but it requires special apparatus not always available, or constant attention for days on end (see p. 139).

Absolute alcohol and acetone, pure glacial acetic acid (see p. 105), saturated neutral salt solutions (for example, sodium chloride and ammonium sulphate), all act primarily as dehydrating agents, although in the case of alcohol, acetone, and acetic acid definite chemical changes may supervene.

II. **Fixation by heat of moist tissues** for micro-chemical research[1]. If heat is to be tried it is best to place the tissue in a test-tube, to just cover it with normal saline solution (0·9 NaCl), and to immerse the test-tube for 5 to 15 seconds in water which has just stopped boiling. Thin wet films (for example, blood) can be fixed by exposure to the heat of steam for 2 to 3 seconds, according to Schäfer's method, or dry films by prolonged action of dry heat.



III. **The fixation by heat of dry proteids** is an exceedingly important method. It was introduced by Ehrlich in 1878. To get good results proceed thus:—(1) Make very thin films of blood, pus, marrow, or areolar tissue on cover-glasses. (The cover-glass method was introduced by Koch for the examination of bacteria.) (2) Dry the films quickly and spontaneously by waving the cover-glasses about in the air. This drying process prevents the coagulation of the proteids, as coagulation can only take place in the presence of moisture. (3) Fix the proteids by exposing the cover-glasses for 1 to 2 hours to a dry heat of about 120° to 140° C. Ehrlich originally used for this purpose a copper plate heated up to the required temperature. The object of fixing by heat is to diminish the normal power of absorbing fluids possessed by all proteids, and thus to prevent the distortion of the shape of cells; secondly, to prevent decomposition of proteids by the action of water, and also the removal of certain constituents (for example, the haemoglobin) from the red corpuscles; and thirdly, to make the cells adhere to the cover-glass.

IV. **Fixation by chemical means** is, however, for ordinary purposes, to be preferred to either of the three methods described above, because in many cases it allows the investigator to study directly, under the microscope, the changes produced by each fixative.

[1] Personally I do not approve of this method, because albumoses are rendered more soluble and salts enter into combination with 'proteids,' altering their reaction.

The methods of employing chemical fixatives are given below.

Living cells are exceedingly liable to undergo, sometimes in a few seconds, changes from which they do not recover; it is therefore essential to bring them as quickly as possible into contact with substances which will fix the various organic constituents, in such a way as to prevent their spontaneous decomposition.

From what has just been stated it follows that by preference only living material should be used. When this is not available it is imperative to obtain tissues as soon after death as possible.

When investigating a warm-blooded animal, the fixing solution should be heated to and be maintained at the temperature of 39° C., while cold-blooded animals are treated with fluids at the ordinary temperature of the laboratory. The amount of the fixing solution required differs according to the procedure we adopt—namely, whether we employ an injection method or whether we place an excised piece of organ into the fixing fluid.

(*a*) **The injection method** was devised by me over ten years ago, and the results achieved by it are much superior to those obtainable by any other process, for the following reasons: (1) within fifty seconds at the latest, every cell in the body is fixed, and therefore we may compare the mutual state of functional activity of the different organs in the body at the time of death. (2) Such exceedingly delicate tissues as, for example, the medulla of the supra-renal, the carotid gland, the pituitary body and the retina are perfectly preserved. (3) The distribution and structure of blood-vessels can be seen in their normal physical state, for we are not dealing with shrivelled and collapsed tubes distorted out of all resemblance to what they must be during life. (4) The natural shape of the hollow viscera in the abdomen is preserved, as is that of the tubules in the testis. (5) Hard structures such as bone and teeth have their cells fixed in such a way as to resist the deleterious action of decalcifying agents. (6) There is absolutely equal fixation of every portion of an organ, while by the ordinary methods the periphery of a block of tissue is fixed in one way, and the centre in an entirely different manner.

From Bolles Lee and Paul Mayer's book I learn that Golgi must have practised the injection method for fixing purposes several years before I did, for he used to inject a half per cent. solution of potassium bichromate through the carotid to fix the brain, and through the aorta if he wanted to preserve the spinal cord [1].

Quervain [2], in 1893, allowed the blood to flow from the carotid artery and then injected the same amount of Müller's fluid heated up to the body-temperature. He takes for dogs 300 to 600 cc., and for cats 100 to 300 cc.

My own procedure, first published in 1894 [3], is described below:

[1] Golgi: *Arch. ital. biol.* 7, 30 (1886).

[2] Quervain: *Virchow's Arch.* 133, 487 (1893), and *Zeitsch. f. wiss. Mikr.* 10, 507 (1893).

[3] Mann: *Zeit. f. wiss. Mikr.* 11, 482 (1894).

Procedure of the injection method.

A simple injection apparatus may be constructed by securely attaching a piece of indiarubber tubing, about two and a half metres long, to a large funnel having a capacity of one litre. Into the lower end of the tubing a glass canula is fitted, bent at an obtuse angle, measuring (for a cat) at its free end about 7·5 mm., and having a distinct constriction or neck to prevent it slipping out of the aorta after the application of the ligature. About 10 cm. above the glass canula a clamp is placed, which can be opened or closed without any loss of time. The apparatus may either be held by an assistant or a simple method be devised for raising and lowering the funnel by means of a pulley and crank.

One litre of normal saline and two litres of the fixing fluid[1] are heated up to 38° C. and are kept ready. The following instruments are arranged in the order indicated from left to right: (1) a solid razor; (2) a large pair of curved scissors, blunt at the point; (3) a strong pair of forceps; and (4) an aneurism needle threaded with thin twine.

The animal is first rendered unconscious and kept in this condition by coal gas, for the following reasons:—(1) pain would cause changes in the nervous system; (2) other anaesthetics, such as chloroform or ether, cause in the cat profuse salivation; (3) coal gas ensures complete dilatation of the blood-vessels; and (4) it retards the coagulation of the blood.

The unconscious animal is fixed to the animal-holder, and the heart is exposed by one incision being made with the razor through the left thoracic wall in the mammillary line. The apex of the heart is then cut off with the scissors, and the upper part of the sternum and the ribs attached to it, up to the region of the mammillary line on the right side, are removed with two incisions. Next, the heart is pulled downwards with the forceps, and the ligature needle with the string passed underneath the aorta. The string is retained in position while the ligature needle is removed.

The funnel is filled with the warm saline, the clamp above the canula is opened, and care is taken to drive out all air-bubbles. The clamp is then closed, and the canula introduced into the aorta through the left ventricle, the ligature tightened, and about 250 to 500 cc. of the normal saline used to wash out the blood-vessels, keeping the funnel 1 metre above the animal. As soon as the last of the saline is seen to be leaving the funnel, the fixing fluid is quickly poured into the funnel, and the latter raised another quarter or half of a metre.

The injection of the fixing fluid takes place, therefore, under an original pressure of $1\frac{1}{4}$ to $1\frac{1}{2}$ metres. After about one litre has been injected, the funnel is lowered to $\frac{1}{2}$ to $\frac{3}{4}$ of a metre, and the second litre passed through under this diminished pressure. The fluids which are injected pass out of the right ventricle, and it may even happen, because of the low pressure used, that the fixative will circulate

[1] Preferably picro-corrosive-formaldehyde (p. 97) for micro-anatomical research, and 75, 90 and 100 per cent. absolute alcohol for micro-chemical investigations. The alcohol must be pure.

chiefly through the head and neck regions, and not flow to the same extent through the lower half of the animal. Using my fixing fluid, which contains picric acid, it is easy to see, by blowing the hairs apart, whether the skin over the abdomen and in the tail is becoming yellow. If within five seconds, counting from the first appearance of the fixing solution in the glass canula, the skin over the abdomen is not stained, then the outflow of the fixing fluid from the right ventricle should be retarded by either clamping the ventricle with a pair of artery forceps or by passing a ligature round both ventricles and tightening it up considerably.

If it be desired to inject only the head and neck of an animal, the thoracic aorta should be clamped, immediately above the diaphragm, with a pair of artery forceps.

After the second litre of the fixative has passed through the body, those parts which are needed may be removed at once, or be left in the animal up to twenty-four hours, without being injured in any way for micro-anatomical work. For cytological or cell research it is, however, as well to remove the organs in the course of one hour, because this will allow portions of them to be placed in further quantities of the same or other fixatives, with the view of preserving everything that can be fixed. For micro-chemical research I pass through a cat 200 cc. of normal saline to distend the blood-vessels, then 500 cc. of 75 per cent. alcohol, 1,000 cc. of 90 per cent. alcohol, and 500 cc. of absolute alcohol. Then I excise the tissues, rapidly cut them into slices 5 to 10 mm. thick, and place these in large quantities of absolute alcohol for 12 hours, then into equal quantities of alcohol and chemically pure benzene, free from thiophane, for 12 hours, then into several changes of pure benzene till the tissues are perfectly clear, and finally into pure liquid paraffin of an oily consistence.

The after-treatment of tissues will be described later (p. 163).

(*b*) The ordinary fixing method without injection.

(1) Solid non-contractile tissues, e. g. liver, brain, kidney . }
(2) Contractile tissues : }
  (*a*) Intestine . . . . . } p. 147
  (*b*) Stomach . . . . . }
(3) Elastic tissues : }
  (*a*) Skin . . . . . }
  (*b*) Lung . . . . . } p. 148
(4) Seminal tubules . . . . . }
(5) Retina . . . . . } p. 149
(6) Cochlea . . . . . }
(7) Olfactory and respiratory mucous membranes . . }

If instead of using the injection method we are compelled to employ the ordinary method of fixation, which consists in transferring pieces of tissue to the fixing fluid, then special care should be paid to the removal of a tissue from the body so as to prevent injury to the more delicate parts. It is, for example, quite easy to do great damage to the medulla of the human supra-renal gland, if any pressure or pull be exerted on its capsule.

The amount of the fixing solution should be about twenty times the bulk of the tissue, and care should be taken that the fixative has free access from all sides. This may be attained by either laying the tissue on absorbent cotton wool, or by suspending it in the fluid by means of a thread or enclosed in muslin.

To allow the fixative to exert its full action, diffusion currents should be set up by placing the vessel containing the tissues on the top of an incubator, provided the temperature does not rise above 40° C. The stream of warm fluid which will be constantly ascending, brings fresh fixing solution in contact with the tissue, and also increases the penetrating power of the fixative.

In thickness a piece of tissue should never exceed 5 mm., while its surface extent may be of any size.

(1) **Solid non-contractile tissues**, such as glands, portions of the nervous system, dead muscle, &c., are cut into slices not thicker than 5 mm., and each piece is laid separately, without touching its neighbour, in a flat glass vessel, on a very thin layer of cotton wool, and is then covered with the fixative. A mistake one is very apt to fall into, is to take a great deal too much cotton wool, which then buoys up the tissues and makes them project out of the fixing solution. Should this occur, a little cotton wool is placed over the pieces, and is then moistened with the solution.

(2) **Contractile tissues** requiring rapid fixation should be treated according to the rules laid down for the intestine and stomach.

(*a*) *The intestine.* If soon after the death of an animal portions be removed from a hollow viscus such as the intestine, special precautions must be taken to prevent the muscular coat from contracting irregularly, and distorting thereby the mucous coat.

The best method under these conditions is to pass a ligature round one part of the gut, to distend it moderately with the fixing solution, to tie a second ligature higher up to prevent the escape of the fixative, and then to either place the intestine in a vessel on absorbent cotton wool, and to cover it with the same fixative with which it was distended, or to suspend it in the fixing solution for twelve hours. Smaller pieces may then be cut out.

(*b*) *The stomach* should be pinned out in the manner stated below on a cork-frame, having an aperture of about 2 × 3 or 1½ × 2 cm. To prevent the fixing solution from entering the cork, and the tannin of the cork from transfusing into the tissues, each frame should be thoroughly dried, and then be soaked for thirty minutes in paraffin (56° C.) heated up to 80° C. When cool the frame is ready for use, and may be used repeatedly.

The cork frame is laid on the unopened stomach, and the muscular coat in contact with the frame is pinned to the sides of the frame by first securing the four corners and then introducing about twenty hedgehog spines, to counteract any subsequent tendency of the piece of stomach to curl outwards. Now with a sharp razor, or with a pair of scissors, the frame with the attached portion of the stomach is excised. The mucous membrane is rinsed with normal saline to remove all traces of food, the cork is floated on the fixative with the

mucous coat in direct contact with the solution, and the little chamber formed by the muscular coat and the sides of the frame is also filled with the solution. After remaining twelve hours in the fixative, all danger of the stomach curling up will be past; and now it may be taken off the frame, and those portions through which the spines have passed be removed.

(3) **Elastic Tissues** must be fixed in such a manner as to represent the appearance shown during life.

(*a*) *Pieces of Skin* should be pinned out on a cork frame, similar to that used for the stomach (see above), and should not be removed from the frame till they have been completely fixed.

(*b*) *The Lung* should be filled with the fixing solution so as to obtain the air-vesicles fully distended. Proceed thus:—Open the thorax carefully, to avoid puncturing the lung. Tie a small funnel into the trachea and carefully dissect out the lung and bronchus. Then fill the funnel with the fixing solution, which will run into the lung and distend it to its full physiological condition. Ligature the bronchus, transfer the lung to a portion of the fixing fluid, and keep it moving up and down for a few minutes. Then cut it into pieces 1 to 2 cm. thick, and cover the pieces with absorbent cotton wool, and leave for twenty-four hours. On the next day very gradually remove the air by means of an air-pump, to cause the pieces to sink, and to ensure, subsequently, an equal penetration of the imbedding medium.

(4) **Seminal tubules.** The tunics of the testis are so firm as to offer great resistance to the entrance of the fixative, and as, further, the seminal tubules require prompt fixation to preserve the delicate structure of the sperm cells, the following method will be found to answer best:

Divide the testis by a longitudinal cut into halves, and move each of these rapidly about in the fixative for five minutes. The seminal tubules will thus be more or less isolated and instantly fixed.

(5) **The Retina.** The eye, for fixation of the retina, is excised and held in the left hand. With a very sharp hollow-ground razor an incision is made through the sclerotic coat, just posterior to the corneo-sclerotic junction. A sharp pair of scissors is introduced and the cornea and corneo-sclerotic junction are removed, while great care is taken in no way to compress the bulb of the eye. Next, place the eye in the hollow of the left hand and carefully remove the vitreous humour with a pair of forceps, inclining the eye at the same time, and thus helping the humour to flow out. Place the eye on some absorbent cotton wool, half fill it with normal saline, take hold of the sclerotic coat with two pairs of forceps placed on opposite sides, and immerse the eye in a vessel containing the fixing solution. Lift the eye ten times completely out of the solution, and then immerse it again. By this means the retina can be obtained almost as perfectly as if it had been fixed by injecting the animal.

If rapid fixation is considered unnecessary, and sections are wanted for macroscopical demonstration, then the excised eyeball should be placed for forty-eight hours in a large quantity (150 and 300 cc.

for eyes of a cat and ox) of 25 per cent. formol, which means a 10 per cent. solution of formaldehyde. After opening the eye, which may now be done without running the risk of the retina collapsing, a secondary fixation with picro-corrosive-formaldehyde solution (p. 97) gives the best results.

(6) **The Cochlea.** For the study of the organ of Corti it is best to use the guinea-pig or the bat, as in these animals the cochlea projects into the tympanic cavity. To obtain good preparations it is impossible to be too quick in one's manipulations. The animal is killed by beheading, the lower jaw rapidly torn and cut away, the tympanic bulla laid bare and then opened up with a pair of bone forceps. The temporal bone with the cochlea is rapidly transferred to the fixing solution, preferably osmium-sublimate acid (p. 97), or Flemming's strong solution (p. 95), or pure formol; the fenestra rotunda and ovalis are opened under the fixing solution, and a further aperture made on one side near the apex of the cochlea. The bone is now moved slowly about in the fluid for ten minutes, then left therein for twenty-four hours in the dark, and decalcified preferably by means of sulphurous acid (p. 156), or by 5 per cent. sublimate solution containing 1 per cent. of nitric acid. After complete decalcification the cochlea should be washed for twenty-four hours in running water, or be treated with repeated changes of 50 per cent. alcohol to remove the acids thoroughly.

(7) **The olfactory and respiratory mucous membranes** are best fixed along with the bones to which they are attached. The olfactory mucous membrane is thicker than the respiratory portion, is yellow in colour, and will be found to line the posterior turbinated bones and the upper posterior part of the nasal septum. After fixation for twenty-four hours in osmium-sublimate it is easy to detach the membranes from the bone, and to treat them without disarranging the glandular and nervous structures.

PART II.

CHAPTER X.

On Bleaching, Isolating, and Decalcifying.

1. Bleaching.

For bleaching animal tissues by removing the pigment occurring in the cell, the following methods are the best:

1. Paul Mayer's free chlorine or oxygen method[1], which acts both on normal pigment and also on the blackening due to reduction of osmium tetroxide (see p. 83).

2. Seiler's mixture[2], consisting of 1 per cent. chromic acid 70 parts, nitric acid 3 parts, and water 200 parts. Decolourize in the dark and wash out the chromic acid in the dark.

3. Rawitz[3] recommends alcoholic soda solution for the pigment on the mantle edge of mussels. Take 3 to 9 drops of the officinal NaOH solution to 15 to 20 cc. of 90 per cent. alcohol.

2. Isolating.

By isolating a tissue, we mean retaining it for histological investigation at the expense of the other elements with which normally it is bound up in any organ. Thus in some instances we may desire to study cellular elements, in others the ground substance, either organic or inorganic, and so forth.

Isolation may be brought about by mechanical or by chemical means, the latter including also putrefactive changes. Thus it is easy to tease the sciatic nerve of the frog, and to spread out the individual fibres with needles, by Stöhr's method, or to obtain epithelial cells by scraping the mucous membrane lining the mouth.

Two other mechanical methods, introduced by His, consist in either carefully brushing sections, to remove epithelial and endothelial cells, or in getting rid of loose cells in sections of the spleen and lymph glands by shaking them in a test-tube half filled with water. Similar results are obtained by injecting animals with normal saline, when the adult red and white corpuscles of the spleen and lymph glands are readily washed out.

The chief chemical means of isolating fibres are:

1. *Ranvier's one-third Alcohol and its Modifications.*

(*a*) *Ranvier* (1868) recommended the use of 90 per cent. alcohol 1 part, and distilled water 2 parts (= 28 cc. of absolute alcohol and 72 cc. of water (Böhm and Oppel)). Leave tissues for 24 hours in

[1] P. Mayer: *Mitth. Zool. Stat. Neapel*, 2, 8 (1880).
[2] *Fol's Lehrbuch*, 112.
[3] Rawitz: *Leitf. Hist. Unters.* 2. Aufl., Jena, 25 (1895).

ten times their bulk of the alcohol, and change every 24 hours till isolation is possible. For ordinary epithelial cells, one day will be found sufficient, while the endocardium should be left three days or longer. Thin, in 1879, recommended one quarter alcohol for the isolation of retinal fibres, and Rawitz recommends the same strength for the central nervous system of evertebrates and for ordinary epithelia.

(*b*) *Felix*, and also Rausch and Martin Heidenhain, use Ranvier's one-third alcohol saturated with salicylic acid[1]. The latter was originally added to prevent putrefaction, but it also acts as a fixative. This solution gives the best results, if tissues are macerated for twice the length of time indicated above for Ranvier's alcohol.

(*c*) *Rausch* adds to 90 cc. of Ranvier's alcohol 10 grams of sodium or potassium carbonate for the demonstration of the cells in stratified epithelium.

(*d*) *Ewald* (1897) uses Ranvier's alcohol, and subsequently fixes the isolated cells in $\frac{1}{2}$ per cent. osmic acid.

(*e*) *Gage* (1890), by combining picric acid with alcohol, obtains a macerating fluid which can also be recommended. It consists of 95 per cent. alcohol 250 parts, distilled water 750 parts, and picric acid 1 gram. Macerate 2 to 24 hours.

2. *Acids.*

(*a*) *Chromic acid* was first advocated by Max Schultze in strengths of 1 : 1,000 and 1 : 10,000 in distilled water. Use large quantities. In 24 to 48 hours, epithelia and non-striped muscle may be separated into the component parts.

Arnold's method for peripheral ganglia I find with Rawitz to give good results. Leave the ganglia for 5 to 10 minutes in 1 : 1,000 acetic acid to swell the connective tissue, then transfer directly for 24 to 48 hours into 1 : 1,000 chromic acid. Tease, impregnate with 1 : 1,000 gold chloride solution 5 minutes, and expose in acidulated water to direct sunlight for 24 hours.

(*b*) *Glacial acetic acid* (Unna) is used either pure for isolating the cells of the stratum corneum (2 days), or in 25 per cent. strength to separate the epidermis from the dermis (4 to 5 days).

Haller was the first to use it in combination with glycerin and water, while Siehler uses it in conjunction with 1 per cent. chloral hydrate to dissolve connective tissue.

Siehler's macerating fluid for muscle is composed of

Ordinary (33 per cent.) acetic acid . . .	1 part
Glycerin	1 ,,
1 per cent. watery chloral hydrate . . .	6 parts

(*c*) *Hydrochloric acid* (Behrens) in 12 per cent. solution, specific gravity 1·060, isolates glandular elements and their ducts in 10 to 24 hours. It gives good results with the kidney.

[1] This salicylic acid alcohol is also the best method for studying the anatomical arrangement of muscles in the frog, the muscular substance becoming white while the tendons remain transparent.

(*d*) *Nitric acid* in 20 per cent. solutions was first used for muscle by Reichart; also in combination with potassium chlorate by Kühne. Apáthy combines it with acetic acid for the isolation of nerves in leeches. Apáthy uses

Nitric acid	3 parts
Acetic acid	3 „
Glycerin	20 „
Absolute alcohol	20 „
Water	20 „

Macerate leeches for 24 hours, and then transfer them directly to 70 per cent. alcohol, in which they swell up.

Hopkins leaves portions of the stomach and intestine in 20 per cent. nitric acid till the mucous coat separates from the muscular one. The latter is then transferred to a saturated solution of potash alum, and preserved till needed. The non-striped muscle-fibres are readily isolated.

(*e*) *Sulphuric acid* in concentrated solutions answers for horny substances, such as nails and hairs. Heat the hairs in sulphuric acid till they begin to curl; transfer them into distilled water; wash carefully, and scrape them for the medullary cells. To demonstrate the cuticle of the hair, simply place it after it has commenced to curl on a slide, cover with water, and press on the cover-glass. The cuticle will detach itself in large flakes.

(*f*) *Sulphurous acid* is used by Sandmann for the isolation of muscle-fibres. In combination with cane-sugar, it was first recommended by Klebs, who adds to 1 cc. of 5 per cent. cane-sugar one drop of sulphurous acid. Usually in 1 to 2 hours epithelial cells separate readily; sometimes they take, however, much longer, and therefore for class purposes I have slightly modified the method. Take 9 cc. of 10 per cent. cane-sugar and 1 cm. of sulphurous acid. Keep epithelial and glandular tissues of the frog for 15 to 30 minutes at a temperature of 40° C.; tease and examine in the macerating fluid.

3. *Alkalies.*

(*a*) *Sodium carbonate* in combination with Ranvier's alcohol has been mentioned above (Rausch's method).

(*b*) *Sodium* and *potassium hydrate* in 35 to 50 per cent. solutions were introduced by Moleschott. Max Schultze used potassium hydrate in 28 to 40 per cent. and sodium hydrate in 20 to 22 per cent. solutions[1]. It is now customary to use a 33 per cent. solution of caustic potash, which answers well, as it dissolves the connective tissue without destroying the other elements. Tissues, if not treated previously with formaldehyde, should be examined either in the 33 per cent. potash solution or in 10 per cent. formol solution, which latter fixes the tissue elements and allows them to be examined in water, or, after washing out the potash and neutralization with acetic

[1] Bolles Lee and Mayer, 254 (1898).

acid, the fibres may be stained in the usual way. The usual method[1] of neutralizing the alkali with acetic acid or transferring the tissues at once to a 60 per cent. solution of potassium acetate, acidulated with acetic acid, is not so good.

4. *Salts.*

(*a*) *Potassium bichromate*, 1 : 1,000 (Deiters' method), allows of ready isolation of the motor cells in the spinal cord and the antler cells of the cerebellum. Place slices of the cord and cerebellum about 1 cm. thick on absorbent cotton wool, and cover them with at least 20 times their bulk of bichromate solution. Change the solution at least four times in the course of two days. Stain in a 1 per cent. sodium carminate solution.

(*b*) *Landois' mixture* contains

Saturated watery solution of ammonium chromate	5 parts
„ „ „ potassium phosphate	5 „
„ „ „ sodium phosphate .	5 „
Water	100 „

Macerate slices of the central nervous system, 1 cm. thick, 2 to 6 days in large quantities of this fluid. Then transfer them to a 1 per cent. solution of van Wijhe's carminein (p. 248) for 24 hours and tease. This method gives better results than the simple potassium bichromate method mentioned above.

(*c*) *Sodium chloride*, in 10 per cent. solutions, is used by Ballowitz for the maceration of spermatozoa, which may or may not be fixed previously in osmic acid vapour (*Zeitsch. f. wiss. Zool.*, 329, 1890, and *Internat. Monatssch. f. Anat. u. Physiol.* 11, 218, 245, 1894).

(*d*) *Chloral hydrate*, first recommended by Lavdowsky (1876), in 2–5 per cent. solutions acts well for all delicate cells, such as salivary glands. Its combination with acetic acid in Siehler's fluid is given above (p. 151).

5. *Digestion methods.*

The idea of subjecting tissues to the action of gastric juice originated with Beale in 1858 (*Arch. of Med.* 1, 276), although he did not actually isolate any tissue elements. His method was used for tracing nerves. Stirling (1873), while working in Ludwig's laboratory, optically isolated the elastic fibres in the skin by digesting the latter with artificial gastric juice[2], which renders all other elements, excepting nerves and nuclei, indistinct.

Ewald and Kühne[3] treated various organs with a slightly alkaline glycerin extract of the pancreas, and obtained results which have been compiled by Böhm and Oppel (*Taschenbuch d. mikr. Technik*, 1900[4]):

'(1) Tendons break up into fasciculi and fibrils; of all the other constituents only shrivelled nuclei remain, which become easily detached.

[1] Behrens, Korsel, and Schieffer: *Das Mikroscop*, 156 (1889).

[2] *Journal of Anat. and Physiol.* (1875).

[3] A. Ewald and W. Kühne: 'Die Verdauung als histologische Methode,' *Verh. naturhist. Ver. Heidelberg* (N.F.), 1, 451–456.

[4] I was unable to procure the original paper.

(2) Alveolar connective tissue of the mesentery behaves like tendon. The endothelium is dissolved, with the exception of the nuclei.
(3) Reticular connective tissue is obtained quite pure.
(4) Hyaline cartilage : cells, except the nuclei, are dissolved. The ground substance forms a granular network behaving like collagen.
(5) Elastic cartilage behaves like the hyaline variety. The elastic fibres disappear.
(6) Elastic tissue is dissolved.
(7) So-called structureless basal membranes are dissolved.
(8) The liver is completely digested, with the exception of the nuclei and the fibrillar connective tissue which extends to the vena intralobularis.
(9) Muscle is completely digested, except the connective tissue.
(10) The epithelium of mucous membranes disappears, with the exception of the nuclei.
(11) In stratified epithelium the rete Malpighii is affected first, then the prickle cells become isolated, and, lastly, the cells of the stratum corneum, which appear as if hollow and with a double contour.
Therefore tryptic digestion is a means of isolating in any organ the collagenous or white fibrous tissue, the cells of the stratum corneum and nuclei.'

On the opposite page I give F. Mall's results in a tabulated form [1]. The reticulin is a special kind of connective tissue occurring in the spleen, lymph glands, mucous membranes, liver, lung, and kidney. It differs from ordinary connective tissue in not being converted into gelatin on boiling.

To obtain the sheaths of elastic fibres, boil the ligamentum nuchae for a short time in strong hydrochloric acid, and then pour the contents of the test-tube into cold water. Sometimes a longitudinal striation can be made out in these sheaths, especially after staining in a dilute solution of fuchsin (magenta) in 50 per cent. watery glycerin. White fibrous tissue and reticulin, which in their normal condition contract at 72° C., do not shrivel after being fixed in a ½ per cent. osmic acid solution till the temperature is raised to 95° C.

Treated with the natural gastric juice of the dog, tendons do not dissolve more quickly than elastic fibres, but with artificial peptic digestion the tendons are acted upon first, then the reticulin, and lastly the elastic fibres.

To demonstrate the elastic fibres in the lung, skin, &c., I have used the following method for some years. Fresh tissues are placed on the freezing plate of a Williams or Cathcart microtome, they are surrounded if necessary with gum to steady them, and are then frozen and cut. The sections are transferred directly to alcohol, and fixed for ten minutes ; they are now stained in orcein (orcein 1 gram, hydrochloric acid 1 cc., water 10 cc., absolute alcohol 90 cc.) for twelve hours, and subjected to gastric digestion at a temperature of 37° C. Very instructive preparations are obtained in this way, especially if thick sections are cut.

E. Höhl [2] (1897) fixes paraffin sections by Gulland's water method to slides, removes the paraffin with xylol, the latter with absolute alcohol, and then, to get rid of all traces of fat, treats the slides in a

[1] F. Mall : *Sächs. Ges. Wiss., math.-physik. Classe*, 17, 299-338 (1891) ; and *Johns Hopkins Hospital Reports*, vol. i (1896).
[2] Höhl : 'Zur Hist. d. adenoid. Gewebes,' *Arch. f. Anat. und Physiol., anat. Abtheil.*, 133-152, 2 plates (1897).

well-stoppered bottle for twenty-four to seventy-two hours, at 37° C., with benzene. (I find this unnecessary.) The slides are now passed through absolute alcohol, 90 and 70 per cent. alcohol, then washed

RESULTS OBTAINED BY F. MALL.

Reagents, percentage of.	*White fibres.*	*Reticulin.*	*Yellow fibres.*
Acetic acid up to $\frac{1}{20}$	No swelling.	. . . . .	Not dissolved in 20 per cent. boiling acid, but fibres become brittle.
$\frac{1}{2}$—25	Swelling.		
25	Solution in 24 hours.		
100	No swelling.		
Hydrochloric acid, $\frac{1}{100}$—3	Swelling.	Swelling.	10 per cent. = no change at 20° C.
3—6	Swelling.	Unaltered.	
6—10	. . . . .	Unaltered.	
6—25	Unaltered for some time.		
25—100	. . . . .	Solution in 24 hrs.	50 per cent. = solution in 7 days, but sheath of fibres more resistant.
100	Solution.	. . . . .	Solution in 2 days + boiling = rapid solution of centre of fibre.
Caustic potash weak sol.	Swelling, but no solution at ordinary temperature.	Behaves like white fibrous tissue.	Solution takes place
1	. . . . .	. . . . .	after months.
2	. . . . .	. . . . .	,, 1 month.
5	+ boiling = rapid solution, conversion into alkali albumin.	. . . . .	,, 3 days.
10	. . . . .	. . . . .	,, 1 day.
20—40	. . . . .	. . . . .	,, hours.
Peptic digestion	Converted into gelatin peptone.	. . . . .	Centre of fibre is dissolved, leaving the sheath.
Tryptic digestion	No action on normal tissue, but if shrivelled by heat, both are quickly digested.		Quickly dissolved.
Heat	Contracts at 72° C.; after treatment with acid dissolves quickly.	Contracts at 72° C.; after treatment with acid dissolves slowly.	No action.
Putrefaction	Does not attack either of these if taken from the body. If left in the body, at 37° C. they quickly decompose.		Quickly destroys centre, leaving the sheath.

for fifteen minutes in running water, digested for ten to twenty-four hours with trypsin and again washed in water, taking special care to prevent the sections leaving the slide. The sections are mordanted for one to twenty-four hours in a ½ per cent. watery solution of ammonio-ferri tartrate, rinsed in water, and stained for three to twenty-four hours in a ripe ½ per cent. haematoxylin solution[1]. If the reticulum is not sufficiently stained, transfer the slide for twenty to thirty minutes to some freshly prepared iron solution, then dehydrate, clear and mount in balsam.

3. On decalcifying and macerating bone.

The chief point to be borne in mind is that good decalcifying methods are useless if the bone has not been properly fixed to begin with.

The best fixative for embryonic bone up to the time of birth is picro-corrosive formaldehyde (p. 97). With older bones, which are dense, and cannot be injected by my method (p. 145), use 20 per cent. formol in 0·9 per cent. sodium chloride solution. Embryonic bones should be fixed, according to size, for twenty-four to forty-eight hours, and older bones for three days. The best results with adult bone are, however, obtained by injecting the whole animal with picro-corrosive formaldehyde. To decalcify, up till last year, I used either Squire's mixture of glycerin (95 parts) and hydrochloric acid (5 parts), in which the glycerin plays the same rôle as the saturated salt solution in von Ebner's fluid, by preventing a swelling of the bone—or 1 per cent. nitric acid in a 2½ per cent. sublimate solution. Now I use Ziegler's method[2], which I have tried on adult bones of cats, rabbits, and guinea-pigs, on the temporal bone of human embryos, and teeth. It acts much more quickly than the ordinary methods, that of phloroglucin nitrate excepted, and preserves tissues better than the phloroglucin methods[3].

Ziegler's method. Fix bones preferably in formol (see above). Decalcify in commercial 5 per cent. sulphurous acid, when the insoluble tricalcium phosphate, which along with carbonate of lime forms the main bulk of the inorganic constituents, is converted into the readily soluble monocalcium phosphate

$$Ca_3(PO_4)_2 + 4SO_2 + 4H_2O = Ca(H_2PO_4)_2 + 2Ca(HSO_3)_2.$$

The humerus of an adult guinea-pig is decalcified in one to two days at the ordinary temperature, and in twelve hours in an incubator at 35° C. A frog's femur requires five hours, and large adult human bones up to one week.

After the decalcification is completed wash in water for twenty-four hours. Dehydrate gradually, and imbed embryonic bones in paraffin and adult bones in celloidin.

[1] A quarter per cent. haematein answers well.

[2] Paul Ziegler: *Festschrift z. 70. Geburtstag v. Kupffers*, 49–52.

[3] An admirable synopsis of the older methods employed for investigating bony tissues will be found in Joseph Schaffer's article in the *Zeitsch. f. wiss. Mikr.* 10, 167–211 (1893).

Maceration, for isolating the inorganic constituents of bone, is best performed as follows:—Either entire bones, or pieces of them, are placed in warm water, after the marrow has been removed if possible. As soon as putrefaction has started vigorously, the water is changed every two or three days, partly to remove the blood completely, and also not to interfere with the bacterial action by the accumulation of their products. After two to three weeks the bones are laid on clean sand and watered regularly twice a day, when all fat will be drawn out of the bones by capillary attraction. I find this method[1] better than extracting fats with ether and alcohol, especially as it does not interfere with bacterial action should the latter be still required.

CHAPTER XI.

On Injecting Blood and Lymph Vessels.

Blood-vessels. Whenever possible, whole animals should be injected, because all the blood-vessels being filled, a larger surface is exposed to the pressure of the injection apparatus, and thus any mistake the operator may make in determining the right pressure is minimized. Warm-blooded animals may be completely injected in four to five minutes, provided the injection is performed immediately after death. If, however, rigor mortis has supervened, no attempt at quick injection should be made.

Animals which have become cold, cold-blooded animals or their organs, must be heated up to 39° C., if gelatin injection masses are to be used. This is best done by placing the tissues in normal saline at a temperature of 40° C.

To obtain good results, only a slight pressure is used to begin with, but gradually towards the end it should be raised. A satisfactory injection of a sheep's eye will take fifteen to twenty minutes, and sometimes even longer.

For injecting whole animals, down to the size of guinea-pigs and rats, I use the same apparatus which is employed for the injection of fixing solutions (p. 145). For smaller animals, such as mice, frogs, and newts, it is preferable to have a pressure-bottle fitted to the tap of the water-pipe and connected, by means of a T-shaped glass canula, both with a manometer, and also with the flask containing the injection mass.

Solid Injection Masses.

1. *Carmin-gelatin* mass (first recommended by Robin, 1871).

Take 250 grms. of the best gelatin, soak it in 750 cc. of distilled water, and dissolve it on a water-bath after it has become soft. When it is completely melted, add 150 grms. of best carmin dissolved in 75 cc. of distilled water containing 25 cc. of ammonia.

Add the ammoniacal carmin to the boiling hot gelatin, stirring all the time. As soon as the carmin and gelatin have been thoroughly mixed, add 50 per cent. glacial acetic acid in quantities

[1] I heard of this method in the Anatomical Department, Edinburgh.

of at first 1 ccm., and later in drops. Insure thorough mixing of each newly-added portion of the acetic acid with the whole of the gelatin carmin. Do not add any more acid if five minutes after the last addition the originally transparent colour has been permanently changed into a distinctly opaque one. Ranvier tells the point of neutralization by smell, which I am unable to do, for to me the boiling gelatin appears to give off ammonia, and to make red litmus paper blue at a time when the gelatin has already acquired its permanent opaque appearance. Filter the carmin-gelatin through a square piece of flannel, measuring 50 cm. (18 inches) along each side, which has been attached by its four corners to a metal ring. Let the hot solution run into a litre-bottle containing 2 grms. of carbolic acid. This mass keeps for years.

2. *Lead chromate-gelatin* (*Thiersch's formula*, 1865).

Soak gelatin in distilled water for 2 hours; let the superfluous water run off; melt the gelatin on a water-bath and divide it into equal parts *A* and *B*. To every 40 cc. of the portion *A* add 30 cc. of a 10 per cent. solution of lead nitrate, and to every 40 cc. of *B* add 10 cc. of a 10 per cent. potassium-chromate solution.

While both solutions *A* and *B* are heated to the boiling-point add *B* to *A*, stirring constantly. Filter through flannel and preserve.

I make up this solution either in the evening or in the photographic dark room, to prevent the light rendering the gelatin-bichromate mixture insoluble.

3. *Barium sulphate-gelatin* (first recommended by Frey, 1866).

It will generally be found best to prepare the barium sulphate separately and then to add it to the melted gelatin. I add to a 10 per cent. boiling solution of barium chloride 10 per cent. sulphuric acid; let the sulphate settle for 24 hours; syphon off the water containing the hydrochloric acid; shake up the barium sulphate with distilled water and allow it to settle again. After repeating this process of washing once again the sulphate is dried and kept till required.

Gelatin, soaked in water for 2 hours, is melted in the water it has absorbed, and, when boiling, is poured into a mortar in which the required amount of barium sulphate has been converted into a thin paste with distilled water. Ten grams of barium sulphate for every 100 ccm. of injection mass will be found sufficient.

The advantages of the above methods are: all three solutions run with great ease, especially the lead chromate-gelatin, and they fill even the finest capillaries; the carmin-gelatin mixture is transparent, and, either by itself or in preparations stained slightly in Ehrlich's acid haematein (p. 239), makes very instructive preparations; the lead chromate injections appear equally effective whether they be viewed with transmitted or reflected light; the barium sulphate mixture is especially to be recommended for photographic purposes.

4. The *silver nitrate* injection methods are given under the lymph-vessels (pp. 160–162).

The method of filling the blood-vessels of a warm-blooded animal with an injection mass. 1. See that the apparatus, and especially the pressure bottle, is in good order. 2. Warm, according to the size of the animal (rabbit or cat), 500 to 1,000 cc. of normal saline, and a similar amount of the injection mass, to 40° C. 3. Kill the animal with coal gas to produce a complete dilatation of the blood-vessels[1]. 4. Fill the injection tube and canula with normal saline, taking great care to get rid of all air-bubbles. 5. Inject the animal according to the procedure given on p. 145. Wash out its vascular system with normal saline under a low pressure, then raise the pressure just before the gelatin injection mass enters the body. 6. Blow the hairs apart to see whether the skin has turned of a red colour all over the body, as it should do in 5 to 10 seconds. 7. When the injection mass is seen to be pouring out of the right ventricle, pass a ligature round both ventricles and tighten it up.

To obtain good results, introduce into the vascular system a quantity of injection mass equal to at least 5 times the normal amount of blood; thus a full-grown rabbit will require 450 to 500 cc. of injection mass, the greater part of which lodges in the abdominal vessels.

If it be desirable not to inject the whole animal, any district of blood-vessels is readily eliminated by ligatures or artery forceps, the injection mass entering, as stated above, through the aorta.

It will be seen that in injecting a whole animal, the tissues of which are surviving, I adopt a plan the opposite to that given above for the treatment of excised organs. The reason for starting with a high pressure is to get all the bigger vessels of the arterial system filled, before the irritation caused by the injection mass will allow them to contract and to offer a great resistance to the inflow of the fluid.

In dealing with the lung, inflate it to its normal size; ligature or clamp the trachea; insert the canula into the pulmonary artery through the right ventricle, and clamp the left ventricle after the blood has been washed out by the injection fluid. To inject the heart, tie the canula, looking towards the heart, into the aorta. Both in the case of the lung and the heart use gentle pressure so as not to damage the fine capillaries or the aortic valves. Inject, therefore, for 20 minutes under a low pressure.

Lymph-vessels are best injected with solutions remaining fluid at the ordinary temperature, similar to those enumerated below. It will generally be found advisable to first inject the blood-vessels with, for example, a red mass, and after this has cooled to fill the lymph-vessels with a blue solution. To obtain good injections of lymph-vessels requires very fine glass canulae, an injection mass containing no granules, experience, and patience. The slower lymph-vessels are injected, the better will be the result.

Having chosen an organ, for example a stomach or kidney, fill

[1] Amyl nitrite has been recommended for this purpose, but I do not use it for personal reasons.

an ordinary injection syringe with the mass to be injected; tie the fine glass canula, held in a short stout piece of indiarubber tubing, to the syringe; invert the syringe so that its point looks upwards; gradually push in the piston to drive all air out of the glass canula; insert its point just beneath the serous coat and inject very gently. If a bleb or bulla forms, or if the zone round the point of the canula is very much deeper in colour than the more outlying parts, another place should be chosen, and the process repeated, because the injection mass is not entering the normal lymph channels.

Fluid injection masses.

1. *Soluble Prussian blue* was first used, according to Brücke, by Schröder van der Kolk, and subsequently by Ludwig, Ranvier, and others. Paul Mayer prepares it as follows:

A	Potassium ferrocyanide . .	20 grm.
	Water	500 cc.
B	Liquor ferri sesquichlorati . .	10 cc.
	Water	500 ,,

Add slowly *B* to *A*, stirring all the time. Let the precipitate settle overnight. Syphon off the supernatant liquor. Transfer the precipitate to a filter, and wash repeatedly with small quantities of distilled water for 1 to 2 days, till the filtrate is turning deep-blue. Now dissolve the whole of the precipitate in one litre of water, or dry it for future use. This soluble Prussian blue must be used in a saturated solution, and to prevent it fading in contact with alkaline tissues, acidulate it with acetic acid (Rawitz).

For injecting blood-vessels 5 per cent. of gelatin may be added to it.

2. *Indian ink* after Grosser's method[1] gives very good results, provided a good sample of Indian ink is obtained. Rub the stick on a glass plate till the ink is of such concentration that it will not run when placed on blotting-paper.

3. Tandler[2] uses *gelatin* which remains fluid at the ordinary temperature. His injection mass is prepared as follows: Wash gelatin with distilled water to remove all salts; dry it; place 5 grms. of this pure gelatin in 100 cc. of water; let it swell and then melt it; now add soluble Prussian blue according to the depth of tint required, and, finally, very gradually add 5 to 6 grms. of potassium iodide, because addition of salts to gelatin always lowers its temperature of setting. This mass remains fluid at 17° C., and keeps indefinitely if a few thymol crystals are added.

4. *Silver nitrate* solutions are best used according to Ranvier's directions. The simplest, but least satisfactory method is to inject a silver-nitrate solution (1 in 300 to 500 cc.) into either blood-vessels or lymph-vessels. A better method for blood-vessels, and the best for lymph-vessels, is to use a mixture of gelatin 30 grms., water 90 cc.,

[1] Grosser: *Zeitschr. f. wiss. Mikr.* 17, 178 (1900).
[2] Tandler: *Zeitschr. f. wiss. Mikr.* 18, 22 (1901).

1 per cent. silver nitrate 20 to 40 cc.—The best, although most tedious, method for blood-vessels is to use in the first instance a quarter per cent. solution of silver nitrate, allowing it to act for 3 to 5 minutes, and then to wash out this solution with 10 per cent. gelatin, to which a mere trace of carmin has been added. The object of either combining the silver nitrate with the gelatin, or of using the latter subsequently, is to get the vessels in their normally distended shape. Good results are also obtained by washing out the silver nitrate with 10 per cent. formol, and keeping the vessels dilated with this solution till the tissues are fixed.

The lymph-vessels in the central tendon of the diaphragm.

To inject the central tendon of the diaphragm the following special methods have been used :

1. Recklinghausen (Stricker's *Handbuch*, p. 222) kills a rabbit, opens the chest, tilts up the lungs and heart, and introduces a round cork frame, which rests on the diaphragm. The abdomen is next opened, and the central tendon pinned over the aperture of the cork. Then the cork, with the tendon attached to it, is excised, placed on a microscope, and the peritoneal surface of the tendon covered with a few drops of milk diluted with sugar solution. On examination with a low power little eddies and vortices are seen. On washing off the milk after a few minutes, milk globules are seen in the lymphatics of the diaphragm. Therefore solid particles may be absorbed from the peritoneum.

2. Ludwig and Schweigger-Seidel (1867) kill a rabbit by bleeding ; open the abdomen and occlude by one ligature the openings of the oesophagus, aorta, and vena cava ; cut off the posterior half of the rabbit, carrying the incision behind the diaphragm. They then fix a canula into the trachea and connect it with a pair of bellows ; pass three or four pieces of string through the skin of the rabbit close to the diaphragm, with which to suspend the rabbit from a metal ring, with its head hanging down ; the peritoneal surface of the diaphragm is then covered with a solution of water-soluble Prussian blue, and artificial respiration is practised by means of the bellows for about ten minutes ; the diaphragm is then washed in water to remove the superfluous blue and is finally fixed in alcohol.

For examination the tendon is excised, cleared in acidulated glycerin, and mounted on a glass plate.

3. Ranvier's method (*Traité technique d'Histologie*, 1875–1882). Kill a rabbit, carefully excise its diaphragm and tie it, with the peritoneal surface outermost, over one end of a glass cylinder, after the manner of a dialyser. Then fill the cylinder to the extent of one inch with a 1 : 1,000 silver-nitrate solution, and immediately immerse the peritoneal surface of the diaphragm into 1 : 500 silver nitrate[1]. Keep the diaphragm for half an hour in a dark place,

[1] Ranvier does not state whether he keeps the diaphragm in the dark, nor whether he washes it before reduction takes place. The above method gives the best results.

when by endosmosis all the lymph-vessels will be filled. Now wash the diaphragm in several changes of distilled water, fix in alcohol and expose to light in 50 per cent. spirit. When slightly brown, dehydrate it and mount in balsam.

Another method of Ranvier's consists in rapidly washing the diaphragm in distilled water; transferring it for one hour to 1 : 1,500 silver nitrate in the dark; washing it again in distilled water; macerating it for fifteen to twenty hours in distilled water, to which a few drops of a 1 per cent. carbolic acid are added to prevent putrefaction, and finally brushing off the endothelium, or simply detaching it by washing in water. This method shows the same appearance as that produced by Ludwig and Schweigger-Seidel's process.

PART III.

CHAPTER XII.

Methods of obtaining Sections.

Encasing Methods.

In the early days of Histology the only method available for cutting sections was to hold the tissue either between one's fingers or to encase or surround it by some medium of sufficient consistency to support it securely, without crushing. The media usually employed for this purpose were waxy liver, obtained from the post-mortem room, or ordinary liver hardened in alcohol; further, carrots, elder-pith, wax, and similar substances. The tissue and its case were cut together, and the latter removed before mounting the sections permanently. A method still to be recommended for rapid orientation.

In Cambridge[1], the following methods are used:—A piece of brass tubing, about one foot long, measuring 2 cm. internal measurement, and provided with a piston, is oiled inside, and then filled with a melted mixture of paraffin (2 parts) and vaseline (1 part). The tube and contents are cooled under the tap; the solid rod of the paraffin mixture pushed out and cut into lengths of about 4 cm.[2] Use the paraffin-blocks as follows:

Heat a stout wire over a Bunsen flame, and with it melt the centre of one end of the paraffin-block. Place the tissue (which has been previously dried with cigarette paper, to remove the superficial moisture) in the melted paraffin; with a hot needle arrange it in the desired plane, remove any air-bubbles which may be in the paraffin with the hot needle and allow the mixture to cool.

An older method, which is also very efficacious, consists in using elder-pith. It should be divided longitudinally with a sharp scalpel, and a groove or hollow be cut into one or both halves of the pith for the reception of the tissue. When the latter has been placed in position, a thread is tied round the two pieces of pith, and they are immersed in spirit for fifteen minutes to cause them to swell.

Sections are cut in the following way: Hold the encased tissue between the thumb and the distal joint of the index finger of the left hand; keep the second phalanx of the index finger in a horizontal plane; rest the heel of a sharp razor for its support firmly on the index finger, and with the edge looking towards you, gradually pull the razor from heel to point through the pith and the tissue.

[1] Foster and Langley: *Elementary Practical Physiology and Histology.* Macmillan, 1896.

[2] In an emergency cheap paraffin candles answer well.

To prevent the sections adhering to the razor, it should be covered on its upper surface with a pool of normal saline (¾ per cent. NaCl), when dealing with fresh tissues—or with a layer of strong alcohol if the tissues have been fixed. If it be found that the salt solution has a tendency to drop off, remove all fat carefully before commencing to cut sections. For this purpose I find the following method best:—Heat a little spirit in a test-tube to the boiling-point, let it run over the razor, and while the latter is still hot, drop some ether on it, taking care that the ether runs off. On now placing the razor into water, it should, on withdrawal, be wetted quite uniformly.

The freezing method.

Yet another method, which gives tissues such consistency as to allow of sections being cut easily, is that discovered by Stilling on January 25, 1842. This great neurologist having left a piece of spinal cord on the window-sill, found it had frozen hard at a temperature of −13°R. While it was in this condition Stilling made a transverse section through it, and examined the section under a magnification of 15 diameters. 'When I saw the magnificent radiating bundles, and the central tracts of nerves, I realised that I had discovered the key which would open up the chambers of the wonderful structure of the spinal cord. Not more gladly did Archimedes call out his εὕρηκα, than did I shout out on beholding that sight!'

Since this time also dates the making of serial sections.

Imbedding Methods.

An advance in cutting sections was made, when instead of simply encasing tissues, they were permeated by or, as it is termed technically, imbedded in the supporting medium.

A. Watery media.

With Fol[1] we may divide the processes of imbedding into two classes, namely, the tissues are either impregnated with watery media, such as sugar and gum (Hamilton, 1878), soap (Kadyi, 1879), gelatin (Sollas, 1884), albumin (Fol, 1896), or they are thoroughly dehydrated and then passed into celloidin, paraffin, wax, or other fatty substances.

Of the watery media just mentioned, Hamilton's mixture is frozen; the soap is allowed to evaporate till it becomes sufficiently hard; the gelatin is coagulated either by alcohol, chromic acid (Klebs), formol (Nicolas), or it is frozen (Sollas); the albumin is coagulated either by heat or alcohol.

For general work a mixture of equal parts of cane-sugar, gum-arabic and one to two parts of water will be found the most convenient. The gum is placed in a muslin bag, and suspended in water heated on a water-bath, till it has completely melted; then the sugar is treated similarly in a fresh bag, and allowed to diffuse into the gum solution. While still hot filter the gum-sugar mixture through flannel, and add to the filtrate either phenol (carbolic acid) or thymol

[1] *Lehrbuch d. vergleich. mikr. Anatomie*, 115 (1896).
[2] For particulars see Lee and Mayer, *Grundzüge d. mikr. Technik*, 91–94 (1898).

in the proportion of 1 : 1,000. Tissues should be left in this imbedding mass, according to size, from one to three days or even longer. Ford Robertson recommends a 20 per cent. pure dextrin solution, which I find answers also very well.

B. Dry imbedding methods.

Whatever good results have been obtained, and are still obtainable, by the wet imbedding process, there is always a great risk of damaging the sections during the process of mounting, and the difficulty in obtaining serial sections of uniform thickness is almost insurmountable.

These difficulties are overcome by the two dry methods of celloidin and paraffin imbedding, both of which require that all traces of water be removed from the tissues by the process of dehydration.

On dehydrating, dealcoholating, and clearing.

Dehydration. To dehydrate tissues without causing an undue shrinkage, it is necessary to transfer them gradually to alcohols of increasing strength. How many changes of alcohol are required depends on whether the alcohol, in addition to dehydrating, has also to extract the reagents which were used for fixing the tissues.

In practice the following changes will in most cases be found sufficient:—Three changes of 60 per cent. alcohol to extract the greater part of the fixative; two changes of 75 per cent. and one change each of 85 and 95 per cent. alcohol; and finally, for complete dehydration, at least three changes of absolute alcohol. It is this last stage which is usually hurried over, and in consequence much greater shrinkage results in the paraffin process than would otherwise be the case.

The table on p. 166 indicates how to obtain alcohol of any given strength.

The figures are calculated to express what amount of water has to be added to 100 cc. of alcohol of any given strength (top horizontal line) to obtain a desired percentage of alcohol (left vertical line). Thus to make 50 per cent. alcohol from 75 per cent. alcohol, we take 100 cc. of 75 per cent. alcohol, and add 52·43 of distilled water. The volume on the left gives the specific gravity of the different strengths of alcohol, pure absolute ethyl alcohol having a specific gravity of 0·749.

Dealcoholation. After complete dehydration, it is essential, if the paraffin process is to be used, to get equally thoroughly rid of the alcohol (dealcoholation [1]).

This removal of the alcohol by some medium which will mix with it, and also with paraffin, is not of necessity accompanied by what is usually called 'clearing' a tissue or section.

Clearing a tissue means to render it transparent, which may be done by any medium having a refractive index similar to that

[1] I am encouraged in introducing this word by some remarks which Lee has made in the first edition of Lee and Mayer, p. 63.

possessed by the tissues themselves. Both water and alcohol have a low refractive index, while the tissues have a high one, in conse-

GAY-LUSSAC'S TABLE FOR DILUTING ALCOHOL.

Specific gravity at 15° C.	*Desired strength.*	*Original strength of alcohol.*												
0·816	95	95	90	85	80	75	70	65	60	55	50	45	40	35
0·833	90	6·50												
0·84	85	13·36	6·56											
0·863	80	20·16	13·79	6·83										
0·877	75	29·66	21·89	14·48	7·20									
0·889	70	39·16	31·05	23·14	15·35	7·64								
0·901	65	50·66	41·53	33·03	24·66	16·37	8·15							
0·913	60	63·16	53·65	44·48	35·44	26·47	17·58	8·76						
0·923	55	78·36	67·87	57·90	48·07	38·32	28·63	19·02	9·47					
0·934	50	96·36	84·71	73·90	63·04	52·43	41·73	31·25	20·47	10·35				
0·943	45	117·86	105·34	93·30	81·38	69·54	57·78	46·09	34·46	22·90	11·41			
0·951	40	144·86	130·80	117·34	104·01	90·76	77·58	64·48	51·43	38·46	25·55	12·8		
0·958	35	178·86	163·28	148·01	132·88	117·82	102·84	87·93	73·08	58·31	43·59	27·6	14·3	
0·965	30	224·4	206·22	188·6	171·1	154·3	136·04	118·9	101·7	84·5	67·5		33·4	16·8

quence of which the light is refracted and reflected from the heterogenous mixture, while the tissue, with all its interspaces filled with a highly refracting medium, forms one homogeneous mass which does not refract and reflect the light. A table of refractive indices will be found on p. 377.

When therefore the alcohol is replaced by a solvent of paraffin, the tissue may either become quite transparent if the medium has a high refractive index, as for example xylene or benzene, or it may retain its opaque look if chloroform or carbon-disulphide is used.

The advantages and disadvantages of the Paraffin and Celloidin methods.

Histologists, as far as these imbedding methods are concerned, may be grouped into two classes, namely, those who believe with Apáthy that celloidin is much superior to paraffin, and those who give preference to paraffin. Each method has, however, its advantages and disadvantages. With paraffin, either by itself or in conjunction with celloidin, complete series of sections are more readily obtainable because of the sections adhering to one another, or, as it is termed, forming ribbons. If time is all-important, and a trained assistant not procurable, the paraffin method will allow of quicker work than the original wet celloidin process.

Paraffin used to have the further advantage of allowing thinner sections to be cut, but since the introduction of the dry celloidin methods this difficulty has been overcome. Paraffin sections are easier to fix to the slide than celloidin sections, and while the paraffin can readily be removed, leaving nothing but the tissue, the celloidin cannot be removed without running some risk of the sections leaving the slides. Further, it is impossible to stain sections imbedded in celloidin with strongly basic dyes, because the celloidin, being an acid substance, becomes deeply coloured at the same time.

The great drawbacks to paraffin are the shrinkage which tissues undergo when heated above 45° C., and the hardness acquired by some tissues, such as white fibrous tissues and glycogen-liver. It is further impossible, without special manipulations (p. 171), to cut paraffin sections of the lens, adult decalcified bone, and teeth.

For micro-chemical work the celloidin method in many instances will have the preference, because heat action has been avoided. Both methods should be used, for each has its own special advantages.

On Paraffin imbedding.

The principle underlying the paraffin imbedding process is to dehydrate a tissue with absolute alcohol; to replace the alcohol with a solvent of paraffin; to impregnate the tissue with solid paraffin dissolved in the solvent; to remove the solvent of the paraffin and to obtain the paraffin in a homogeneous firm state so that it may support all the tissue elements.

As paraffin, if we allow the solvent to evaporate at the ordinary temperature, forms a spongy, loose mass, we must have recourse to heat and melt the paraffin; and as it is best not to heat certain tissues beyond definite degrees of temperature, and also to avoid any

cooling of the paraffin, we make use of special warm chambers, called incubators, and provide them with automatic regulators.

Who first impregnated tissues with paraffin, I have not been able to find out. Giesbrecht describes a chloroform-paraffin imbedding method on September 12, 1881, in the *Zool. Anzeiger*, and O. Bütschli and F. Blochmann, on Dec. 28 of the same year, in the *Biol. Centralblatt*, also advocate chloroform, without referring to Giesbrecht's account. Gaule (*American Monthly Micr. Journal*, 1882, 14) is stated to have also discovered the paraffin imbedding method. He passed tissues through clove oil, turpentine, turpentine and paraffin, and ultimately pure paraffin.

The paraffin used for imbedding purposes is a mixture of different hydrocarbons, about 50 per cent. consisting of the compounds $C_{21}H_{44}$ (melting-point 40° C.); $C_{22}H_{46}$ (m. p. 44°); $C_{23}H_{48}$ (m. p. 48°); $C_{24}H_{50}$ (m. p. 51°); $C_{27}H_{56}$ (m. p. 60°); $C_{31}H_{64}$ (m. p. 68°). The lower the melting-point of any paraffin, the greater is the admixture of the lower members of the paraffin series. These evaporate on heating the paraffin mixture, and in consequence the melting-point of what remains is raised; therefore do not heat a paraffin for more than 1 to 2 degrees above its melting-point.

For soft tissues I use a paraffin having a melting-point of 48° to 52° C., and for hard material paraffin melting at 56° C. For structures rich in white fibrous tissue, the fatty paraffin of Graf Spee[1] will be found to answer best. Graf Spee recommended his paraffin originally for readily obtaining sections in ribbons. This fatty paraffin may either be obtained from Hollborn and Grübler, or Alexander Frazer in Edinburgh, or be prepared as follows: Ordinary white paraffin, having a melting-point of 50° C., is heated in a porcelain dish or enamelled iron vessel till disagreeable white fumes are given off. After the lapse of one to several hours, according to the quantity of paraffin taken to begin with, the melted mass becomes brown in colour, and has its melting-point raised several degrees. On cooling the modified paraffin is greasy to the touch.

Paraffin does not mix with alcohol, but we possess many reagents, which are miscible on the one hand with alcohol and on the other with paraffin. Such media are used for replacing the alcohol in dehydrated tissues, and in their turn have substituted for them the paraffin.

The best solvents for paraffin, from the histological point of view, are chloroform, carbon-disulphide, cedar-wood oil, benzene, and xylene. For ordinary purposes benzene and xylene are the best, because they are readily removed from the tissues and the paraffin, but for very delicate work chloroform, carbon-disulphide, or cedar-wood oil are preferable. All traces of chloroform must be removed, or otherwise the paraffin will not form a hard mass on cooling, while a slight admixture of cedar-wood oil is in some cases an advantage (Lee).

The subjoined table indicates in hours the length of time tissues should remain in the third change of absolute alcohol; in a mixture of equal parts of alcohol and xylene (or benzene); in pure xylene or benzene (three changes); in a mixture of equal parts of xylene (or

[1] Graf Spee: *Zeitsch. f. wiss. Mikr.* 2, 8 (1885).

benzene) and paraffin; and lastly in pure paraffin (three changes). The time is calculated for the thickness of the tissue as its surface diameter is immaterial.

Thickness of tissue.	1 *mm.*	5 *mm.*	1 *cm.*	2 *cm.*	
Time.	*Minutes.*	*Hours.*	*Hours.*	*Hours.*	
Alcohol, 3rd change .	15	5	10	24	At the ordinary temperature.
Alcohol + xylene . .	15	2½	5	10	
Xylene, 3 changes each	15	1½	2½	5	
Xylene + paraffin . .	60	5	10	24	In the incubator.
Paraffin, 3 changes each	15	2	5	10	

If chloroform is used instead of xylene, the time given in the table should be trebled, while for inspissated cedar-wood oil it should be doubled.

For rapid work, where the best possible preservation is not required, the times given above may be shortened. For example, a piece of frog's intestine may be taken through the paraffin process comfortably in an hour and a half in this way:

Fix and dehydrate in three changes of alcohol (ten minutes each), clear in two changes of xylene (ten minutes each), transfer to xylene and paraffin equal parts at 40° C. (twenty minutes), and finally into two changes of paraffin (ten minutes each), and 'cast' in a third change of paraffin.

Carbon-disulphide, CS_2, is recommended by Martin Heidenhain[1]. I have given the method a thorough trial during last winter and find it good. Its advantages, as stated by Heidenhain, are these: (1) it penetrates with great quickness and thoroughness, probably owing to its low molecular weight (m. w. 76). (2) The greater part of the imbedding process takes place at a temperature of 41° to 42° C., as at this temperature 3 to 4 parts or even more paraffin are dissolved by one volume of carbon-disulphide. (3) The tissue may be left for a long time at a temperature of 42° C. without being injuriously affected, and therefore the subsequent stay at a temperature from 56° to 57° C. may be shortened to a minimum. (4) The CS_2 does not oxidize, and therefore tissues stained in bulk, for example with haematoxylin, do not suffer. The method was found especially useful for investigating heart muscle.

The chief disadvantage in using CS_2 is its smell, but this difficulty is greatly overcome by the use of well-fitting glass stoppers, and by not agitating the CS_2 unnecessarily, as practically no gas is evolved till the solution is shaken or moved about. When it is desired to move the tissues about in the carbon-disulphide paraffin mixture, go outside, or to a stink-chamber, remove the glass stopper, then mix the contents of the vessel, replace the stopper, and put the tissue back on the top of, or inside, the incubator.

Carbon-disulphide and alcoholic mixtures do not keep indefinitely,

[1] M. Heidenhain: *Zeitsch. f. wiss. Mikr.* 18, 166 (1901).

for both sulphur and carbon particles separate out. Pure CS_2 also occasionally undergoes disintegration, but the CS_2 + paraffin mixture keeps indefinitely.

To get the best possible results proceed thus: Thoroughly dehydrate the tissues; place them in an equal mixture of CS_2 and absolute alcohol, when they will become clear; transfer them to three changes of pure CS_2, when they will become opaque; place them in CS_2 saturated with paraffin at 30° C., when they become clear again; then into CS_2 saturated at 42° C.; and finally into two baths of pure paraffin, each bath lasting for one to one and a half hours. Cast in some pure paraffin.

How to cast tissues.

Proceed thus:

(1) A perfectly clean glass plate is rubbed over with a piece of cloth moistened with glycerin, to allow the paraffin-block when cool to separate readily from the glass plate.

(2) Two rectangular L-shaped pieces of brass (Leuckard's type-metal boxes) are also covered with a thin layer of glycerin, and arranged on the glass plate so as to form with the glass support an enclosed space.

(3) This box is then completely filled with melted paraffin.

(4) When the paraffin in contact with the glass plate is beginning to cool, as is shown by its becoming opaque, the tissue is transferred into the box and arranged with needles in the desired plane, or, as it is termed, is orientated. To prevent the tissue adhering to the needles or pair of forceps, these should be carefully heated up to the melting-point of the paraffin; on no condition should they be so hot as to char the tissue.

(5) The glass plate with the metal box containing the tissue is quickly cooled by being immersed in cold water, care being taken not to let the water get into the still fluid paraffin. Quick cooling is essential because it prevents the slow crystallization of the paraffin, and thereby renders the paraffin more homogeneous.

(6) Should the tissue be so bulky as to project out of the paraffin during the contraction of the latter in the cooling process, the box must be filled up again with melted paraffin, before the centre of the first portion has solidified, and with a hot needle the partly solidified paraffin must be melted again.

(7) When the paraffin has been cooled by immersion in water for 10 to 20 minutes the two rectangular pieces of metal and the glass plate are removed, and the block is inspected. If it has a homogeneous appearance it is all right, but if it has opaque streaks in it or if it looks milky, then some of the solvent of the paraffin has remained behind.

Under these circumstances it is best to remelt the block containing the tissue in the incubator and after a time to recast, using fresh paraffin. Air-bubbles should be removed from a block of paraffin with hot needles, taking care not to injure the tissue. The time so spent will repay itself when cutting the sections.

To attach the paraffin-block to the object-holder of the microtome proceed thus: Cover the object-holder with an even layer of paraffin, removing all air-bubbles; press the paraffin-block containing the tissue down on the object-holder and fix it by inserting a hot scalpel from the four sides; after the block has cooled pare off the superfluous paraffin firstly from the free end; then, placing the object-holder on the table, cut off with a razor, held vertically, thin slices, taking care that two sides of the block are kept parallel to one another. On fixing the object-holder to the rocking microtome, arrange the parallel sides of the paraffin-block in a horizontal plane.

Using the rocking microtome it is easy to obtain long ribbons of sections, as discovered by Caldwell, if the sections are not thicker than 6 micromillimetres [1], and if the sections are cut rapidly. This second factor acts, according to Lee, by heating the paraffin-block during the process of cutting, and in consequence fusing together the edges of neighbouring sections.

Sections 10 μ thick have little or no tendency to form ribbons with 56° paraffin, but ribbons may be obtained in the following way (Caldwell-Paul Mayer method): After the paraffin-block has been cut into the rectangular shape, heat up paraffin having a melting-point of 40° C. to 80°; immerse the paraffin-block for one second in the melted paraffin, and as soon as it has been withdrawn turn it round so that the object-holder is lowermost. The superfluous soft paraffin runs towards the base. Finally remove the soft paraffin from two sides, leaving it, however, on those other two by which we require the sections to adhere to one another.

If it be desired to cut sections of a tissue lying some distance from that part which has been cut last, increase the feed of the microtome to its full extent, replace the good razor by an ordinary razor, and cut off slices. Do not be tempted to remove a thick piece of tissue with one single sweep.

Certain tissues, such as eggs, the lens, skin, &c., become so brittle, when taken through the paraffin process, that special precautions have to be taken to keep the different elements in their place. Mark, in 1885 [2], fell on the plan of collodionizing the surface of the paraffin-block each time a section had been cut. Heider, in 1889, modified this method by using a mixture of equal parts of collodium (dissolved in alcohol and ether) and gum mastic (dissolved in ether). This mixture is thinned with ether till it becomes quite limpid.

With a brush paint a thin layer over the surface of the paraffin-block, taking special care not to let the varnish accumulate on the sides of the block. Then rapidly move your finger over the varnished surface till it emits a high note, indicating that the ether has evaporated. Cut the section and fix it to the slide as you would an ordinary section, but remember that, because of the varnish, a long time is required to ensure complete evaporation of the water on which the

[1] A micromillimetre is the $\frac{1}{1000}$ part of a millimetre, and is expressed shortly by the Greek letter μ. In the rocking microtome each tooth of the wheel is equal to 0·635 μ, or $\frac{1}{40}\frac{1}{00}$ of an inch, therefore eight teeth are equal to 5 μ.

[2] *Amer. Natural.* 19, 628 (1885).

section was floated out. Before staining, the collodium and mastic have to be removed with alcohol and ether.

Rabl uses, instead of the collodium, 52° paraffin heated up to about 75° C. By means of a brush a thin layer is rapidly painted over the surface of the block, and the section is cut as soon as the paraffin has cooled. I consider this method the best, because of both its expeditiousness and cleanliness.

If sections are to be floated out subsequently on water, it is best to use for Rabl's method a paraffin having the same melting-point as that in which the tissue is imbedded.

On Celloidin Imbedding.

Collodium is a solution of gun-cotton or pyroxylin in a mixture of alcohol and ether, while celloidin is a very pure gun-cotton prepared by some special process. The best celloidin is Schering's or Grübler's; it is sold in semi-transparent cakes of such a consistence as to be readily cut into shreds. Celloidin has a faintly acid reaction and therefore stains deeply with all basic anilin dyes. The collodium process of imbedding was introduced by Duval in 1879[1].

To prepare celloidin solutions for imbedding purposes proceed thus: Cut the celloidin-block into shreds and dry these in an incubator till they are perfectly hard and transparent, because the commercial celloidin contains water which must be removed to increase the penetrating power of the celloidin (Apáthy). Make 2·5 and 10 per cent. solutions of the dry celloidin chips in equal quantities of absolute alcohol and ether in the following way: Take the requisite quantity of celloidin and soak it for 24 hours in absolute alcohol, then add the ether, for Elsching has found the celloidin dissolves more quickly if it be treated first with alcohol without the ether. I find that methyl alcohol is preferable to ethyl alcohol, as it dissolves the celloidin more quickly, and as it also allows the latter to penetrate more readily, but care must be taken to procure pure absolute methyl alcohol. I use that of E. Merck.

To imbed, thoroughly dehydrate the object and then place it in a mixture of equal parts of absolute methyl alcohol and ether for 24 to 48 hours, according to size.

Subsequently large objects such as human embryos of the third and fourth month, human brains, or very dense tissues such as bone, teeth, and tendon, are preferably taken through the original long process, while small objects are best treated by either Gilson's or Stepanow's quick methods.

The ordinary long method necessitates three distinct operations, namely, imbedding, hardening, and cutting.

(*a*) *The imbedding process.* Transfer the tissues from the alcohol and ether mixture to the 2·5 per cent. celloidin solution and leave them in it according to their size for 2 to 14 days. A piece measuring 5 mm. in thickness requires seven days if it has such a dense structure as decalcified bone; a two months' human embryo should be

[1] Duval: *Journ. Anat. Phys.* 15, 185 (1879).

left at least one month in this solution. Then the tissues are transferred for an equal length of time to the 5 per cent. and for half that time to the 10 per cent. celloidin solution. Up till now the tissues should have been kept in well-stoppered bottles.

To still further inspissate the celloidin, the alcohol and ether are allowed to evaporate very slowly. This end is achieved by transferring the tissues in the 10 per cent. solution to circular glass vessels with a well-fitting, ground-glass lid (so-called sporulating dishes). The lid must be replaced at once, so that the air-bubbles which are formed during the transference to the sporulating dish may escape before the upper layer of the celloidin becomes condensed.

By placing a strip of paper between the glass vessel and its lid the alcohol and ether are allowed to evaporate slowly, the rule being that the bigger the tissue the slower should the inspissation take place. Therefore with bulky tissues the sporulating dishes are best placed partially covered by their lids under a bell-jar, and the latter is lifted once or twice a day to remove the ether which has evaporated.

When the tissues are first placed in the sporulating dishes they should be covered by the 10 per cent. celloidin. When the latter by evaporation diminishes in bulk and the tissues commence to project out of the imbedding mass, new solution must be added. If the surface of the inspissated celloidin feels dry to the touch, moisten it with a few drops of ether before adding the new solution. The imbedding process is completed when the celloidin is of such a consistence that it cannot be indented with the tip of one's finger (not the finger-nail). Now the tissue, surrounded by the celloidin, is removed from the sporulating dish, and the superfluous celloidin cut off in such a way as to leave a square block containing the tissue in its centre. Do not pare off too much of the celloidin, as otherwise the block will bend during the process of cutting and sections of unequal thickness will be the result. As yet the celloidin-blocks have not acquired sufficient firmness to be cut into thin sections and therefore must be hardened.

(*b*) *The hardening process.* The reagents commonly used for hardening the celloidin are alcohol, chloroform, or benzene. If alcohol is used, it should be of 85 per cent. strength, as has been determined experimentally by Busse [1]. The time required for hardening objects measuring 1 cm. in thickness is 2 to 3 days, while bulky tissues have to be left a fortnight, changing the alcohol every other day.

Small objects may be hardened by chloroform vapour in 24 hours (Lee), or in benzene (Stepanow). When treated by the last-mentioned methods, the tissues may be preserved indefinitely in chloroform or benzene, and if alcohol was used as the hardening agent the celloidin-blocks should be kept in 75 per cent. spirit, till required.

Either before or after hardening the blocks in alcohol, they must be fixed to pieces of soft wood, and on no condition to cork or other yielding substances. Each piece of wood is covered on one side

[1] Busse: *Zeitsch. f. wiss. Mikr.* 9, 49 (1892).

where its grain is cut across with a 10 per cent. celloidin solution; the latter is allowed to dry completely, and thus the pores in the wood are closed and the escape of air-bubbles prevented, otherwise their presence may prevent a firm union between the celloidin-block and the piece of wood.

If the celloidin-blocks are fixed to the wood directly they come out of the sporulating dishes, they need not be treated in any special way; but if the blocks are taken out of the 85 per cent. spirit which served to harden them, they should first be wiped dry, then some absolute alcohol be poured over the surface which is to be in contact with the wood, and the alcohol in its turn be wiped off. The piece of wood is then covered on its collodionized surface with 10 per cent. celloidin, and the celloidin-block pressed firmly on to it. Wait 15 to 20 minutes to allow some of the ether and alcohol to evaporate from the celloidin acting as a cement, and then complete the hardening by putting the wood block into 85 per cent. spirit for two hours, or by exposing it to chloroform vapour under a bell-jar for one hour.

(*c*) *On cutting sections.*

Celloidin blocks, treated as indicated, may be either cut directly, while they are permeated with the alcohol and opaque-looking, or they may be first cleared according to the method of Bumpus, and then be cut dry as advocated by Lee.

The ordinary wet process, however, is still the best for large sections. These are obtained most satisfactorily with a microtome which allows cutting under spirit, as, for example, Bruce's instrument (p. 179). When such an instrument is not available the surface of both the razor and the celloidin-block should be covered with a few drops of 80 per cent. spirit. The knife is always placed obliquely so as to get a cutting motion, instead of a planing action as in the ordinary process of cutting paraffin ribbons.

Lee's dry method consists in impregnating the celloidin-blocks in an equal mixture of chloroform and inspissated cedar-wood oil, and then letting the chloroform gradually evaporate. Cedar-wood oil is added from time to time to keep the tissues well covered, and sections are cut with a dry razor whenever the celloidin-block has become quite transparent.

Gilson's method. Transfer tissues from the alcohol-ether mixture into pure anhydrous ethyl ether having a boiling-point of 34·9°. When thoroughly permeated by the latter, place the tissues in a short test-tube and cover them with 2·5 per cent. celloidin. Now put the test-tube in a paraffin bath heated up from 35° to 40° C., when the ether begins to boil off. When the tissue is just covered with the inspissated celloidin, add about one inch of 10 per cent. celloidin and continue the boiling. Within 1 to 1½ hours the celloidin will be of the consistency of a thick syrup. Now pour the celloidin and the tissue into a paper capsule, avoiding the formation of air-bubbles, and harden the tissue in the vapour of pure chloroform (Lee[1]) by placing the paper box with the celloidin on a support

[1] Chloroform as a hardening agent for celloidin was introduced by Viallanes in 1883 (*Ann. Sc. N.* (6) 14, 129).

standing in a vessel which contains 2 to 5 ccm. of pure chloroform, and cover the whole with a well-fitting bell-jar.

Leave small objects 3 hours and bigger pieces 24 hours. When the celloidin-block is fairly hard, transfer it to a mixture of equal parts of chloroform and inspissated cedar-wood oil for 5 to 10 hours, then to pure cedar-wood oil till complete transparency of the block has been attained. Finally allow the last traces of chloroform to evaporate by exposing the block to the air (Lee).

Fix the celloidin-block to a piece of wood or to the object-holder of the microtome by the method of Lindsay Johnson, who uses a cement employed by metal-workers, consisting of a mixture of bees-wax one part, and resin two parts [1]. Cover the piece of wood or the object-holder of the microtome, which is to serve as a support for the celloidin-block, with the cement; dry the celloidin-block; heat up the cement and while still hot press the celloidin-block into firm contact. In a few seconds the cement will cool and the celloidin is firmly attached. Cut the sections with a dry knife (Lee).

Stepanow's method [2]. Tissues which are dehydrated are placed in a mixture consisting of

Dry celloidin chips	1·5 grams.
Clove oil (or eugenol)	5·0 cc.
Absolute ether	20·0 ,,
Absolute alcohol up to	1·0 ,,

The purer the ether the greater will be the quantity of absolute alcohol required, but to obtain a clear solution more than the stated quantity of alcohol should not be added. The clove oil facilitates the penetration of objects by the celloidin.

In this mixture the amount of celloidin is greater than 6 per cent., and rises to 30 per cent. after the alcohol and the ether have evaporated. For very delicate work, such as eggs of the Axolotl, the amount of celloidin in the above formula should be only 0·5 gram to begin with.

Tissues remain in the celloidin mixture in a closed vessel for 3 to 24 hours. Then they are transferred with the mixture to a funnel made of very thin tissue-paper, to allow the alcohol and ether to evaporate. The completion of this process is recognized by the tissue, which at first looked opaque, gradually becoming clear. A sufficient quantity of celloidin should be taken to begin with, to safeguard against the tissue at any time projecting out of the celloidin mixture during the period of inspissation.

When the celloidin is of such consistency as to be readily cut with a scalpel, the object is removed from the filter and as much celloidin is pared off as can be done with safety. To still further harden the celloidin, it is exposed to the vapour of chloroform (see above), and

[1] If this cement is used the whole of the tissue should be cut at once, as alcoholic and oily media dissolve the resin, and therefore the cement is loosened if one attempts to preserve the blocks in spirit or in oil.

[2] Stepanow: 'Eine neue Einbettungsmethode in Celloidin,' *Zeitsch. f. wiss. Mikr.* 17, 185–191 (Oct. 1900).

the clove oil which exudes is sucked up by a piece of filter-paper attached to the celloidin. To obtain sections, fix the object to a block of wood as detailed above (p. 174 or p. 175), and cut sections with a dry knife.

Benzene may be used, instead of chloroform vapour, for removing the clove oil and simultaneously hardening the celloidin. If the benzene method be used it is possible to obtain thin sections (2 μ) by imbedding the celloidin objects for 6 to 12 hours in a mixture of 40° C. paraffin (2 parts) and anethol[1] (1 part). This mixture melts at 35° C., and for use should be heated up to 40° C.

After imbedding in the anethol-paraffin mixture, the object is cast in the usual way (p. 170); the greater part of the paraffin is pared off; the celloidin-block is placed in a drop of anethol on the freezing plate of a microtome; the tissue is quickly arranged in the desired plane; the whole mass is frozen and sections are cut. These when placed on water flatten out, and are mounted as if they were ordinary paraffin sections.

Instead of using anethol-paraffin, a pure paraffin (45° to 48° C.) may be used as was originally done by Kultschitzky (1887)[2], who devised this double celloidin-paraffin method to overcome the brittleness shown by certain tissues when imbedded in paraffin alone. Kultschitzky used origanum oil for clearing the celloidin-block, while Ryder (1888) recommends chloroform as the solvent for paraffin.

CHAPTER XIII.

Microtomes.

To cut sections by hand satisfactorily requires a great deal of practice, and even then large sections are not readily obtained. To overcome this difficulty Valentin introduced a double-bladed knife which is called after him.

The Valentin knife consists of two blades, which by means of a screw are so adjusted as to lie parallel and close to one another. The instrument is used in the following way:—The space between the two blades is filled with normal saline solution. The knife is then pressed, and to a slight extent drawn through a piece of tissue till the latter falls into two pieces, or the knife-edge is brought again to the surface of the organ we are dealing with. By this means a thin section is left between the two blades. To prevent the section tearing, the knife is kept immersed in normal saline, while the blades are separated from one another, and then the section is floated off. The section may now be fixed by being transferred to alcohol, formol in normal saline and other fixatives, or may be examined in the unfixed condition, in normal salt solution.

[1] Anethol or aniseed oil, as supplied by Schimmel in Leipzig, has, when unoxidized, a melting-point of 26° C.

[2] Kultschitzky: *Zeitsch. f. wiss. Mikr.* 4, 48 (1887).

With Valentin's knife consecutive sections are impossible, and therefore the necessity of inventing some mechanical process soon made itself felt.

Stirling's microtome. To meet the requirements of Goodsir, who was investigating the minute anatomy of the septum lucidum of the human brain, A. B. Stirling, Curator of the Anatomical Museum in Edinburgh, constructed in 1861 the first microtome or apparatus for cutting serial sections of approximately the same thickness. His instrument consisted of a circular brass well, sunk in a piece of mahogany, and flush at its upper end with a brass plate. The tissues were placed into the well and rested on a plate which could gradually be lifted up by means of a screw (*Stirling's microtome*, see fig. 1).

To ensure a tight fitting of the tissue, a gouge on the principle of a cork borer was used, with a diameter the same as that of the well. With this apparatus cores of fresh organs or tissues fixed in chromic acid, such as brain, liver, and kidney, were cut out and then pushed into the well. Pieces of organs too small to fit the well were firmly encased in portions of a cylinder of white or yellow turnip or a large round carrot, cut out by the gouge, split longitudinally, and hollowed out for the reception of the tissue.

'As an illustration of the precision with which the instrument may be employed I may state that I can cut as many as 300 transverse sections from an inch of spinal cord' (Stirling).

Ranvier then adopted Stirling's idea for his hand-microtome (*Ranvier's microtome*, see fig. 2).

The next great step was made by Rutherford in May, 1871, when he adapted a freezing-box to Stirling's microtome and also introduced a gauge, to determine the thickness of the sections. The freezing mixture was composed of pounded ice and salt (*Rutherford's microtome*, see fig. 3).

'I was aware that the freezing method would in the end largely supersede all other methods of imbedding the tissues for the purpose of making microscopical sections.' Rutherford, for imbedding, used in 1876 a mixture of solid paraffin (5 parts) and hog's lard (1 part), which was melted and poured into the well, the tissues then being imbedded in the fluid mixture and sections cut after the paraffin had set, or, to prevent the paraffin getting mixed up with the sections, he simply froze the tissues in 0·75 salt solution. Later, and up to the time of his death, the tissues were imbedded in gum solution and then cut in his freezing microtome. In this way sections 15–20 μ were obtained, and as to their quality generations of students and his assistants can testify.

The sections, while yet semi-frozen, are placed in a basin of water, where they will float. After an hour they should be transferred to some fresh water, and this procedure be repeated at least four times, to wash out the sugar and the gum. After this the sections may be stained as desired.

For class purposes, or whenever a tissue has to be kept frozen for a long time, the Williams' (Quecket Club) microtome is the best. It

consists of a circular wooden box with an upright metal rod in its centre, to the top of which circular metal disks can be screwed. The lid of the box is made of wood and covered with a plate of thick glass. In the centre of the lid is a hole which is filled by the disk, attached to the central rod, whenever the lid is placed on the box (*Quecket's microtome*, see fig. 4).

For use fill the box with a mixture of equal parts of pounded ice and salt, 'taking care not to allow the mixture to touch the under surface of the cover[1].' The metal disk projecting slightly beyond the glass plate of the lid, is covered with the gum or gum-sugar solution with which the tissues have been impregnated; the tissues are placed in position and within a few minutes will be firmly fixed. As the freezing progresses add gradually more gum solution so as to surround the tissues on all sides by a coat of gum at least 5 mm. thick.

Sections are cut, not by lifting the tissue towards the razor as in Stirling's microtome, but by lowering the razor. The latter is supported by a semicircular holder resting on three screws, one of which is placed opposite the edge of the razor. By lowering this front screw the razor is gradually depressed, and a section is readily obtained by pushing the holder over the frozen tissue.

Should the latter be frozen too hard, breathe on it once or twice before cutting a section. To make the three screws of the razor-holder travel smoothly, rub some vaseline on the glass plate.

Instead of using ice for freezing purposes, Bevan Lewis, of the West Riding Asylum, introduced the ether spray. To him also, I believe, belongs the credit of having introduced a glass plate for the support of the razor while cutting sections (fig. 5).

In Cathcart's freezing microtome, which is the best instrument for quick work, the ether spray and the glass support for the razor have been adopted from Bevan Lewis.

For cutting paraffin sections I prefer the Cambridge rocking microtome and the different instruments evolved from it. In the small rocker, manufactured by the Cambridge Scientific Instrument Company, the razor is fixed, and the tissue is brought towards the razor, because the rod carrying the tissue is attached eccentrically to the axle of a lever, which rotates round its own axis. Because of this movement each section is slightly thicker along its upper edge, and further, each section is cut as an arc instead of a plane, owing to the lever-like action of the rod to which the tissue is attached (*Cambridge microtome*, see fig. 6).

These two disadvantages are negligible when cutting small objects, but in cutting a long piece of tissue they make themselves felt. To overcome these objections the same firm has devised a large rocking microtome for cutting plane sections, and I cannot praise this instrument too highly. Its action is perfect, the sections (2 × 3 cm.) never vary in thickness (*Cambridge microtome*, see fig. 7).

For smaller objects a thoroughly reliable instrument, cutting plane sections, is that devised by Leek and manufactured by Pye and Co.,

[1] Stirling: *Practical Histology* (1881).

Cambridge. It has nearly three times greater forward movement than the small Cambridge rocker, and a special arrangement to ensure sections of equal thickness (*Leek's microtome*, see fig. 8).

This instrument also allows a freezing-box to be attached in place of the holder for the paraffin-block, and I have found the freezing arrangement to act well.

Celloidin sections of large tissues, such as the central nervous system, should always be cut by the wet process, and the best instrument for this purpose is that devised by Bruce and manufactured by Alexander Frazer, Edinburgh. It consists of a long shallow trough with a well sunk in its centre: through the bottom of the well passes a screw which is turned mechanically each time a section is cut. The screw has attached to its upper end a plate carrying the block of celloidin. The great advantages this instrument possesses are that the sections are cut under spirit; the knife, attached to a sledge, plays in a groove outside the spirit-well; the cutting edge of the knife can be altered to any obliquity without changing its plane; the well economizes spirit and allows thick tissues to be cut; the tissues are readily orientated by a ball and socket arrangement; and further, owing to the simplicity of construction, it does not get out of order. The instrument may be worked by hand or by a treadle arrangement, by means of which a weight pulls the knife forward.

For medium-sized objects the sledge microtomes of Jung, Schantze, and Minot, may be used. In all of these instruments the knife can be placed at any angle, so that if placed transversely paraffin ribbons are obtainable, while, if placed obliquely, both dense paraffin sections and celloidin material may be cut without exposing the tissues to the risk of tearing.

A full account of many sledge microtomes, of special modifications of knife-holders, of 'heads' for holding celloidin and paraffin blocks, of devices for raising or lowering the temperature of microtome knives, and of travelling ribbons for the reception of paraffin sections will be found in the volumes of the *Zeitschrift für wissenschaftliche Mikroskopie*.

Additional note: According to H. Welcker[1], Oschatz was the first to make a microtome, which Welcker then modified. The latter used his instrument already in 1854 for obtaining serial sections of a calf's spinal cord.

[1] H. Welcker: *Über Aufbewahrung mikroskopischer Objecte, nebst Mittheilungen ü. d. Mikroskop u. dessen Zubehör*, Giessen, 1856, bei J. Ricker; also idem: *Arch. f. Anat. u. Physiol.*, 1894, p. 81. See also Weigert, *Technik*; *Anat. Hefte*; Merkel u. Bonnet, 2te Abth., 3, 7 (1893).

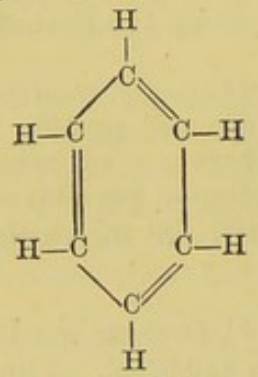
PART IV.

CHAPTER XIV.

General Remarks on the Nature of Dyes.

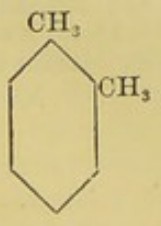
All coloured substances manufactured from coal-tar are derivatives of benzene, C_6H_6, a compound in which six carbon atoms are arranged in a closed ring, forming the so-called benzene ring. In such a carbon ring each carbon atom has attached to it one hydrogen atom; adjacent hydrogen atoms being said to occupy the ortho-position (*o* or 2), alternate ones the meta- (*m* or 3), and opposite ones the para- (*p* or 4) position. If one or more of the original hydrogen atoms are replaced by other chemical compounds, there are formed mono-, di, tri-, &c., benzene derivatives; it is customary in writing constitutional formulae to represent the benzene ring simply as a six-sided figure.

The chief substances from the dye point of view, besides benzene, C_6H_6, are its homologues toluene (toluol) or methyl-benzene, $C_6H_5{\cdot}CH_3$; xylene (xylol) or dimethyl-benzene, $C_6H_4{\cdot}(CH_3)_2$; quinone, $C_6H_4O_2$; naphthalene, $C_{10}H_8$; and anthracene, $C_{14}H_{10}$.

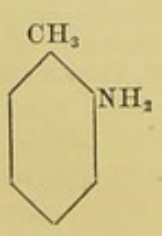


Benzene and its constitutional formula

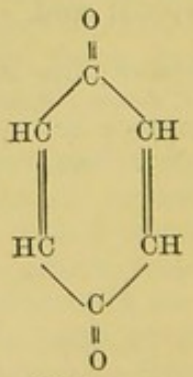
Toluene



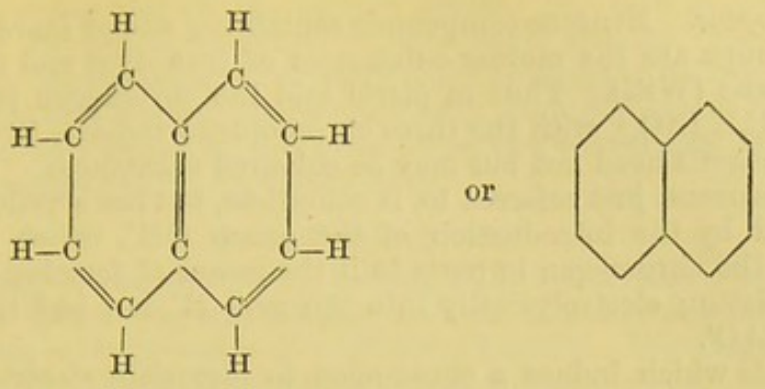
Ortho-xylene



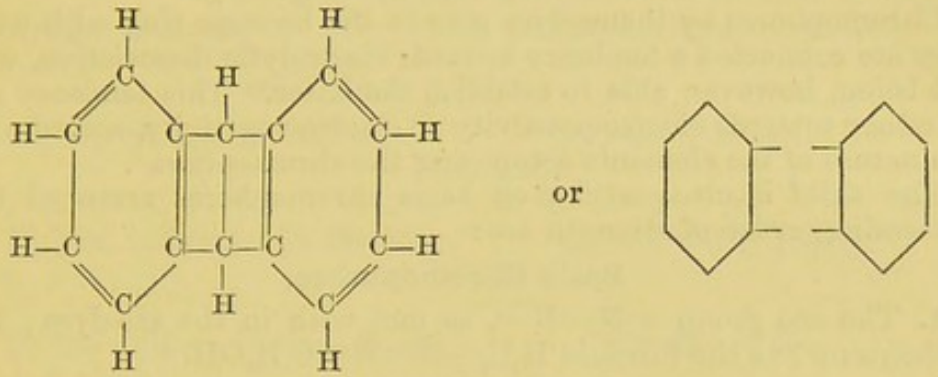
Ortho-toluidin



Quinone

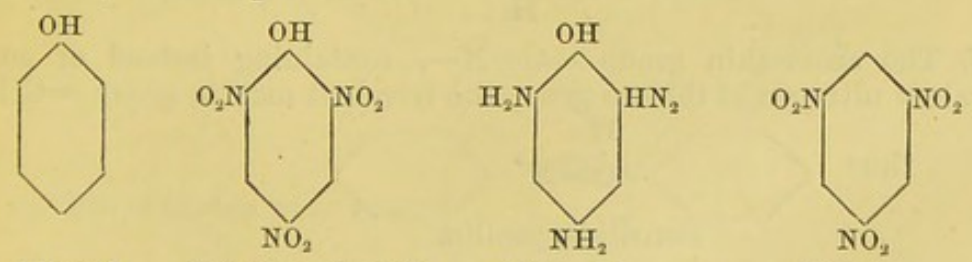


Naphthalene and its constitutional formula



Anthracene and its constitutional formula

Chromophores. Whatever chemical constitution a dye may possess, it always contains in each of its molecules one or more groups which under certain conditions confer on the remainder of the molecule a specific colour. These colour-radicals have been called by O. N. Witt (1876) the chromophores. Thus if in the benzene ring one of the hydrogen atoms is replaced by the hydroxyl group OH, leading to the formation of phenol or carbolic acid, and if of the remaining five hydrogen atoms, three alternate ones are replaced by three chromophoric nitro-groups, NO_2, there results the yellow dye trinitro-phenol or picric acid.



Phenol — Trinitro-phenol or picric acid — Triamido-phenol — Trinitro-benzene

That the colour depends on the NO_2 group is proved by reducing picric acid by means of tin and hydrochloric acid, when the colourless triamido-phenol $C_6H_2(NH_2)_3OH$ is formed.

Chromogens. Benzene compounds containing one or more chromophore groups are the mother-substances of true dyes and are called chromogens (Witt). Thus in picric acid the chromogen is trinitrobenzene $C_6H_3(NO_2)_3$ with the three chromophore radicals NO_2.

Chromogens need not but may be coloured substances. Thus the trinitro-benzene, just referred to, is colourless, but has a yellow colour developed by the introduction of the group OH′, which latter on entering the chromogen imparts to it the power of forming salts, i.e. of dissociating electrolytically into the acid H˙ ion and the an-ion $C_6H_2(NO_2)_3O'$.

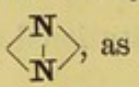
Radicals which induce a chromogen to dissociate electrolytically, or in other words to behave as a salt, are termed salt-formers, and these will be studied more fully afterwards (p. 184).

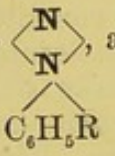
Chromophores by themselves give to the benzene ring with which they are connected a tendency towards electrolytic dissociation, without being, however, able to establish the latter. This tendency may be either towards electro-positivity or electro-negativity, according to the nature of the elements composing the chromophore [1].

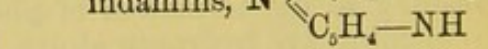
The chief electro-positive or basic chromophores arranged in a descending order of strength are:

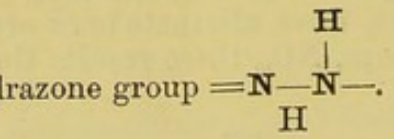
Basic Chromophores.

1. The azo group —**N**=**N**—, as met with in the azo-dyes; thus azobenzene has the formula H_5C_6—**N**=**N**—C_6H_4OH.

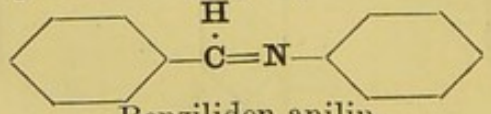
2. The azin group  , as in phenazins .

3. The azonium group  , as in the safranins.

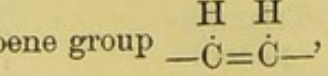
4. The indamin group **N**≡, as found in the indamins, 

5. The hydrazone group  .

6. The azomethin group —C=N—, containing instead of one trivalent nitrogen of the azo group the trivalent methin group =CH,

thus 

Benziliden anilin.

7. The stilbene group  , containing two methin radicals.

The basic nature of the chromophores enumerated above depends on the presence of nitrogen, while the acid nature of the chromophores

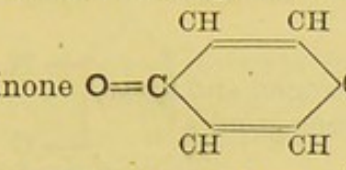
[1] See the table of electrical tensions given on p. 14.

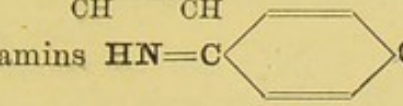
given below depends on the oxygen atoms. All acid chromophores are either oxygen compounds or have been derived from oxygen radicals, by substituting the divalent sulphur or the trivalent nitrogen.

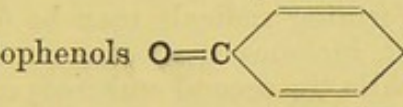
The chief acid chromophores are, firstly, the nitro group NO_2, as in picric acid, and secondly, the ketonic group =C=O and derivatives of it occurring as part of a closed ring.

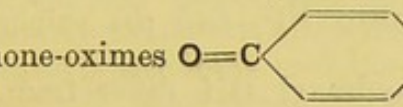
Acid chromophores.

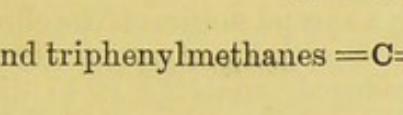
The simplest derivative of ketone is quinone, and it has been argued by Armstrong and Nietzki that all coloured substances owe their colour to the presence of a quinonoid group. The chromophoric radicals in the following formulae appear in heavier type.

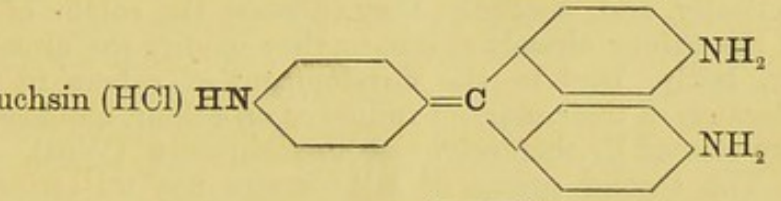
1. Quinone  and its derivatives, namely

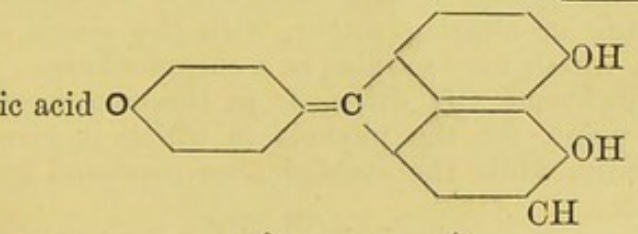
(*a*) the indamins  .

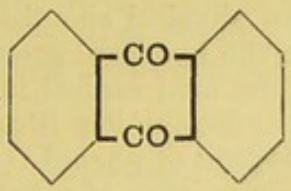
(*b*) the indophenols  .

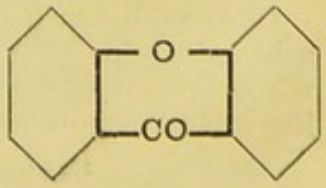
(*c*) the quinone-oximes  .

(*d*) the di- and triphenylmethanes 

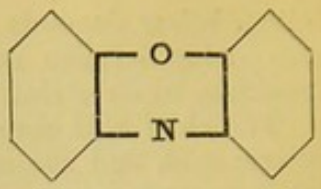
e.g., parafuchsin (HCl) 

rosolic acid 

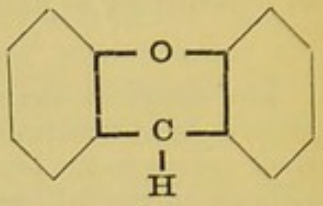
2. The anthraquinone group 

3. The xanthone group, in which one ketone group is replaced by O 

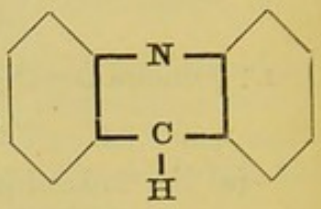
4. The oxazin group, with the one CO group replaced by O, and the other by nitrogen



5. The pyronin group, with one oxygen and one C—H group replacing the two CO groups



6. The acridin group, with one nitrogen and one C—H group replacing the two ketone groups



Salt-formers. The salt-forming radicals may be divided into two groups, of which the first includes the basic amidogen NH_2 and the 'acid' hydroxyl OH, while the second embraces carboxyl CO·OH, the nitrozo NOH, the nitro NO_2 and the sulphonic acid HSO_3 radicals.

Amidogen NH_2 and hydroxyl OH differ from the other salt-forming radicals in having a special action on the chromogens. They, firstly, influence the electro-positivity or negativity of the molecule into which they are introduced, enabling it to dissociate electrolytically, and, secondly, they increase the colour of the chromogen, if the colour already exists, or they modify the already existing tint, or, lastly, lead to the development of colour in the chromogen. Because of their marked influence on colour, amidogen and hydroxyl are classed by themselves as 'auxochromes' (Witt).

The second group of salt-formers has no action in producing, strengthening or altering colour, with the single exception of the NO_2 group, which may act also as a chromophore.

The auxochrome OH differs from the auxochrome NH_2 in one important point, for the phenols to which it gives rise dissociate electrolytically, while the amido-bodies produced by introduction of NH_2 do not.

In the chapters on physical chemistry it was shown that all basic reactions are due to the hydroxyl group OH', and if in the following pages OH is called the acid radical it is because of the dissociation into $RO' + H^{\cdot}$, the hydrogen ion liberated in this way causing for example the acid reaction of phenol $C_6H_5O' + H^{\cdot}$.

Chromophores being either of a basic character (p. 182) or of an acid nature (p. 183), and the auxochrome NH_2 being a strong base, while the auxochrome OH is a comparatively feeble acid, the following interactions between chromophores and auxochromes take place[1].

[1] Pappenheim : *Grundriss der Farbchemie*, 1901.

(1) basic chromophore + basic auxochrome NH_2 = strong base,
(2) acid chromophore + acid auxochrome OH = strong acid,
(3) acid chromophore + basic NH_2 = feeble base,
(4) basic chromophore + acid auxochrome OH = feeble acid.

The most strongly acid or basic dyes are therefore those in which both chromophore and auxochrome have the same tendency towards acidity or basicity.

If a dye consisting of a strong basic chromophore + the basic NH_2 group has introduced one or more hydroxyl groups, its strongly basic nature becomes changed into a feeble one; and, similarly, if an acid chromophore + the acid auxochrome OH has introduced the strongly basic NH_2, then the dye loses its acid character and becomes a feeble base.

Dyes having two amido groups are more basic than those containing only one, and therefore on the same principle rosanilin, a triamido-triphenylmethane, is more basic than malachite-green, which is a diamido-triphenylmethane.

Analogously, increase in the number of hydroxyl groups means increase in acidity. The chief function of the auxochromes is, however, to increase the colour of the chromophores; their action as salt-formers is subsidiary. The auxochrome OH, if it meets with an acid chromophore, can play the part of a salt-former, inasmuch as the H' is split off electrolytically; but should it meet with the auxochrome NH_2, then its powers of dissociation are interfered with.

The auxochrome NH_2 itself never dissociates electrolytically, but it influences other nitrogen atoms which may be present, and gives them the power of linking on to the tissues, as in toluidin-blue (p. 404) and safranin (p. 412), in which the imide radical NH attaches itself to the tissue. For example, the triphenylmethane compound rosanilin (p. 425) contains the chromophore =C—R=NH, the basic reaction of which depends on the imide NH. There are, however, in addition two auxochromic amidogen groups NH_2, increasing the basic character of the substance. That the imide and not the amide links on to the tissue is proved by suspending in the colourless rosanilin base a skein of silk, when the latter turns a red colour owing to the formation of the deep-red mono-acid rosanilin salt. This explanation is correct, for if the amido groups were concerned in the salt formation the silk should become yellow, as rosanilin, in which the amido groups are satisfied, is yellow.

The salt-forming groups, being strong acids, have the power of overcoming, in part or completely, that basicity of a dye-molecule which depends on the presence of a basic auxochrome or chromophore.

Thus in rhodamin (p. 188) and in chrome-green (p. 423) the CO·OH group weakens the action of the amido auxochrome, but the chromogen being basic the dye-molecule as a whole still acts as a base. The carboxyl radical in combination with acid chromophores, or with auxochromes into which oxygen has been introduced, produces acid dyes, e.g. fluorescein (p. 429).

The salt-forming NO_2 group occupies a peculiar position, for besides

being a strongly acid radical it also acts in some dyes as a chromophore, as pointed out in connexion with picric acid (p. 181). The sulphonic group HSO_3 resembles NO_2 in being such a strongly acid radical that it impresses an acid character on the dye-molecule, whatever other basic groups may be present. Thus basic fuchsin becomes acid fuchsin on introducing SO_2OH (p. 425). It also increases the solubility of dyes, by rendering even those which are quite insoluble in water, soluble; for example, basic anilin-blue is water-insoluble, but after sulphonation it forms the acid water-soluble 'water blue' or methyl-blue (p. 426). The tendency of acid radicals to increase the solubility of a dye is not restricted, however, to the sulphonic group, but is of general occurrence, and this increase of solubility goes hand in hand with a diminution in the intensity of staining power.

That dyes can only act in virtue of salt-forming radicals is especially well seen in the case of azo-benzene, $H_5C_6—N=N—C_6H_5$, an intensely coloured body, containing the chromophore —N=N—. It is a neutral compound, and does not act as a dye till either basic or acid characters have been given to it by the introduction of, for example, the basic amidogen radical, NH_2, when amido-azo-benzene is formed, $H_5C_6—N=N—C_6H_4NH_2$, or of the acid radical ·OH, leading to the formation of oxy-azo-benzene.

Acid dyes, according to the nomenclature of Ehrlich, are those which contain an acid staining principle, as does, for example, eosin, which is the sodium salt of the coloured acid tetrabrom-fluorescein. Basic dyes analogously possess basic colour radicals; for example, methylene-blue is the chloride of the coloured base tetramethyl-diphenthiazin. 'Neutral' dyes Ehrlich calls those in which a colour base is joined to a colour acid, as in the eosinate of methylene-blue or in the picrate of rosanilin. It is better, however, to group Ehrlich's neutral dyes under the general heading of salt dyes, meaning by this term that the coloured compounds are taken up by tissues as salts without having undergone a preliminary dissociation into the an- and kat-ion radicals of which they are composed.

The radicals in 'salt dyes' need not, as in the special case of Ehrlich's 'neutral dyes,' be both coloured, for most 'salt dyes' are acid sodium salts of the sulphonic acids of benzidin (p. 395), of primulin, or of oxystilbene (p. 397). These dyes and their action being discussed in the special chapter on the theory of staining (p. 327), it will now suffice to point out, firstly, that all of them, including Ehrlich's so-called neutral dyes, are true salts containing definite salt-forming groups; and secondly, that although they have the power of dissociating electrolytically, the molecule seems to act as a whole in many reactions. Thus methylene-blue is the chloride of a dye belonging to the quinone-imides, and dissociates normally into the colourless an-ion chlorine Cl' and the remainder, which is the coloured kat-ion; eosin is the sodium salt of tetrabrom-fluorescein, and in a watery solution breaks up into the colourless kat-ion sodium and the coloured an-ion tetrabrom-fluorescein, while the 'salt-dye' eosinate of methylene-blue, formed by the union of the methylene-blue kat-ion with the coloured tetrabrom-fluorescein an-ion, is insoluble in water

and does not dissociate. It can be made, however, to dissolve by either using alcohol as the solvent, or by having a slight excess of the methylene-blue kat-ion or the eosin an-ion.

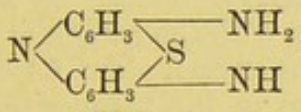
From such solutions the eosinate of methylene-blue may be taken up as a whole, for example by the neutrophil granules of certain leucocytes which stain in the mixed colour of methylene-blue and eosin, namely violet; while other tissues with different affinities select either the methylene-blue or the eosin radical, and thereby become stained blue or red.

Salt dyes are employed extensively commercially for dyeing cotton and woollen wares, and are also used for histological purposes (p. 441).

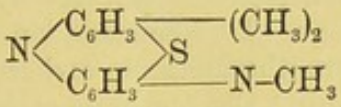
It frequently happens in the case of dyes, as in other aromatic compounds, that a hydrogen atom may be replaced by a fatty or aromatic radical without there being produced any considerable alteration in the character of the compound.

Side-chains. When the replaced H atom forms part of a benzene ring, then the substituted alkyl (methyl or CH_3; ethyl or C_2H_5) or aromatic phenyl group (C_6H_5OH) is called a side-chain.

Thus thionin becomes toluidin-blue by the introduction of alkyl groups CH_3 into the auxochrome amido group NH_2 and also by replacement of the H atom in the imide group NH.



Thionin



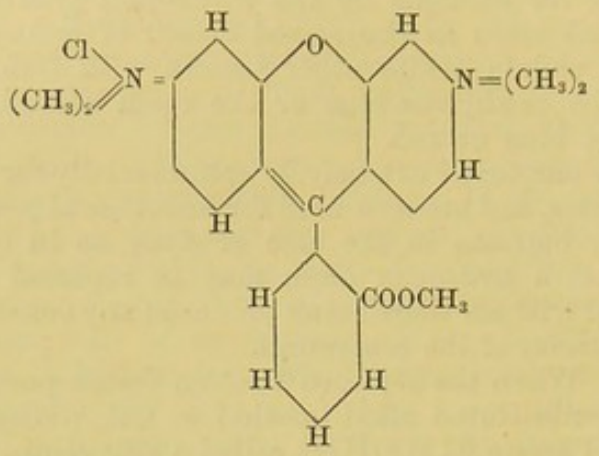
Toluidin-blue

The alkyl and phenyl groups do not materially interfere with the chemical constitution of a dye-molecule, but they serve to deepen its colour, and also to increase its size. An $O{\cdot}CH_3$ compound is, for example, darker and slightly less acid than an O·H group, and a $NH{\cdot}C_2H_5$ radical darker and slightly less basic than NH_2 (Pappenheim). Alkyl groups thus modify the tint of the dye-molecule, while the sulphonic acid radical $SO_2{\cdot}OH$ does not do so.

An alteration in the tint of a dye may further be induced by the introduction of halogen radicals, or by the substitution of one halogen for another; for example, mono- and dibrom derivatives of fluorescein are yellowish-red, while tetrabrom compounds are bluish-red (p. 430), and tetra-iodine-fluorescein (erythrosin) is more bluish-red than tetrabrom-fluorescein (eosin). Generally speaking it may be stated that dyes which have all their carbon in benzene rings, possess a yellow colour, and that by the addition of auxochrome groups, or by the accumulation of carbon by means of side-chains (see above), the colour increases in depth, passing through red into violet and brown. For example, picric acid containing three NO_2 groups is yellow, while aurantia containing six NO_2 auxochromes is orange. The simple azo-dyes are yellow and red, while blue tints only result from the accumulation of several azo groups in one molecule, as met with in the dis- or tetrazo compounds (p. 388).

How colours are produced we know, but not why.

Rhodamin (p. 430), in addition to containing four methyl groups (CH_3) which have replaced four hydrogen atoms of the two auxochrome radicals NH_2, possesses also a methyl group introduced in the salt-forming carboxyl group CO·OH.



It may be useful to analyse this last formula, and to recapitulate what has been stated on the previous pages.

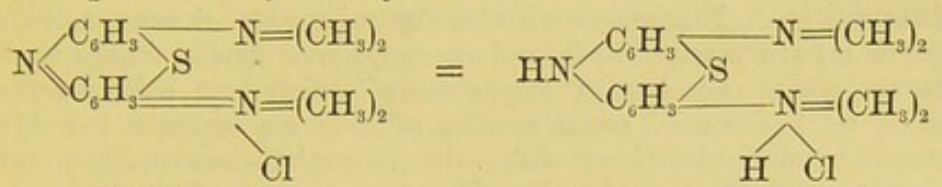
Rhodamin is a triphenylmethane derivative, and contains:

1. The basic chromophore =C= (p. 183) connected up with three benzene rings. Two benzene rings, along with the carbon atom of the chromophore (the methane carbon) and an oxygen atom, form a fourth ring containing the methane carbon atom in the paraposition to the oxygen atom, reminding one of the chromophore =C=O of quinone (p. 183). It is for this last reason that rhodamin may be said to possess a quinone-like or quinoid character.
2. A chromogen radical consisting of the chromophore =C=, three benzene remainders, and the 'paraquinoid' fourth ring.
3. The remainders of two auxochrome groups (NH_2) occupying the paraposition to the methane carbon atom.
4. The remainder of the salt-forming carboxyl group CO·OH in the third benzene ring.
5. Five methyl groups CH_3, of which four replace the four hydrogen atoms of the two auxochromes $(NH_2)_2$, while the fifth methyl radical has taken the place of the hydrogen in the carboxyl group.
6. The rhodamin dye-molecule taken as a whole is a base, owing to the basic chromophore and the two basic auxochromes together being stronger than the single salt-forming carboxylic group. The commercial rhodamin not being the free base but a salt, and usually the chloride, the chlorine is shown attached to a pentavalent nitrogen atom of the original auxochromic NH_2 group.

A dye retains its colour only as long as its possible affinities for hydrogen have not been satisfied; for example, the yellow picric acid, when brought in contact with nascent hydrogen, becomes the colourless triamido-phenol (p. 181). Similarly, all other coloured benzene

compounds, by taking up one or two molecules of hydrogen, become colourless, white, or leuco-compounds.

If by the absorption of hydrogen atoms no deep change is produced in a dye, then it is always possible by oxidation to reconvert the leuco- into the colour compound. Thus methylene-blue is readily reduced by boiling a watery solution with sodium carbonate, Na_2CO_3, and grape-sugar, $CH_2(OH)—CH(OH)_4—CHO$, the aldehyde radical of the latter, CHO, acting as the reducing agent. If the colourless solution be divided between two test-tubes, the fluid in one of the test-tubes being covered with hot oil, and if then both test-tubes be cooled, after the lapse of a few minutes a distinct blue colour will be seen in that test-tube where the access of the oxygen of the air has not been prevented by the layer of oil.



Methylene-blue = Leuco-methylene-blue
(according to quinone theory)

From what has just been stated, it follows that all coal-tar dyes will be readily decolourized by contact with nascent hydrogen, which for experimental purposes is obtained in sufficient purity by the action of 1:8 of sulphuric acid or 1:2 of hydrochloric acid on granulated zinc.

The question of pseudo-bases (see p. 25) and real bases has also to be considered, for dyes will stain only if they are real bases, and in this connexion we have to bear in mind the effect of accentuators (see p. 212) which are added to the stains, for by them it is possible to convert the pseudo into the real base.

Amongst the dyes in ordinary use we may with Hantzsch and Osswald distinguish two groups, those which do and those which do not form pseudo-bases.

(1) *Dyes forming pseudo-bases* are the bases of the di- and triphenyl-methane series (crystal-violet, pararosanilin, brilliant green, and auramin), also the azonium dyes (rosindones and rosindulins), and lastly flavindulin.

All these are so constituted as to allow the (OH) group to wander from the nitrogen to the carbon (or to another nitrogen group as in rosindulin); $R{\equiv}N'—OH'$ becomes $HO—R{\equiv}N$, and in consequence of this change their electrical conductivity is soon reduced to almost zero.

Primarily crystal-violet, pararosanilin, brilliant green, and auramin are very strong, completely dissociated bases, analogous to alkalies or completely substituted ammonium hydrate; but secondarily, and usually soon, the coloured substance is changed into a colourless derivative by the migration of the (OH) group:

$[H_2N—C_6H_4]_2C=C_6H_4=NH_2—OH$ becomes $(H_2N—C_6H_4)_3C·OH$.
pararosanilin pseudo-pararosanilin carbinol.

(2) *Dyes not forming pseudo-bases* comprise safranin (p. 412) and thiazin (see p. 403), as in them no isomeric formulae possessing the hydroxyl in a different group are possible.

It is probable that the safranins and thiazins are stronger bases than the triphenyl derivatives because there is no tendency to form pseudo-compounds; anyhow histologically they possess great advantages over rosanilin, as is readily shown by staining identical sections in toluidin-blue and basic fuchsin.

CHAPTER XV.

The History of Staining.

Introduction. Nowadays when every microscopist uses stains, and most of us are pleased to introduce some new dye to which we take a fancy, either because it happens to satisfy our colour idiosyncrasies, or because of some special affinity we suppose the dye to possess for particular tissue elements, we ought occasionally to reflect on how much was done by the older histologists without the aid of stains.

It is a sad fact that microscopists almost without exception rely only on coloured sections, and that because of the very ease with which certain structures stand out, preparations are examined less carefully than of old, when microscopy in itself was a science. What can be done without stains may be realized by studying old books on histology, such as Kölliker's *Microscopic Anatomy*, 2, 1850–1854, and Gerlach's *Handbook*, Mainz, 1854.

The first decade of staining was characterized by a conservatism still noticeable, for the teaching of botanists was neglected by the investigators of animal tissues. An observer belonging to the latter class, as early as 1851, gave an account of having used 'a dilute carmin stain' for investigating the structure of the internal ear. His paper published in one of the first German periodicals attracted no notice. It was written in French.

Later, in 1854, another observer having been led, by a fortunate accident, to the conception of the idea that present ways may not always be the best, by a second equally fortunate occurrence was enabled to discover a reliable staining method. By publishing the latter, a seed was sown, which owing to a number of further fortunate circumstances commenced to germinate. The method of staining, once having taken root in the animal histologist, grew and grew, till to be an histologist became practically synonymous with being a dyer, with this difference, that the professional dyer knew what he was about, while the histologist with few exceptions did not know, nor does he to the present day.

During this prodigally liberal production of new staining methods, the advantages accruing to histology from the use of dyes were but little, for most of the discoveries attributed to the use of special stains could have been made by a skilled microscopist on perfectly unstained sections with almost, if not quite, equal ease.

On reading treatises published especially within the last decade, I cannot help thinking that now we have lapsed into a state of degraded conservatism, since men in the name of justice haggle about their rights of priority, and in the name of nationality do injustice to their neighbours. Knowingly, or let us charitably suppose in crass ignorance, there are published, almost daily, articles supposed to contain new ideas or facts which time and again will be met with in older writings.

It is time that a stop was put to this kind of literature, for compelling others to read productions devoid of any new ideas or facts means robbing workers of their time, and keeping back science.

Notwithstanding that Ehrlich in 1879 founded the science of staining as regards anilin dyes, that Böhmer, Frey, Weigert, and Paul Mayer have explained the action of haematoxylin, that Unna proved what can be done by a systematic research, that Gierke, Griesbach, Rawitz, Benda, Michaelis, and others have also shown the way how to proceed,—notwithstanding all this, the majority of histologists still prefer to dabble in stains in an unscientific manner, owing to the baneful principle that 'Experimentation is preferable to study.'

It is not enough to regard dyes simply as acids or bases, as oxidizers or reducers, but we must aim at micro-chemical methods, and endeavour to know the composition of the dye and the tissue, to apply tests in a definite purposive way. Not till then will progress be made in the most difficult of all branches of Physiology, namely micro-chemistry. If two dyes are found to give identical staining reactions, it is permissible to choose that one which either gives the sharpest pictures with artificial light, or which, for aesthetic reasons, harmonizes best with the other colours. I do not, however, hold with Rawitz that it is immaterial whether one use, for example, eosin or erythrosin, simply because these substances are chemically closely related to one another. With erythrosin it is easy to demonstrate the intercellular channels of nerve-cells, while with eosin it is very difficult. If I had not discarded erythrosin because of its glaring effect, I might have generalized my first observation on the Malopterurus cell; instead of which the credit of having demonstrated the general occurrence of a canalicular system in nerve-cells belongs to Holmgren, who, erythrosin apart, has been using my methods for both fixing and staining.

The following pages are essentially based on the genial articles by Gierke, in the first and second volumes of the *Zeitschrift für wissenschaftliche Mikroskopie*. Here the older staining methods up to 1884 have been chronicled in a masterly manner, and the student of history is especially recommended to peruse his account, because of the full references to literature.

Historical review. The first attempt at histological staining, apart from the use of iodine, which seems to have been used very early, was made by the botanists Göppert and Cohn in 1849, who used carmin to render more evident the rotation of the cell contents in *Nitella flexilis*. Their researches stimulated Hartig, 1854–1858, to

employ carmin for staining the chlorophyll granules of plants. He also attempted to throw light on the functions of the nucleus, by using solutions of carmin, litmus, black ink, copper sulphate, &c. He was the first to observe that the nucleus of a cell stains with dyes only after it has been killed, and that albumin and gelatin are readily stained by carmin, while gum, mucus, &c., do not stain. Hartig is the discoverer of histological staining, but his work attracted no attention, and we must regard Gerlach as the founder of the practice of staining.

Quite independently of Hartig, Gerlach was led to the discovery of carmin staining by observing in 1854 that tissues after injection with badly prepared carmin gelatin became coloured. He naturally proceeded to stain with ammoniacal carmin, but failed for a long time to get satisfactory results, till a fortunate accident occurred in 1858. A section through a cerebellum, hardened in potassium bichromate, was left by an oversight in an exceedingly dilute solution of ammoniacal carmin, and when found 24 hours later was beautifully stained, the nerve-fibres and nerve-cells being differentiated. Previous failures had been due to the use of too concentrated carmin solutions.

The apothecary Maschke, continuing the work of Hartig, in addition to carmin, also employed, for the first time, indigo (1859). He divides all organic substances into two classes, namely, proteid substances which stain readily (horn, albumin, gelatin), and the cellulose group which has no affinity for dyes (cellulose, vegetable membranes, starch, sugar, and mucus), and predicts: 'Pigment solution, I am convinced, will in future be quite as indispensable as iodine solution, and both will share with the knife of the anatomist a place of honour beside the microscope.' In 1882 Maschke recommended, instead of ammonium carminate, the sodium carminate, a salt still in use at the present day.

Thiersch, in 1865, was the first to employ carminates in combination with borax and with acids, namely with oxalic acid, while Schweigger-Seidel introduced a combination with acetic acid (1868). In the same year Ranvier used carmin in combination with picric acid as a double stain, after Schwarz in the previous year, 1867, had stained material first in carmin, and then in picric acid by the following procedure: Tissues were first boiled for one minute in a mixture of creosote (1 part), acetic acid (10 parts), and water (20 parts); then allowed to dry completely; next cut into thin slices and macerated for one hour in dilute acetic acid, washed in distilled water, stained in very dilute ammonium-carminate for 24 hours; washed again in distilled water, and stained in 0·066 gram picric acid in 400 ccm. water for 2 hours, and finally mounted in dammar balsam. By Schwarz's method the results are the same as those obtainable by Ranvier's picro-carmin stain: muscle-fibres, gland-contents, vessels, and nerves are yellow, while connective tissue and nuclei are stained red.

Haematoxylin in the form of a watery extract of logwood was first tried by Waldeyer in 1863, who endeavoured, but without

success, to obtain by its means a sharp staining of axis-cylinders. Only by the publication of Böhmer's thoroughly scientific formulae (1865) can haematoxylin be said to have been introduced into histology (compare p. 231). Böhmer used, as did practical dyers, haematoxylin crystals in combination with alum, the latter acting as a mordant (p. 225). Later it was pointed out by Frey, in 1868, that the addition of alum to the haematoxylin solution could be dispensed with provided the tissue had been fixed by a reagent acting as a mordant for haematoxylin, such fixing reagents being, for example, copper sulphate, chromic acid and its salts. These facts seem to have led Alleyne Cook, in 1879, to combine with alum-haematoxylin a second mordant, namely copper sulphate (logwood extract 6 parts, alum 6 parts, copper sulphate 1 part, distilled water 40 parts, thymol a small crystal).

The first to use alum-haematoxylin in an acid solution was Ehrlich (1886), the idea underlying the addition of acetic acid being to prevent thereby the dissociation of the alum into free sulphuric acid and basic aluminium compounds, which latter, by combining in the staining solution with the haematoxylin, lead to the precipitation of the insoluble aluminium-haematoxylin lake.

Anilin dyes became commercial articles in 1856, when Perkin's mauveine made its appearance, and two years later Hofmann's Anilin-Roth, or what is now termed rosanilin or fuchsin, was on sale.

Already in 1862 Beneke recommended for histological purposes acetic acid coloured with a commercial lilac anilin, probably the mauveine or anilin-violet, and in 1863 Frey and Waldeyer used, independently of one another, fuchsin (or anilin-red), which the latter stated would stain nuclei and nucleoli more deeply than the cell-plasm, and the axis-cylinder more quickly than the medullary sheath of nerves. Waldeyer also employed anilein (or anilin-violet) and anilin-blue (or Parisian blue).

To Böttcher (1869) belongs the credit of having introduced the method of differentiating sections. He procured a sharp staining of the nuclei, while the cell-plasm became decolourized, by staining sections in watery or glycerin solutions of rosanilin nitrate, differentiating in alcohol, clearing in creosote, and mounting in dammar or Canada balsam.

The method just described is generally but erroneously attributed to Hermann, for he did not publish his results till 1875. Hermann recommended to fix tissues in chromic acid, to harden them in alcohol, and not to use anilin dyes which were water-soluble. The solution he found most useful consisted of rubinfuchsin 0·25 grm. dissolved in 20 cc. of 96 per cent. alcohol, to which subsequently 20 cc. of water were added.

Flemming (1881), not knowing of Böttcher's work and attributing the method of differentiating to Hermann, endeavoured to extend it, by trying, quite indiscriminately, acid and basic dyes, with the result that he found certain dyes such as eosin, ponceau, and orange did not answer (all these belong to the acid group), while Magdala red,

dahlia, and especially safranin (these dyes belong to the basic group) gave good results. Flemming also introduced 1 : 1,000 hydrochloric acid alcohol for facilitating the process of differentiation, after Lawson Tait in 1875 had already employed 4 per cent. nitric acid to render the nuclei in haematoxylin preparations of a reddish-brown tint, while the cytoplasm became a cherry-red colour.

It is impossible to give here a full account of all the stains which have been advocated up till now, but the more important ones are given in the following table:

TABLE INDICATING WHEN AND BY WHOM THE MORE IMPORTANT DYES WERE FIRST USED.

Carmin.

1849. By Göppert and Cohn.
1851. 'A dilute carmin solution' by Corti.
1854. Ammonium carminate by Hartig.
1865. Borax carmin by Thiersch.
1866. Glycerin-alcoholic carmin by Beale.
1868. Picro-carmin by Ranvier.
1870. Acetic acid carmin by Frey.
1876. Alcoholic carmin by Hoyer.
1879. Alum-carmin by Grenacher.
1882. Sodium carminate by Maschke.
1884. Carminic acid by Dimmock.
1900. Oxidized carmin ('carminein') by van Wijhe.

Litmus.

1854. Litmus by Hartig.

Indigo.

1859. Indigo by Maschke.
1864. Indigo-carmin by Chrzonszczewski, for intra vitam staining.
1865. Indigo-carmin in oxalic acid solution by Thiersch.

Anilin Dyes.

1862. A lilac anilin dye (?) is used, in an acetic acid solution, for the first time in histology by Beneke.
1863. Picric acid by Roberts, in blood investigations.
Fuchsin by Frey and also Waldeyer.
Anilin-violet and anilin-blue by Waldeyer.
1867. Picric acid after staining in carmin by Schwarz.
1868. Parma blue by Frey.
1874. Dahlia by Huguenin, and also by Zuppinger.
Cyanin by Ranvier.
1875. Eosin by Fischer.
Iodine-violet by Jürgens.
1876. Safranin by Ehrlich.
Anilin blue-black by Sankey.
Colin-black by Luys.
1877. Methyl-green and indulin by Calberla.
1878. Bismarck-brown by Weigert.
Methyleosin, aurantia, indulin, nigrosin, tropaeolin, Bordeaux ponceau, naphthylamin yellow, Aechtgelb, by Ehrlich.
1879. Acid fuchsin and methylene-blue by Ehrlich.
1880. Methyleosin, malachite green, and Biebricher scarlet by Schwarz.
1881. Gentian-violet and Magdala red by Flemming.
Sulphonic acid anilin-blue by Frey.
Nigrosin by Errera.
1882. Violet B (?) by S. Mayer.
Iodine-green by Griesbach.
1883. Metanil-yellow by Griesbach.

1884. Orange G by Ehrlich.
1885. Thionin by Ehrlich.
Anilin-green (?) by Schiefferdecker.
1886. Victoria blue by Lustgarten.
Congo-red by Griesbach.
1887. Metaphenylene-diamin + sodium nitrite by Unna.
Thiophene green by W. Krause.
1888. Benzoazurin and Deltapurpurin by Zschokke.
Water-blue by Mitrophanow.
1890. Toluidin-blue by Hoyer.
1891. Light-green SF and acid violet by Benda.
1892. Polychromic methylene-blue by Unna.
1893. Patent blue (carmin blue) by Janssens.
1894. Neutral red by Ehrlich.
1898. Kresyl-violet R by Ehrlich and Lazarus.
1899. Caerulein S by Lenhossék.
1900. Kresofuchsin by Röthig.
Oxonin by Ehrlich.
Janus-green by Michaelis.
1901. Para-dimethyl-amido-benzaldehyde by Ehrlich.

Haematoxylin.

1863. Logwood extract by Waldeyer.
1865. Alcoholic solution of alum-haematoxylin by Böhmer.
1876. Calcium chloride + alum-haematoxylin by Kleinenberg.
1879. Alum-copper-haematoxylin by Alleyne Cook.
1881. Glycerin-alum-haematoxylin by Renaut.
1886. Acid alum-haematoxylin by Ehrlich.
1891. Haematein substituted for haematoxylin by P. Mayer.

Alcanna.

1863. Alcanna by Waldeyer (a watery extract).
1882. An alcoholic extract by Dippel.

Alizarin.

1864. Alizarin by Lieberkühn for intra vitam staining.
1880. Alcoholic alizarin by Benczur.
1895. Alizarin used adjectively by Rawitz.

Other Dyes.

1874. Purpurin by Ranvier.
1875. Extract of red cabbage by Lawson Tait.
1878. Orseille by Wedl.
1886. Orcein by Israel for general histological purposes.
1891. Orcein for elastic fibres by Tänzer-Unna.
1884. Extract of *Vaccinium myrtillus* by Lavdowsky.
1887. Kernschwarz by Platner.

CHAPTER XVI.

Classification of the Staining Methods.

A systematic classification of the different staining methods will be possible only after the laws have been determined which govern the processes of dyeing. Theoretically it is easy to make a scheme, but the fitting into this scheme of certain methods is in many cases quite impossible, as we do not know the extent to which either chemical or physical, or both, factors come into play.

For practical purposes the classification of Bancroft into 'substantive,' or direct, and 'adjective,' or indirect, methods is the most useful. The direct staining process implies that either the chemical or the physical nature of both the tissue and the stain are so inter-

related that by the end of the dyeing process the tissue has acquired the colour of the dye. The indirect or adjective staining method on the other hand requires, in addition to the dye and the material to be dyed, the presence of a third substance which brings about the union between tissue and stain. How this is done will be shown later (p. 227).

The direct methods are most readily subdivided, according as to whether purely physical or physico-chemical factors predominate during the act of dyeing. A further subdivision may be made by separating those dyes which stain after having undergone electrolysis or hydrolysis, from those which stain by the whole undissociated dye molecule combining with the tissue. Closely allied to this last group are also the dyes which form additive compounds owing to a process of oxidation.

Still another subdivision may be made by distinguishing the dyes which bring about the desired result at once, and those which require a special process of 'differentiation.'

The term 'differentiation' implies that the primary staining effect obtained by acting on a tissue with a dye is modified, secondarily, by some special after-treatment, which induces only certain tissue-elements to remain stained or to stain in different degrees.

Where it is desired to stain a tissue by more than one dye, the various stains may be used simultaneously or successively, and the dyes so employed may be mixtures of colour bases or of colour acids, colour acids followed by colour bases or vice versa, or colour salts, the latter term including Ehrlich's 'neutral' dyes.

Table showing methods of staining.

I. Physical union takes place between dye and tissue because the chemical affinities of the latter are either already satisfied or cannot be satisfied by the dye.

A. The factors necessary to produce physical staining:

1. The state of the tissue with respect to
   (*a*) moisture.
   (*b*) power of swelling.
   (*c*) texture and permeability.
   (*d*) size of objects.
   (*e*) presence or absence of the fixative.
2. The state of the stain with respect to
   (*a*) solubility.
   (*b*) concentration.
   (*c*) rate of diffusion.
   (*d*) power of staining deeply (covering-power).
3. The nature of the solvent.
4. Time.
5. Temperature.

B. Effects produced by the interaction of the factors enumerated under A:

1. By staining with single dyes.
   (*a*) The result of interrupting the process of diffusion into the tissue.

(*b*) The result of differentiating:
(1) By causing the diffusion outwards, of the dye already in the tissue,
(α) through physical means.
(β) through chemical means.
(2) By allowing other dyes to diffuse inwards.
2. By staining with a mixture of dyes.
(The subdivisions as under 1.)

II. Chemical union between dye and tissue takes place, after the stain has been brought in contact with the tissue by physical means.
A. Direct chemical union takes place between the tissue and the stain. (Bancroft's substantive method of staining.)
1. No differentiation is required, the dye being offered in such a manner that only certain tissue-elements absorb it.
(*a*) Staining with single substances:
(α) colour acids.
(β) colour bases.
(γ) colour oxides.
(*b*) Staining with several dyes:
(1) Simultaneous application of
(α) colour acids.
(β) colour bases.
(γ) colour salts.
(2) Successive application of
(α) colour acids followed by colour bases.
(β) colour bases followed by colour acids.
2. Differentiation is required because the dye is offered in such a way that more than one substance is imbibed by it. The removal of the stain from tissue elements which we desire to be colourless or to stand out in one particular tint is brought about by chemical agencies.
(*a*) After staining with one stain:
(1) colour acids.
(2) colour bases.
(*b*) After staining with more than one stain.
B. Indirect chemical union between the tissue and the stain is brought about through the agency of a third substance called a mordant. (Bancroft's adjective method of staining.)
1. The mordant is an acid.
2. The mordant is a base.
3. The mordant is a salt.
C. Chemical union takes place between the stain and substances with which the tissue has previously been impregnated:
(*a*) impregnation with fixatives.
(*b*) impregnation with amido acids.
(*c*) impregnation with tannin.
(*d*) impregnation with proteids:
(α) nucleic acid.
(β) albumose.

CHAPTER XVII.

Physical Methods of Staining.

I. A. Physical factors which determine staining.

1. The state of the tissue.

(*a*) Moisture.

Ehrlich[1], in 1878-9, drew special attention to the physical aspect of fixing by pointing out that certain granules, which show an affinity for the dye indulin or nigrosin, on heating lose this power, being now only stainable with eosin. The indulin, β-, or amphophil granules he held at that time to be young eosinophilous or α-granules and to differ from the adult form in containing more moisture, which, by careful heating, could be driven off (compare also with p. 199). Ehrlich further noticed that a number of dyes will not stain, even if used in saturated solutions, provided the solvent be anhydrous. Thus dahlia and fuchsin in absolute alcohol do not stain the γ-granules of mast-cells. Similarly, other dyes dissolved in pure glycerin or glacial acetic acid also fail to act as stains.

This observation, along with the fact that watery solutions overstain, has led Ehrlich to add glycerin, and in some cases acetic acid, to his staining mixtures. The credit of originating the idea of combining extractives with the staining solution seems, however, according to Westphal[2], a pupil of Ehrlich's, to rest with Hermann, who used alcohol, and with Schäfer, who employed glycerin.

(*b*) The power of swelling up.

Ehrlich, according to the testimony of his pupil Schwarz[3], was the first to point out that a diminution of staining capacity is always accompanied by a swelling up of the tissue, while an increased affinity for dyes depends on coagulation, because coagulated albumin possesses greater affinity for many stains than does uncoagulated albumin.

Gierke[4], following up the ideas of Ehrlich, also stated (l. c., 213) that viscous substances cannot be stained permanently as long as they are in a viscous state; that it is essential to limit the power of swelling up, and that, generally speaking, the firmer a tissue is, the better it will stain. For this reason he supposed living tissues, in which metabolism is going on, not to stain at all or only very temporarily; newly dead material to stain fairly well, while that quite dead stained best, as the molecules had settled down into a more rigid system.

There is, however, a more important factor, namely, that the proteid, when living, is in the pseudo-acid or basic state, in which it cannot react chemically. To ensure permanent staining, no matter whether chemical or physical, it is necessary either to coagulate moist tissues

[1] Ehrlich: *Verh. d. physiol. Gesellsch.*, Berlin, 20 (1878-9).

[2] Eugen Westphal: 'Dissertation 31st January, 1880'; P. Ehrlich: 'Farbenanalytische Unters. z. Hist. u. Klinik d. Blutes,' *Gesammelte Mitth.*, Hirschwald, Berlin (1891).

[3] Schwarz: 'Inaugural Dissertation' (1880); see also Ehrlich's *Gesammelte Mittheilungen.*

[4] Gierke: 'Färberei zu mikroskopischen Zwecken,' *Zeitsch. f. wiss. Mikrosk.* 2.

by chemical or physical means, and thereby to render them only partly soluble, or to allow the proteids to lose their moisture by dehydration and then subsequently to modify their power of re-absorbing water.

This latter end may be achieved in two ways, namely, either by dissolving the stains in strong alcohol, glycerin or similar substances, which act also as dehydrating agents; or by employing Ehrlich's principle of heat-fixation, which renders tissues more or less water-insoluble according to the length of time or the temperature at which the dry heat is allowed to act.

The most likely explanation, when we are dealing with chemical interaction between tissue and stain, is to assume that the side-chains (p. 187) of either interact, satisfying mutual desires. No interaction is supposed to take place when the dye stains physically, as will be explained more fully afterwards.

(*c*) Texture.

Ehrlich having conceived, in 1878–9, the idea that moisture is essential for staining was, of necessity, led to the view that loss of water or dehydration, as practised at ordinary temperatures, must produce a shrinkage of the proteid, and thereby a diminution in the size of the intermolecular spaces. Ehrlich had observed, firstly, that red blood-corpuscles, containing haemoglobin, have an affinity for all the members of the nitro-group of dyes (p. 383); secondly, that without having been heated the red corpuscles stain both in aurantia, which is a dye having a large molecule, $(NO_2)_3C_6H_2NHC_6H_2(NO_2)_3$, and diffusing therefore slowly, and also in picric acid, which has a much smaller molecule, $(NO_2)_3C_6H_2OH$, and diffuses rapidly; and thirdly, that the more highly the red corpuscles are heated the less they will stain with aurantia, till if heated up to 180° C. they will even cease to stain in picric acid. The explanation offered is that by dehydration the intermolecular channels become so close as to interfere at first with the entrance of the large-moleculed aurantia; and, finally, also with the diffusion into the corpuscle of the small-moleculed picric acid. That different tissues react differently to heat was shown by the fact that the *α*-granules of the eosinophilous granules still retained their power of being stained when the red corpuscles had already lost their power.

Similarly the *β*-granules in the amphophilous leucocytes of the rabbit were found to stain according to the amount of heat-fixation in a glycerin mixture of indulin, eosin, and aurantia, successively black, black-red, red, reddish-yellow, and finally pure yellow. The purely neutrophil or *ε*- granules of man, monkey, dog, and pig by overheating do not stain in the neutral tint of the triacid mixture (p. 220), but red, becoming pseudo-eosinophil; and from the neutrophil methylene-blue-eosinate mixture they also take up under these conditions the eosin radical[1].

[1] The best temperature for heating blood films is 120°–140° C. for one to two hours; as thereby the red blood-corpuscles are rendered quite insoluble, and the white corpuscles retain their staining reactions. Heating up to 160° C. causes the eosinophilous granules to fuse together into wax-like masses (Westphal).

How the texture of objects modifies the staining result is further shown by impregnating albumose granules with platinum chloride; —carefully washing out the fixative which was used;—reimpregnating the granules with new albumose solution during 20 hours, and then finally by precipitating the freshly introduced albumose inside the old granules with 1 per cent. chromic acid. The comparatively loose albumose-platino-chloride granules by this secondary impregnation and fixation become so dense, that using the Ziehl-Neelsen method for staining tubercle bacilli, the granules may now be left for five minutes in 5 per cent. sulphuric acid without becoming decolourized, while without impregnation they very quickly lose their stain (A. Fischer).

(*d*) The cubic capacity of objects.

The size or cubic capacity of objects to be stained is a factor which must never be lost sight of in staining, as has been done only too frequently in the past. Personally I had realized the importance of the size of objects quite early in 1890, while working at nuclear changes in plants, and had then arrived at conclusions similar to those which have been put forward by Raciborski[1], Heine[2], Galeotti[3], and Fischer[4].

Raciborski stated that staining depended—(1) on the method of fixation and subsequent manipulations; (2) on the nature and saturation of the dye, and the length of time it was allowed to act; and (3) on the texture of the tissue. He also stated that the double staining with an iodine-green and fuchsin mixture depended not on chemical reactions, but was due to differences in surface attraction.

Heine has similarly pointed out that only physical factors determine whether out of a mixture of methyl-green and safranin, this or that dye shows special affinities for certain tissue-elements; and Galeotti, speaking of acid fuchsin, says that in using the term 'fuchsinophil' he only means to imply that the acid fuchsin is taken up by certain elements owing to their special size or density.

Fischer[4] has made the most exact investigation into the physical causes of staining, and to his work I shall be constantly alluding when discussing the effects of physical staining; but I do not hold with him that all staining depends on physical factors, which latter he supposes to be much more subtle than the coarser chemical affinities (l. c., 119).

For his researches into the physical aspect of staining Fischer required granules varying greatly in their size, and obtained them in the following way: Osmium tetroxide, potassium bichromate, or Altmann's fixative are all neutral in their reaction and do not precipitate a 40 per cent. neutral albumose solution[5]. On acidifying

[1] Raciborski: 'Über d. Chromatophilie d. Embryosackkerne,' *Anzeiger d. Akad. d. Wiss. Krakau*, 1893.

[2] Heine: 'Die Mikrochemie d. Mitose, &c.,' *Zeitsch. f. physiol. Chemie*, 21 (1895).

[3] Galeotti: 'Beitrag z. Kenntniss d. Secretionserscheinungen,' *Arch. f. mikr. Anat.* 48 (1897).

[4] Fischer: *Fixirung, Färbung u. Bau d. Protoplasmas* (1899).

[5] W. A. Osborne has failed to get a 40 per cent. albumose solution, as he tells me. I have used a 10 per cent. solution for my experiments.

the neutral solution, however, a precipitate is formed, the nature and amount of which is determined by the quantity of acid added and the time the latter is allowed to act.

To obtain granules of different sizes Fischer proceeds thus: Add to 8 cc. of a very slightly acid almost neutral solution of 40 per cent. albumose solution, 16 cc. of a 2·5 per cent. solution of potassium bichromate, mix the two solutions well, and pour the mixture into a Petri-capsule, or any flat glass dish. After 24 hours the mixture will have become milky in appearance owing to the formation of very minute granules, but as yet no sediment is deposited. Now add 1 cc. of glacial acetic acid and mix quickly, when there is thrown down a heavy precipitate consisting of granules varying in size from 0·7 to 8 μ.

A second method, depending on the principle that a weak fixative forms minute granules while a strong fixative produces large ones, is as follows:—To 10 cc. of a 40 per cent. albumose solution add 1 cc. of 10 per cent. 'platinum chloride' (see p. 78), and mix well. After 24 hours a primary precipitate consisting of minute granules varying from 0·4 to 3 μ will be seen. Now add 10 cc. of 10 per cent. 'platinum chloride' to completely throw down all the albumose. The second precipitate formed in this manner consists of granules composed of the primary and secondary precipitates up to 10 μ in diameter, so that in the sediment granules are to be found of all sizes from 0·4 to 10 μ.

These sediments are then distributed on cover-glasses, the latter are allowed to dry, are then fixed by heat, and may be used directly after a slight rinsing in water to remove the excess of the fixative outside the granules, or may be thoroughly washed so as to extract from the granules any fixative which is not united to the albumose as a firm chemical compound. In many cases it is preferable to wash the precipitate as a whole and to make cover-glass preparations with the washed material. What use these cover-glass preparations are put to is fully described on p. 206.

(*e*) The importance of removing the fixative has long been recognized, because insufficient removal always leads to great difficulties being experienced in staining. Fischer, who represents the physical school of dyers, believes that the fixative, if not washed out, blocks the intermolecular spaces, and thereby prevents the dyes from entering. I believe that fixed tissues, after the removal of the so-called excess of the fixative, contain certain unsatisfied side-chains (p. 187) which bring about the union with the dyes, a view which will be discussed more fully later (p. 367); and also that these side-chains, as long as the fixative is present, are satisfied by it and therefore do not show any affinity for the stains. (For the interaction of stains and fixatives, see p. 256, where also the removal of the fixative is discussed.)

2. The state of the stain.

(*a*) The solubility of anilin dyes in water varies greatly, but no accurate determinations have been made up till now. Generally speaking basic dyes are much less soluble than acid ones. The least soluble amongst the basic dyes is fuchsin (solubility about 0·5 per

cent.), and the most soluble is methyl-green (solubility about 8 per cent.), while methylene-blue, with about 4 per cent., is intermediate. Solutions freshly prepared seem to be much more saturated than old solutions: basic fuchsin, especially, and also methylene-blue are apt to form sediments, although to begin with the solution may have been complete. Amongst acid dyes the least soluble is picric acid (solubility at 20° C. about 1 per cent.), and eosin has a solubility of at least 2 per cent., while orange G and acid fuchsin have respectively the solubilities of about 14 and 77 per cent., according to Paul Mayer (p. 221). All other sulphonic acids resemble acid fuchsin in also being extremely soluble.

(*b*) The saturation of dyes.

The saturation or concentration of dyes is a very important factor in physical staining, for it determines the depth of the stain, the amount of the diffusing dye being always proportional to its saturation.

On mixing two clear solutions of two dyes it does not follow that in the mixed solution the dyes will be in the proportion in which they were mixed, as either both or one of them may be partially or completely precipitated, or even, although in solution, may not be dissociated electrolytically. Whether precipitated or undissociated the dye in question will have its staining power and staining properties greatly altered.

Thus on mixing clear solutions of two basic dyes one may be partially precipitated, as is the case with basic fuchsin which is thrown down by both methylene-blue and methyl-green, or with safranin which has its solubility diminished by methylene-blue. On mixing, however, safranin and gentian-violet solutions, the latter is partially precipitated (Fischer). Analogously, on mixing the two acid dyes eosin and picric acid, the former is rendered partially insoluble, and mixing colour acids with colour bases leads, as will be mentioned repeatedly, to a precipitation of the neutral insoluble colour salt (see p. 442).

On adding an alum solution to a solution of eosin a yellowish-orange precipitate is thrown down, which the French school, as represented by Ranvier and others, believe to be the free colour acid, tetrabrom-fluorescein; in Germany, Fischer in special believes the eosin to be precipitated as such. I have made the following experiment, which shows Ranvier is in the right:—A watery 1 per cent. eosin was precipitated with a saturated alum solution; the precipitate was filtered off and washed in five changes of distilled water, the filtrate gradually becoming of a distinctly red colour, showing that the precipitate was slightly soluble in water. The sediment which remained on the filter was dried at 40° C., and when completely dry extracted with pure chloroform in which the same eosin, as a dry powder but not acted upon by alum, was quite insoluble. The chloroform was coloured a deep yellow tint with the eosin, which had been treated with alum, showing the presence of the free tetra-brom-fluorescein (pp. 214 and 429). When therefore Fischer gives eosin as an example of a dye which stains more deeply after the addition of alum. simply because it is rendered thereby less soluble,

he is mistaken, and consequently his physical explanations of staining with supersaturated solutions, as far as eosin is concerned, are wrong.

He seems, however, to be in the right when he gives indulin as an example of an acid dye which is rendered less soluble by the addition of sulphuric acid. It is possible to precipitate this dye inside the platino-chloride granules of thymus-nucleic acid, by allowing the dye to diffuse into the intermolecular spaces, and then, by the addition of sulphuric acid, to render the indulin insoluble and thus to cause it to be retained by the granules. Fischer adds to 19 cc. of 2 per cent. indulin solution, in the course of 10 minutes, gradually, 14 drops of 5 per cent. sulphuric acid. I find if this same procedure be used that very instructive preparations of areolar tissues can be obtained, resembling the well-known negative silver-nitrate specimens. Proceed thus:—Make a film of areolar tissue on a slide, fix it with alcohol, remove the superfluous alcohol with cigarette paper and cover quickly with a few drops of 2 per cent. indulin 19 cc. + 5 per cent. H_2SO_4 1 cc. Remove the dye in 10 minutes by rinsing in water and let the preparation dry spontaneously. In this case we are dealing with a purely physical staining effect, the dye being precipitated in all elements excepting the very dense winged connective tissue-cells.

(*c*) The rate of diffusion of dyes.

Griesbach[1] was the first to analyse the difference in the rate of diffusion between azo-blue and gold-orange by means of the diffusion apparatus commonly used in physical research: but the best and most thorough investigation we owe to Fischer[2], who chose gelatin as being a suitable medium for the study of the phenomena of diffusion, for in all colloid substances diffusion goes on at the same rate as in water[3]; and further, any salts which may be present in the gelatin also do not interfere with the travelling of the dye molecules, because one salt diffuses through the solution of another one, almost as quickly as in pure water[4]. A third law, formulated by Graham, must also be kept in mind:—From a mixture of salts each component will diffuse with its own specific speed, the faster one being slightly accelerated while the slower one is slightly retarded[5].

Fischer performed his experiments in the following manner:—A clear 10 per cent. gelatin, just perceptibly acid, or in other experiments very faintly alkaline, was poured over a number of slides in approximately the same thickness, and was allowed to solidify; neutral gelatin was not used because it is apt to become acid on its own account, and would therefore vitiate the experiment. Platinum wire 0·4 mm. thick was bent round a circular glass rod measuring 4 mm. in thickness so as to form a perfectly round loop,

[1] Griesbach: 'Weitere Unters. ü. Azofarbstoffe, &c.,' *Zeitsch. f. wiss. Mikrosk.* 3, 366 (1886).

[2] Alfred Fischer: *Fixirung, Färbung u. Bau d. Protoplasmas*, 122 (1899).

[3] Ostwald: *Die wissenschaftlichen Grundlagen d. analyt. Chemie*, 687 (1894).

[4] Ostwald: *Lehrbuch d. Chemie*, 1, 675.

[5] Ostwald: *ibid.*, 1, 692; Winkelmann: *Lehrbuch d. Physik*, pp. 616, 617; Walker: *Electro-chemistry* (1901).

which, not taking the menisci into consideration, holds about 5 cmm. of fluid; the dyes were employed in strengths of 0·5 per cent., and as differences in temperature greatly alter the rate of diffusion all the dyes were used in each set of experiments, the average temperature being 20° C.

By means of the platinum loop a drop of the stain was placed in the centre of the slide covered with gelatin; the slide was put at once under a bell-jar made air-tight with water so as to prevent the gelatin drying; and every two hours the total diameter of the coloured area was measured by placing the slide over a millimetre scale.

The alkaline gelatin above referred to was used for determining the rate of diffusion of acid dyes, such as eosin and the salts of the sulpho-acids, acid fuchsin and light-green. As sulpho-acids in the presence of alkalies become colourless, the amount of diffusion must be determined by holding the slide momentarily over the mouth of a bottle containing acetic acid, and thus restoring the colour, or a watch-glass with a few drops of acetic acid may be placed under the bell-jar containing the alkaline gelatin.

Taking the rate of diffusion of light-green as unity, the relative rate of diffusion of anilin dyes is as follows:—Indulin, 0·9; light-green, 1; acid fuchsin, eosin, fuchsin, safranin, methyl-green, methylene-blue, 1·3, and picric acid, 2·1.

By subtracting from the total diameter of the coloured area the diameter of the original drop, namely 5 mm., and dividing the remainder by 2, the absolute rate of diffusion was readily determined.

The following table calculated in this way indicates the distance, in millimetres, a 0·5 per cent. solution of a dye will travel every ten minutes, 2, 4, and 24 hours after commencement of the experiment. It will be noticed that the rate of diffusion gradually diminishes owing to a diminution in the concentration of the dye; that eosin at first diffuses more quickly than safranin, and that similarly methylene-blue also travels at first faster than do the other basic dyes, but that after 24 hours the rate has become much more uniform.

TABLE INDICATING THE DISTANCE COVERED EVERY TEN MINUTES BY A 0·5 PER CENT. DYE SOLUTION IN 2, 4, AND 24 HOURS.

0·5 *per cent. dye solution.*	2 *hours.* *mm.*	4 *hours.* *mm.*	24 *hours.* *mm.*
Indulin	0·08	0·06	0·02
Light-green	0·125	0·08	0·03
Acid fuchsin	0·17	0·125	0·05
Basic fuchsin	0·17	0·125	0·05
Safranin	0·17	0·125	0·05
Methyl-green	0·17	0·104	0·055
Eosin	0·208	0·125	0·05
Methylene-blue	0·208	0·125	0·045
Picric acid	0·38	0·25	0·1

Fischer has further determined the rate of diffusion on filter-paper,

a method which originated with Schönbein[1], and which was also employed by R. Hertwig[2] in 1892.

If solutions of acid dyes are dropped on filter-paper the rate at which the dye and the water diffuse is the same, but when a drop of a basic dye is taken then the dye will be noticed to spread only slightly, while the water, diffusing readily, forms a broad uncoloured rim round the coloured area. The reason assigned by Fischer to this phenomenon, is that capillarity produces a separation of the comparatively insoluble basic dye from its solvent, while it has no such effect on the more solid acid stains.

TABLE SHOWING THE AMOUNT OF CAPILLARY DIFFUSION IN FILTER-PAPER (AFTER FISCHER).

Dyes in 0·1 per cent. strengths.	*Observed.*		*Calculated, taking light-green = 1.*	
	total area.	*coloured area.*	*total area.*	*coloured area.*
1. Acid fuchsin	28	28	1	1
2. Light-green	30	30	1	1
3. Methyl-green	36	20	1	0·55
4. Fuchsin	36	16	1	0·42
5. Methylene-blue	33	15	1	0·45

Mixture of dyes.	*total area.*	*coloured area.*	*red dye.*	*blue or green dye.*	*Mixture of both dyes.*	
6. Light-green + acid fuchsin	33	33	1	1	33	33
7. Light-green + basic fuchsin	27	27	0·52	1	14	27
8. Acid fuchsin + methyl-green	29	29	1	0·5	29	15
9. Acid fuchsin + methylene-blue	26	26	1	0·5	26	13

In this table the acid and basic dyes in the mixtures Nos. 6, 7, 8, 9, are seen to differ greatly in their power of diffusion. In No. 6, composed of the two acid dyes light-green + acid fuchsin, each component spreads over the whole area, while the basic dyes in Nos. 7, 8, and 9, namely basic fuchsin, methyl-green, and methylene-blue, cover a much smaller area than do their acid companions.

(*d*) The staining power of dyes.

The staining intensity of a dye, or its power of producing a certain depth or saturation of colour, varies greatly. Professional dyers state that acid fuchsin, for example, produces tints only half the intensity of those obtainable with basic fuchsin[3]. To overcome this drawback free sulphuric acid is added to solutions of acid dyes, and should the result even then be wanting in uniformity—in other words, if the cloth is spotty—then sodium sulphate is used in addition to the acid.

Thus the sulpho-acids (acid fuchsin and light-green) are used in

[1] In Mierzinski's *Theerfarbstoffe*, Leipzig (1878).

[2] R. Hertwig: 'Über Befruchtung u. Conjugation,' *Verh. d. Deutsch. Zool. Gesellsch.* 2 (1892).

[3] Knecht, Rawson, and Löwenthal: *Handbuch d. Färberei d. Spinnfasern*, Berlin (1895).

baths containing 2 to 4 per cent. of pure sulphuric acid and 20 per cent. of sodium sulphate; picric acid also requires 2 to 4 per cent. of sulphuric acid, while indulin requires much less acid, and stains best if the acid is added very gradually during the course of the dyeing operation. When staining with eosin, it is customary to add 5 to 10 per cent. of alum.

Fischer has adopted these procedures to stain various granules, as, for example, nucleic acid precipitated with 'platinum chloride,' which otherwise would not stain with acid dyes. The conclusions he has based on these results are fully discussed in the chapter on the theory of staining (pp. 334–340).

The factors of time and temperature are incidentally referred to in the following pages while discussing the effects of staining.

B. When dealing with the physical aspect of staining in the following pages I have taken it for granted that the granules obtained by the methods described on p. 201 are chemically inactive; that they have the same chemical nature, and in consequence of this the same physical structure. As to the chemical nature of all the granules being the same there can be but little doubt, but the two other factors are by no means certain. Reasons for believing that the albumose-platino-chloride granules act chemically are discussed later, but now it will suffice to point out that the physical structure of quickly formed precipitates is in all probability looser than that of slowly formed ones; that large granules, therefore, are looser than small ones. The reader is, however, to forget everything except that there are given three factors, namely:

(α) A number of spherical granules varying in size, with their surfaces being to their cubic capacity in the same proportion as the square is to the cube of their diameter, thus—

Diameter . . .	1	2	3	4	5	6	7	8	9	10
Surface of granules .	1	4	9	16	25	36	49	64	81	100
Cubic capacity of granules	1	8	27	64	125	216	343	512	729	1000

(β) A number of dyes in more or less saturated solutions, and

(γ) Each dye having its own specific power of diffusion.

Effect of interrupting the Process, of staining with one Dye.

On placing a cover-glass with albumose-platino-chloride granules (p. 201), varying in diameter from 1 to 10 μ, into a solution of such basic dyes as methyl-green or methylene-blue[1], the small granules having a cubic capacity of 1 will be stained optimally long before the large granules with a cubic capacity of 1,000 will have been penetrated by the dye. In consequence of this, interrupting the staining process at an early period by taking the cover-glass out of the methylene-blue solution and rinsing it in water to remove the superfluous dye, drying it and mounting directly in balsam, will result in leaving the small granules stained deeply, while the large ones are pale.

If the stain is allowed to act till the large granules are impreg-

[1] Albumose-platino-chloride granules have little affinity for acid dyes (Fischer).

nated throughout their thickness with the dye, then, after rinsing in water, drying and mounting in balsam, the large granules will appear almost black, the depth of the staining being proportional to the diameter of the granule, while even the smallest granules will be of a deep-blue colour.

Effect of Removal of Dye.

On endeavouring to remove the stain from the granules either by purely physical or by chemical means, the methylene-blue is extracted from the small granules much more readily than from the large ones. In consequence, the small granules, being uncoloured, might escape our notice if during a microscopical investigation we were in the habit of studying only coloured structures.

Physical Double Staining.

Suppose, after being stained as deeply as possible in methylene-blue, we firstly remove the colour from the small granules, or 'differentiate' and then stain the preparation in a red basic dye such as fuchsin, we should obtain the small granules stained red, while the big ones would be practically pure blue, provided the staining with fuchsin was interrupted at a time when the minute granules were already fully impregnated while the big granules had not yet been invaded to any extent by the fuchsin.

The fact of not having taken into consideration the physical aspect of staining has led many observers to describe the existence of chemically different substances, while in reality they were dealing with the same chemical compound occurring in differently sized aggregates. Fischer, following the example set by Raciborski, Heine, and Galeotti (p. 200), has in this respect done good in drawing attention to the importance of the physics of histology.

Formation of Bull's-eyes.

There is still another point which Fischer has made very clear, namely, the production of 'bull's-eyes,' or as he terms them, Spiegel-bilder[1]. Given a cover-glass preparation of albumose-chromate (p. 201) fully stained in acid fuchsin, it is possible, by differentiating with picric acid, according to Altmann's method, to obtain at a certain time this picture: All small granules are yellow, because the picric acid has turned out the fuchsin and has usurped its place; the middle-sized granules are essentially yellow, but with a minute red spot in the middle, owing to the fact of the picric acid not having reached as yet to the centre and the fuchsin not having as yet diffused out, while the very large granules have a broad red centre surrounded by a narrow yellow rim, resembling the bull's-eye in a circular target.

By using picric acid first and acid fuchsin secondarily, the colour effects just stated cannot be reversed, but it is possible to get the reverse effect by the use of a mixed solution of picric acid and acid fuchsin, as will be described later on (pp. 208 and 209).

On the same principle on which the two acid dyes, picric acid and acid fuchsin, interact, it is possible to use two basic dyes. We may, for example, stain albumose-chromate granules by means of Flemming's

[1] The German word 'Spiegel' means mirror, but also a target with its bull's-eye.

safranin acid-alcohol gentian-violet method in such a manner as to obtain the big granules red and the little ones violet. In this case by using gentian-violet first and safranin subsequently, the colour-effect is reversed, the big granules being now violet, while the little ones are red.

Whenever differentiation comes into play we must beware of the pitfalls opened up by physical causes; for not only do we get into difficulties with anilin dyes, but also with various haematein and carmin staining methods. These, when followed by chemical differentiation, are liable to make objects appear smaller than they are in reality. It is the realization of this fact which makes me insist on the examination of unstained specimens and the examination of preparations in mounting media differing in their refractive indices (p. 337).

By the 'covering-power' of a dye we mean its ability to hide from our eyes the presence of another colour; thus picric acid cannot cover light-green or fuchsin, but light-green to a considerable extent hides the red colour of fuchsin. The importance of the covering-power of different dyes will become more apparent immediately.

Effect produced by staining with a mixture of Dyes.

By interrupting the process of diffusion into the tissue and thus producing simultaneous double staining, with

1. Homogeneous mixtures of (α) acid and (β) basic dyes.
2. Heterogeneous mixtures of acid and basic dyes.

1 (α). A picric acid + acid fuchsin mixture allowed to stain albumose-chromate granules for six minutes, produces according to the relative and absolute concentrations of the two colour acids the results given in the accompanying table, which has been abridged from Fischer's book, p. 135. In all the experiments the cover-glasses at the end of the staining process were rinsed in water to remove the superfluous dye, then dried and mounted in balsam.

TABLE SHOWING FISCHER'S METHOD OF PRODUCING PHYSICAL DOUBLE STAINING.

No.	*Relative concentration.*		*Absolute concentration.*		*Albumose-chromate granules.*	
	Picric acid.	*Acid fuchsin.*	*Picric acid.*	*Acid fuchsin.*	*Large granules.*	*Small granules.*
1	1	8	0·055	0·44	deep orange	red
3	1	2·5	0·14	0·36	bull's-eye golden yellow, margin red	red
6	1	1	0·25	0·25	yellow with margin showing traces of red	red, frequently with yellow bull's-eye
9	1	0·5	0·33	0·17	completely yellow	faintly red, yellow bull's-eye
11	1	0·5	0·1	0·05	yellow bull's-eye, margin red	yellow bull's-eye, margin red
13	1	0·25	0·13	0·03	completely yellow or with red margin	red
15	1	0·17	0·15	0·025	completely yellow	only the minutest granules still red, all others yellow

The relative rate of diffusion of picric acid and acid fuchsin being as 1·6 to 1, the granules will be occupied by the picric acid long before the red dye has had time to diffuse inwards, even if there be present eight times more fuchsin than picric acid. It is for this reason that the granules are stained a deep orange tint in No. 1. In No. 3 the red margin, or, to be more accurate, the red mantle covering the central yellow core, indicates that the picric acid, although only in the proportion of 1 to 2·5 of the fuchsin, has diffused right through the whole thickness of the granule at a time when the acid fuchsin just commenced its invasion. In No. 9, containing only one-half the amount of acid fuchsin, the large granules after six minutes are still completely yellow, while the small ones show already an indication of the slower acid fuchsin making its way into the albumose-chromate. The appearance presented by No. 13 is the exact reverse of that produced by staining according to Altmann's method, who uses, first, acid fuchsin and subsequently picric acid.

Results analogous to those just described were obtained by Fischer with mixtures of picric acid + light-green, acid fuchsin + light-green, acid fuchsin + indulin, and eosin + light-green.

To obtain physical double staining with these mixtures is only a question of properly apportioning the relative and absolute concentrations of the two colour acids, and of interrupting the staining process at the proper time.

No.[1]	*Relative concentration.*		*Absolute concentration.*		*Albumose-chromate granules.*	
	Picric acid.	*Light-green.*	*Picric acid.*	*Light-green.*	*Large granules.*	*Small granules.*
1	1	16	0·03	0·47	all green	all green
2	1	12	0·038	0·46	yellow bull's-eye, green margin	green
3	1	8	0·055	0·445	pure yellow	green, frequently with yellow bull's-eye
6	1	0·25	0·13	0·03	pure yellow	bright green
	Acid fuchsin.	*Light-green.*	*Acid fuchsin.*	*Light-green.*		
5	1	4	0·1	0·4	deep red	bluish-green
	Acid fuchsin.	*Indulin.*	*Acid fuchsin.*	*Indulin.*		
1	1	15	0·017	0·25	deep red, often with blue margin	deep steel-blue
4	1	1	0·05	0·05	red	indulin colour
	Eosin.	*Light-green.*	*Eosin.*	*Light-green.*		
1	1	19	0·025	0 475	red with greenish-blue shade	bluish-green
2	1	19	0·005	0·095	deep red, often with green margin	green or bluish-green
5	1	1	0·05	0·05	red	red

[1] The numbers on the left side refer to Fischer's original tables.

Homogeneous mixtures of basic dyes.

The first to draw attention to the danger of mistaking physical staining effects for chemical ones in using these mixtures was Heine (p. 347). Albumose-platino-chloride granules (p. 201), stained with ordinary commercial methyl-green, exhibit the large granules stained green, while the small and very small granules have a distinct violet shade, owing to the presence of traces of methyl-violet in the methyl-green. As stated on p. 424, methyl-green may be obtained pure by shaking out the methyl-violet with amyl alcohol.

To obtain albumose-platino-chloride granules doubly stained with a methyl-green + basic fuchsin mixture Fischer had to mix 10 cc. of a 0·5 per cent. purified methyl-green and 3 drops of a freshly prepared 0·1 per cent. fuchsin (= 0·14 cc. of fuchsin of 0·1 per cent., for 21 drops = 1 cc.). It requires therefore 357 parts of methyl-green to 1 part of fuchsin to stain the large granules green and the small ones red. If instead of three drops six drops, or double the stated quantity of the 0·1 per cent. fuchsin, are taken, then no double staining results, as the fuchsin, possessing much greater staining power, makes all granules appear red.

Watching the process of staining under the microscope reveals that both big and small granules are at first stained green, and that only secondarily the small granules turn red within 8 minutes. As the rate of diffusion of equivalent amounts of the two basic dyes in question is the same, the physical double staining is produced by the great differences in their concentrations, the more concentrated methyl-green with its higher osmotic pressure diffusing, proportionately to its concentration, more quickly.

A methylene-blue + fuchsin mixture stains analogously to the methyl-green + fuchsin mixture.

That colour base, which is in the greatest amount in the solution, on entering the granules in proportion to its concentration will at once take possession of the intermolecular spaces. The larger the granules are, the more difficult will it be for the other colour base, present only in small amount, to exert its physical rights within a given time; for it is not only a question of the numerically weaker dye, with its low tension, diffusing into the granule, but also of the numerically stronger dye already in possession of the granules diffusing outwards against its own high tension, to make room for the molecules of the other dye present.

The smaller the granule the greater is its surface relative to its bulk, and for this reason the more readily will it be entered by the colour base present in the lesser concentration, as is shown by the observation that although all the granules stained by the above mixture are blue to begin with, the small granules very soon assume the red colour. Why the red granules are redder than the mixture itself Fischer does not say, but it appears from his views expressed in other parts of his book that he explains the fact as due to 'specific mechanical attractions.'

As the result of the experiments just stated, Fischer has arrived

at the conclusion that every double staining in histology can be simply accounted for by differences in the rate of diffusion of the dyes and their relative and absolute concentrations: but he has completely overlooked the very important factor of time. Although it is possible to produce the different colours in granules, as indicated in the above tables, by staining for 6 to 8 minutes, it is impossible to get the same results by staining cover-glasses for 30 minutes, far less allowing them to remain in contact with the dyes for 24 hours. For example, albumose-chromate granules left for 24 hours in any of the mixtures of picric acid and acid fuchsin given in the table on p. 208, are all stained red, because within this time the acid fuchsin, notwithstanding its slower rate of diffusion, gradually makes its way through all the granules and 'covers' the yellow picric acid stains, if it does not also force the picric acid out, at least partly.

Being convinced that the results obtained under very special circumstances are generally applicable, Fischer has left the study of the different granules of leucocytes to medical men (l. c., 96).

If instead of doing this he had used the acidophil mixture of Ehrlich (p. 217), and had stained red-marrow films of rabbits and his albumose granules for 24 hours, he would have found the latter all stained uniformly while the different leucocytes would have been stained differentially.

Another point which has not been taken into account by Fischer in making his generalizations is the enormous difference in the absolute and relative size of the albumose (1 to 10 μ) and the leucocyte (0·25 to 2 μ) granules. He has endeavoured to explain also on purely physical grounds the staining of tubercle bacilli by the Ziehl-Neelsen method, but has overlooked the relative size of the objects to be stained. Thus albumose granules stained red by this method have at least five times the diameter of the small granules which were counter-stained, secondarily, in methylene-blue. If, however, a mixture of tubercle bacilli and the ordinary concomitant bacteria are stained in exactly the same way, the very opposite result is obtained, for the more slender tubercle bacilli are red, while the more bulky remaining organisms are blue. It is, however, still possible to explain the tubercle staining on purely physical grounds by assuming the tubercle bacilli to have a very dense structure, similar to that of albumose granules reimpregnated with albumose and fixed for a second time (see p. 200). The effect of dissolving dyes in such media as will physically alter the texture of the tissue has already been referred to on p. 198 (see also p. 212). Later the question of the co-efficient of solutions will be discussed (pp. 329, 331).

CHAPTER XVIII.

Chemical Methods of Staining.

A chemical union between dye and tissue cannot take place till these two have been brought together by such physical means as have been discussed in chapter xvii, and even then in many cases, to ensure

a chemical union, the addition of certain substances to the dye-bath or staining fluid becomes essential. These substances are not true mordants, as they do not participate in the union between the tissue and the stain, but by acting in some not always explainable manner on the material or the stain, they thereby produce or accentuate a chemical tendency.

Such substances, which, for want of a better term, we may call accentuators, are either bases or acids.

The chief basic accentuators are :

1. Caustic potash. It was first used by Koch in the staining of tubercle bacilli with methylene-blue. *Koch's methylene-blue* solution consists of a saturated alcoholic solution of methylene-blue 1 cc., 10 per cent. caustic potash 0·2 cc., and water 200 cc. *Löffler*, instead of using a 1 per cent. caustic potash solution, used 1 : 10,000 solution, but for general bacteriological as well as histological purposes a 1 : 1,000 solution answers best, especially if the staining fluid contain 25 to 50 cc. of alcohol. (Löffler's solution, according to Behrens' tables, contains: methylene-blue 0·5 grm., alcohol 30 cc., caustic potash (1 : 1,000) 100 cc.)

2. Borax or sodium pyroborate, $Na_2B_4O_7$, is generally used in strengths of 1 per cent., as in *Unna's borax methylene-blue*: methylene-blue 1 grm., borax 1 grm., water 100 cc.,—or in *Harris's toluidin-blue.*

3. Anilin (amido-benzene or phenylamin), $C_6H_5—NH_2$, acts as a base because of the amido group NH_2. *Ehrlich's bacterial stain* consists of gentian-violet 1, alcohol 15, anilin 3, and water 80 parts. Anilin is also used when staining according to *Gram's method* (p. 223), the anilin water being obtained by dissolving 3 grms. of anilin in 100 cc. of water, by shaking, or by taking a larger quantity of anilin, shaking and then filtering.

4. Pyridin is a tertiary amin and a strong basic substance of the formula $C_5N + H_5$, the 5 carbon and 1 nitrogen atoms forming a ring analogous to the benzene ring; the 5 hydrogen atoms are attached to the carbon atoms, while the N has an unsatisfied affinity. As watery solutions are strongly basic, turning litmus paper blue, the N unites probably with a hydrogen ion derived from the hydrolysis of the water, there being formed the an-ion $C_5N + H_6^{\cdot}$ and the kat-ion OH'.

5. Quinolin, C_9H_7N, has a constitution analogous to that of naphthalene (p. 181), consisting of a benzene ring linked on to a pyridin ring.

6. Methylal, $CH_2(OCH_3)_2$, introduced by Ehrlich and Lazarus (*Die Anaemia*, see also the English edition, translated by W. Myers, Cambridge, 1900), is a pleasant-smelling liquid, boiling at 42° C., readily soluble in water and easily decomposed by oxidizing agents into its constituents formaldehyde OCH_2 and methyl alcohol $2CH_3OH$. How it acts is not known, but in all probability the formaldehyde radical is the active principle.

The *Ehrlich-Lazarus formula* is as follows :

Use a freshly prepared mixture of the reagents stated below, because fresh solutions stain far more intensely and more variously

than do older ones. The blood-films should have been fixed very carefully by heat (see footnote, p. 199):

1 per cent. watery eosin	10 cc.
Methylal	8 cc.
Saturated watery solution of methylene-blue .	10 cc.

Unna's combination of caustic potash and anilin for staining plasma-cells fixed in alcohol: Methylene-blue 1 grm., caustic potash 0·05 grm., and water 100 cc. diluted with 10–100 cc. of anilin water.

7. Soap has been used in combination with methylene-blue by *Nissl* for staining the basophil granules of nerve-cells. Sections of material fixed in alcohol are placed in a watch-glass, covered with a stain composed of 15 parts methylene-blue and 7 parts Venetian soap in 4,000 parts of water; the stain is next heated up to from 65 to 70° C., and afterwards the sections are differentiated in a mixture of pure anilin (1 part) and 96 per cent. alcohol (9 parts).

8. Formaldehyde (p. 88) has also repeatedly been used to increase the staining power of anilin dyes, but as in no case precautions were taken to use neutral formaldehyde it is difficult to know how in these cases the reagent acted; because pure neutral aldehyde by itself has a distinct action on the tissues, and so has the free formic acid always present in commercial formol.

The chief acid accentuators are[1]:

1. Acetic acid (p. 73), first employed by Ehrlich for staining mast-cells specifically. *Ehrlich's solution* consists of glacial acetic acid 12·5 cc., absolute alcohol 50 cc., water 100 cc., and dahlia up to nearly saturation. The use of acetic acid in combination with methyl-green is described on p. 215. *Friedländer* uses acetic acid for staining the capsules in bacteria; for example the pneumococcus, by either combining the acetic acid with the stain (fuchsin 1 part, glacial acetic acid 2 parts, alcohol 5 parts, water 100 parts), or first staining with a 2 per cent. watery solution of gentian-violet and subsequently differentiating with 1 to 2 per cent. acetic acid.

2. Carbolic acid or phenol (pp. 181, 417) is used especially for staining tubercle bacilli by the *Ziehl-Neelsen method.*

3. A combined acetic acid and phenol staining is obtained by the following *bacteriological method*, which I have seen employed at Oxford: Thionin 1 grm., phenol (1 : 40) 100 cc., is to be diluted for use with 3 parts of water and to be filtered. Stain five minutes or upwards. Wash very thoroughly in water to prevent a deposit of crystals and then decolourize in glacial acetic acid 1 cc. and water 250 cc.

4. Oxalic acid (p. 86) is used in *Weigert's fibrin method*: Make a hot saturated solution of methyl-violet in 75 per cent. alcohol, allow the solution to cool and pour off the alcohol from the undissolved methyl-violet. To 100 cc. of this alcoholic solution add 5 cc. of a 5 per cent. watery solution of oxalic acid.

The conversion of pseudo-acids and pseudo-bases into real acids and bases by chemical action is discussed in the chapter on the theory of staining (p. 337).

[1] Sulphuric acid is discussed on pp. 203, 205, 332, 364.

I. Staining methods requiring no differentiation.

A. Direct staining with single dyes.

With colour acids.

1. *The Mylius-Ehrlich reaction for determining alkalinity.* F. Mylius, in 1889[1], described a method for determining the alkalinity of a given glass by means of 'Jodeosin.' This substance (see p. 429) iodine-eosin, according to its name, is the free colour acid radical tetraiodo-fluorescein, its sodium salt being called erythrosin. To obtain the free acid add a few drops of either strong acetic or hydrochloric acid to a test-tube full of one per cent. or stronger erythrosin solution, when the orange-coloured free acid, being insoluble in water, is precipitated. Filter off the precipitate, wash it repeatedly in distilled water to remove all traces of the acid which was added, dry the precipitate and make an ethereal or chloroform solution[2].

Wherever the free acid comes in contact with a base the deep-red salt erythrosin is formed, which, being insoluble in ether or chloroform, remains in situ.

To obtain the reaction proceed thus: Carefully clean slides, cover-glasses and all other glass vessels to remove the alkali with which all are coated. The tissue, film, membrane or section, fixed either by drying or in absolute alcohol and quite free from water, is immersed in the ethereal solution of iodine-eosin and left for five minutes. The preparation is now transferred to some fresh ether and washed to remove all free colour acid, leaving because of its insolubility the coloured salt in the tissues. Finally, transfer the tissue to a slide, or, if already on a slide, cover it at once with neutral balsam (see p. 378), and not with ordinary acid balsam, as otherwise the stain disappears.

Ehrlich, to whom we owe so much, was the first to apply the Mylius reaction to histology, showing that the cytoplasm of leucocytes and the blood platelets stained deeply, while the red blood-corpuscles and the nuclei of the white corpuscles remain colourless[3]. Three years ago I demonstrated the basic nature of pure albumin crystals prepared by the Hofmeister-Hopkins reaction (see p. 124), and also examined different tissues of a rat which had been fixed by the injection of absolute alcohol. Since then A. C. Hoff, following the advice of Ehrlich, has investigated the distribution of alkalies in plant tissues[4].

2. *Pappenheim* states that benzylated Nile blue BB is even a more delicate test for the presence of alkalies than iodine-eosin.

3. *Weigert's Bismarck-brown* method[5]. Personally I like Bismarck-

[1] Mylius: *Ber. d. deutsch. chem. Gesellsch.* 22, 310 (1889).

[2] On adding equimolecular quantities of any acid the washing-out process is obviated, but the above procedure is readily performed. Iodine-eosin may further be bought from Grübler and Co., or Alexander Frazer in Edinburgh.

[3] Ehrlich and Lazarus: *Die Anaemie*, Wien (1898).

[4] A. C. Hoff: 'Topik d. Alkalivertheil. in pflanzl. Geweb.,' *Bot. Centralbl.* 83 (1900).

[5] Weigert: *Arch. f. mikr. Anat.* 15, 258 (1878).

brown greatly, as it does not overstain and as it forms a good contrast with haematoxylin. It is especially useful for staining the mucus-cells of the intestine and the basophil cytoplasm of the lymphocytes in lymph follicular tissue. Its chief disadvantage is that watery solutions are apt to form a precipitate, and therefore one-half to 1 per cent. solutions in 25 per cent. methyl alcohol should be used. The chemistry of Bismarck-brown is complicated and is given on p. 390.

4. Carnoy's method about to be detailed, strictly speaking, does not belong to this class at all, but should be given under II (p. 222), for the methyl-green is employed with an excess of acid, which latter prevents it from acting as it would do if it were employed by itself. In reality we are differentiating, only the processes of staining and differentiation go hand in hand. This method is therefore not the counterpart of the Mylius-Ehrlich reaction (p. 214).

Carnoy's methyl-green acetic acid method for nuclei. Various proportions of the dye and the acid are given, but a half per cent. methyl-green in 1 per cent. acetic acid will be found useful for fresh tissues (see below). The acid serves to kill the cell and also prevents the cytoplasm from staining. *Mathews*[1] objects to the use of the acid, but he is not dealing with living cells. *Lee's* plan[2] has the advantage of giving more permanent preparations. It consists in fixing the fresh tissues for not more than one-half to two minutes in the vapour of osmic acid; in staining for five minutes in the methyl-green acetic acid mixture; mounting in a drop of Ripart and Petit's mixture[3], and fixing the cover-glass with a ring of balsam. Another method which is also given, consists in teasing a piece of tissue directly in Ripart and Petit's mixture to which some osmium has been added, and then staining in methyl-green.

The principle of using a basic dye either by itself or in conjunction with acids is also applicable to tissues which have been previously fixed by any of the usual methods.

5. *Toluidin-blue acetic acid* mixture. Strongly basic dyes, such as toluidin-blue, must be used in acid solutions if only nuclein-compounds are to be stained. It is thus possible to obtain a sharp and precise staining of nothing but the nuclei, or nucleo-proteids such as Nissl's granules in nerve-cells. The amount of acid to be added is determined by the fixing method which was previously used. I prefer toluidin-blue to all other basic dyes of the thiazin group (p. 403).

B. Direct staining with several substances applied simultaneously.

(*a*) Colour acids:

[1] Albert Mathews: 'A contribution to the chemistry of cytological staining,' *American Journal of Physiology*, 1 (July, 1898).

[2] Lee and Mayer: *Grundzüge d. mikrosc. Technik*, Berlin, 295 (1898).

[3] The mixture of Ripart and Petit, according to Carnoy, *Biol. cellulaire* (1884), p. 94, consists of camphor water not saturated, 75 parts; distilled water, 75 parts; glacial acetic acid, 1 part; copper acetate, 0·3 part; copper chloride, 0·3 part.

6. *Ranvier's picro-carmin* stain, containing the two colour acids, carminic and picric acid (see p. 246).

7. *Mann's* eosin-methyl-blue[1] mixture (1892[2]).

The chemistry of methyl-blue and eosin is discussed on pp. 426 and 429.

The stain contains two colour acids in the following proportions:

1 per cent. methyl-blue in distilled water . .	35 cc.
1 per cent. eosin in distilled water	45 cc.
Distilled water	100 cc.

The proportions are chosen in such a manner as to avoid precipitation of the methyl-blue[3], for the latter has in this respect as well as in some others certain resemblances to colour bases such as methylene-blue, although constitutionally it is an acid dye. Eosin also in some respects approaches basic dyes, and therefore my mixture would lead one to expect marked peculiarities in its staining properties, and this is fully borne out.

Sections may be stained in the above mixture either by the 'short' or the 'long' method.

The 'short' method consists in placing sections from water into the staining mixture, and leaving them there five to ten minutes according to the special effect desired and also according to the thickness of the section and the method of fixation.

The methyl-blue component is insoluble in alcohol, and therefore it is essential to rinse sections in water, both before placing them in the staining bath and also after the staining has been completed.

Sublimate sections, after the removal of the mercury by means of iodine + potassium iodide and rinsing in water, may be treated with the eosin-methyl-blue mixture while still yellow with iodine,—or the iodine may be removed with alcohol, but on no condition with ammonia or alkalies. All chrome-fixatives, as for example Zenker's solution or my chromic acid + sublimate mixture, can be specially recommended in connexion with this stain. The white fibrous connective tissues and mucus-cells are stained a deep-blue colour and more precisely than by any other method known to me.

Fresh tissues are best stained by diluting the methyl-blue-eosin mixture with ten times its bulk of distilled water.

This method has been used during many years by me for class purposes, as it is certain in its action, and as the preparations are permanent.

The 'long' method of using methyl-blue-eosin[4] comprises staining sections for twelve to twenty-four hours; rinsing in distilled

[1] Not methylene-blue.

[2] In our joint paper 'The Histology of Vaccination,' by S. Monckton Copeman and Gustav Mann, Local Government Board, *Annual Report of the Medical Officer* (1898-9), 509, I have called the mixture a 'biacid' mixture, which I herewith retract because Ehrlich has used the term 'triacid' in an entirely different manner (p. 221).

[3] It may happen that after some months a slight blue precipitate is formed which may be neglected.

[4] I thought it best to describe this method here, instead of later, under the heading of indirect staining.

water for half a minute; thoroughly dehydrating with absolute alcohol and differentiating in a vessel containing caustic alcohol prepared as follows: to absolute alcohol 30 cmm. are added five drops of a one per cent. solution of caustic potash in absolute alcohol[1]. The reason for dehydrating and differentiating in absolute alcohol is to prevent the caustic from acting on the cell.

After the sections attached to slides by my albumin-method (p. 373) have turned a reddish tint in the caustic alcohol, withdraw the slide, wash it very carefully in absolute alcohol to remove the alkaline alcohol, and place it in distilled water to discharge the eosin from all the tissue-elements except the red blood-corpuscles which stand out bright red, and the neutrophilous leucocytes and nucleoli which remain purple.

Should the blue colour not be deep enough, then it may be intensified by transferring the slide to a vessel containing two drops of glacial acetic acid to 100 cc. of water. I know of no better method for showing the chromatin-segments or nuclein-constituents of the nuclei of nerve-cells, gland-cells and connective tissue elements, although the methyl-blue does not fix on to the nucleic acid radical. The red reaction obtained with red corpuscles, especially after picro-corrosive fixation, is so distinct that even with the A lens of Zeiss every corpuscle is readily seen. Many years ago I noticed that the same red reaction is obtained with the blood-vessels of the octopus and Loligo. The ganglion stellatum, for example, appears as if injected with carmin-gelatin; and I am told that my long method is being used in Liverpool for mapping out the circulatory system of fishes, because of the readiness with which blood-corpuscles are traced. Haemoglobin and haemocyanin occurring in muscle, especially the striped variety, explains why, after the long method, muscle is also stained red.

8. *Ehrlich's acidophil mixture* 'C,' containing three colour acids, namely eosin, aurantia, and indulin (or nigrosin), in the proportion of two grams of each of the three salts in thirty grams of glycerin (according to Grübler after Lee). Ehrlich stains blood-films in this mixture, to obtain the acidophil or α-granules stained red by eosin; the haemoglobin stained orange with the aurantia; the amphophile or β-granules and nuclei stained bluish-black with the indulin. Nikiforoff[2] and Lee use the stain for sections of tissues fixed in Flemming's solution. They stain sections for twenty-four hours.

Staining by the formation of additive compounds.

The methods of Max Schultze (9), Daddi (10), and Michaelis (11), for staining the oleic series of fatty compounds are given on pp. 81 and 307. What is common to all these methods is the formation of additive compounds.

[1] To 100 cc. of absolute alcohol add 1 grm. of solid caustic potash: place the bottle on the top of an incubator to set up diffusion currents in the alcohol, and after twenty-four hours filter the alcohol to remove the KOH which has not been dissolved. Although called a 1 p. c. caustic alcohol the solution does not contain 1 grm. of KOH in 100 cc. of alcohol.

[2] Nikiforoff: *Zeitsch. f. wiss. Mikr.* 8, 189 (1891), and 11, 246 (1894).

(β) Direct staining with colour bases.

12. *Ehrlich has used combinations* of any two of the following dyes: basic fuchsin, methyl-green, methyl-violet and methyl-blue, and has even made a mixture containing the three colour bases: basic fuchsin, Bismarck-brown, and chrome-green.

13. *Ehrlich's Janus-green* or diethyl-safranin-azo-dimethyl-anilin (p. 444) may also be mentioned amongst colour bases, as it contains the basic azo-group and also the basic safranin group. It stains nerves intravitally[1], and has been used extensively by Michaelis for staining living gland-cells[2].

14. *Gibbe's double stain* is prepared as follows: Triturate magenta (i. e. fuchsin) two grams and methylene-blue one gram, then add slowly a mixture of anilin oil three cc. dissolved in rectified spirit (i. e. 84 per cent. alcohol) fifteen cc., and finally add distilled water fifteen cc.

(γ) Direct staining with colour salts.

This group includes some of our best staining media, and in it should be placed, theoretically, only those dyes which contain colour acids joined to colour bases in correct molecular proportions.

The idea of uniting colour acids to colour bases with the view of obtaining 'neutral' colour salts originated with Ehrlich in 1879[3], and led him to the discovery of the neutrophil or ϵ-granules in the leucocytes of the human blood. As examples of 'neutral dyes' or colour salts, Ehrlich mentioned rosanilin picrate, methylene-blue + acid fuchsin and methyl-green + acid fuchsin.

These colour salts being insoluble in water, one of the following plans must be adopted to render them soluble: (1) add an excess of the acid dye (Ehrlich), or the basic dye (Rosin); (2) dissolve salt in pure methylic alcohol (Jenner), or (3) procure a temporary solution by prolonged boiling (Laurent).

The relative merits of these methods are not easy to determine. Ehrlich's procedure of dissolving the colour salt in an excess of the colour acid may be perhaps less exact, for the following reason: it is possible to suppose that the excess of the acid radical might favour the corresponding radical in the colour salt; thus if methylene-blue eosinate is rendered soluble by adding a trace of free eosin, it might be argued that the sections will tend to stain to a greater extent in eosin than they would if no excess of eosin had been added.

On the other hand, I believe it is also possible to argue that the eosin added in excess has no action whatever on the tissue, provided it is only added in such quantities as to just render the insoluble methylene-blue eosinate soluble. In this case the whole of the energy of the eosin, added in excess, is employed in converting the non-dissociated colour salt into the dissociated colour-ions, on the same principle that the slight ionization of perfectly pure distilled water is

[1] P. Ehrlich: *Sitzungsb. d. Vereins für innere Medicin* (1 Dec. 1898).
[2] Michaelis: *Arch. f. mikr. Anat.* 55, 558 (1900).
[3] P. Ehrlich: 'Methodologische Beiträge z. Physiol. u. Pathol. d. verschiedenen Formen d. Leucocyten,' *Zeitsch. f. klin. Medicin*, 1.

probably the factor which produces both electrolytic and hydrolytic dissociations. In practice I have been unable to detect any difference between the results obtained by Ehrlich's or Laurent's methods, as long as both stains were used in saturated solutions.

15. (*a*) *Ehrlich's 'neutral' mixture of methylene-blue + acid fuchsin.* It is used for blood-films which have been fixed for one to two hours by careful heating at 120° C. The neutrophil or ϵ-granules of leucocytes are stained violet.

(*b*) *Romanowsky's* methylene-blue eosinate method. The preparation of the dye is given on p. 441.

(*c*) *Jenner's* method, originally devised for blood-corpuscles, may also be used for sections. The staining solution (p. 442) is used in this way: a thoroughly clean cover-glass (p. 371) is covered with a thin film of blood, which is allowed to become air-dry. Cover the film with seven drops of the methylene-blue eosinate solution; invert a watch-glass over the preparation; after one to three minutes pour off the stain; rinse in distilled water; dry in the air and mount in balsam.

(*d*) *Laurent* stains sections in the following way: The bottle containing the methylene-blue-eosin precipitate and the mother liquor (p. 442) is thoroughly shaken till the precipitate forms a suspension. A given quantity of this mixture is then diluted with four times its bulk of distilled water, and is boiled vigorously for five minutes over a Bunsen burner.

The solution is then cooled under the tap, and cover-glasses or slides are placed in the still warm fluid for half an hour and up to, but not exceeding, five to six hours. The preparations after rinsing in 96 per cent. spirit are differentiated in absolute alcohol. It is imperative to take absolute alcohol, as otherwise methylene-blue is extracted more quickly than the eosin. Two to 10 minutes' differentiation is, as a rule, sufficient, but it may be prolonged up to six hours. After formalin fixation in celloidin sections the following substances are stained red:—collagen (white fibrous connective tissue), the stratum corneum, and eosinophilous granules. The fibrillar structures in the protoplasm are blue, while the stroma is distinctly redder; the cells at the base of Lieberkühn's glands are red, but blue higher up. The theoretical views of Laurent are discussed on p. 340.

(*e*) *G. Maurer's method*[1]. To what an extent the exact relationship of methylene-blue and eosin comes into play, how readily slight differences in the excess of one of the dyes modify the result, and above all what important factors the age of the methylene-blue-eosin mixture and the duration of the staining are, will become apparent on studying G. Maurer's paper on malaria parasites. The best results are obtained by using a 1 per cent. solution of the medicinal methylene-blue from Höchst in 0·5 per cent. soda solution, which has ripened for one month, and a 1 : 1,000 eosin w.g. from Grübler and Hollborn. To stain blood-films (or sections, especially after fixation in alcohol, Mann) dilute 1 cc. (= 15 drops) of the above methylene-blue solution with 25 cc. of water; similarly dilute 1 cc. (= 15

[1] G. Maurer: 'Die Tüpfelung d. Wirthszelle des Tertianaparasiten,' *Centralbl. f. Bacteriol.*, Abth. 1, 28, 114–125 (1900).

drops) of the eosin solution with 25 cc. of water. Pour the eosin solution into the methylene-blue solution and immerse the object to be stained without the least delay in the staining mixture, and examine at the end of 10, 20, 30 and 60 minutes, but do not stain for longer.

16. *Ehrlich's Triple Stain* or the methyl-green, acid fuchsin, and orange mixture, and its modifications :

(*a*) *Ehrlich* originally employed for blood-films a mixture consisting of

Saturated watery orange	125 cc.
Saturated 20 per cent. alcoholic acid fuchsin .	125 cc.
Absolute alcohol	75 cc.
Saturated watery methyl-green	125 cc.

This stain was first used for sections by Babes in 1886 [1].

(*b*) *Biondi's modification.* The solution of Ehrlich given under (*a*) was then modified to meet the requirements of R. Heidenhain [2], by Biondi in 1888, who mixed thoroughly saturated solutions of

Orange	100 cc.
Acid fuchsin	20 cc.
Methyl-green	50 cc.

It is absolutely necessary that the solutions be saturated, and to ensure this, fill a bottle half full with the dye ; fill it with hot water, just leaving a little free space, and shake vigorously for ten minutes ; on the next day shake again ; on the third day take the requisite quantity by means of a pipette from the upper clear layer of the fluid and mix in the order indicated.

(*c*) *Martin Heidenhain* [3] recommends orange G, rubin S, and methyl-green OO to be obtained in a crystalline form from the Actiengesellschaft in Berlin [4]. M. Heidenhain uses the three-colour mixture as sold by Grübler and Hollborn, and acidifies it with the view of accentuating the red colour, according to the following plan: Three parts of Grübler's stock solution are diluted with 200 parts of water. Then a few drops of this dilute solution are placed in a beaker and acidified with 1 : 500 acetic acid till the colour changes from a smoky to a distinctly carmin-red tint. This vessel is used as the test beaker. Now 1 : 500 acetic acid is added gradually to the 3 : 200 solution made from the stock solution, till a few drops of this solution, when placed in a second beaker, show the same carmin colour as does the solution in the test beaker.

Sections, fixed preferably in sublimate [5], are first subjected for a few hours to the treatment of 1 : 1,000 acetic acid to ensure more elective staining; they are then treated for a quarter of an hour with tincture of iodine, to remove the sublimate ; are washed in alcohol and stained for 12 to 18 hours in the 3 : 200 stain.

[1] Babes : *Virchow's Arch.* 105, 511–526 (1886).

[2] R. Heidenhain : 'Beiträge z. Hist. u. Physiol. d. Dünndarmschleimhaut,' *Pflüger's Arch.* 43, Supplement (1888).

[3] *Festsch. Kölliker*, Leipzig (1892), 116, and *Zeitsch. f. wiss. Mikr.* 9, 202 (1892).

[4] Obtainable through Alexander Frazer, Edinburgh.

[5] Alcohol-fixed material answers also well.

When the staining is completed, the sections (fixed to the slides) are rinsed for a short time in distilled water (which may be slightly acidulated), then they are differentiated in absolute alcohol and are mounted in xylol balsam. (It is better to use the neutral balsam of Grübler.)

(*d*) *Israel*[1], on the recommendation of R. Heidenhain, advises the following procedure: Make of the methyl-green-orange mixture sold by Grübler a 12 per cent. solution; dilute 1 cc. of this stock solution with 30 cc. of water, and add 3 cc. of a half per cent. watery solution of acid fuchsin.

(*e*) *R. Krause*[2] prepares the triple stain from dyes made by the Actiengesellschaft, Berlin.

Rubin S : 20 grms. in 100 cc. of distilled water . .	4 parts	stock solution
Orange G : 8 grms. in 100 cc. of distilled water . .	7 ,,	
Methyl-green : 8 grms. in 100 cc. of distilled water .	8 ,,	

Mix the dyes in the order indicated, and dilute 1 cc. of the resulting stock solution with 50 to 100 cc. of water. Treat the sections with dilute acetic acid for a couple of hours previous to staining, and stain twelve to twenty-four hours.

(*f*) *Ehrlich's Triacid Stain.* The name of this mixture indicates that the tribasic methyl-green has its basic affinities satisfied by three acid equivalents of the two other constituents, namely orange G and acid fuchsin.

To prepare this dye Ehrlich proceeds thus:

Saturated watery solutions of the dye (which take days to prepare) are mixed in the following proportions, in the order indicated and using the same measuring vessel throughout:

Orange G	13
Acid fuchsin . . .	7
Water	15
90 p. c. alcohol . . .	15
Methyl-green . . .	12·5
90 p. c. alcohol . . .	10
Glycerin	10

(*g*) *Paul Mayer*[3] tells me that he has analysed Ehrlich's triacid solution, and finds the solubility of

Rubin S (and fuchsin)	to be approximately	1 grm. in 1·3 cc. of water
Orange G	,, ,,	1 grm. in 7 cc. of water
Methyl-green OO	,, ,,	1 grm. in 12 cc. of water

To make Ehrlich's triacid solution P. Mayer first dissolves in the fluid mentioned the colour acids, and subsequently the methyl-green, in these proportions:

Dyes.		*Fluid.*	
Acid fuchsin . .	3 grm.	Water . .	45 cc.
Orange G . . .	2 grm.	in Glycerin . .	10 cc.
Methyl-green . .	1 grm.	Alcohol 90 p. c.	25 cc.

Ehrlich's triacid, if used in full strength, overstains sections fixed in picro-corrosive formaldehyde in two minutes. It is best to dilute

[1] Israel: *Praktic. Path. Hist.*, Berlin, 69 (1893)

[2] Krause: 'Beiträge zur Histol. d. Wirbeltierleber,' *Arch. f. mikr. Anat.* 42, 53–82.

[3] Paul Mayer: *Grundzüge d. mikroscop. Technik*, 212 (1901).

the stock solution in the proportion of 1 to 4 with a mixture of glycerin 10 cc., water 15 cc., and 90 per cent. ethyl-alcohol 25 cc.

I find it best to stain with the 1 : 5 solution for 2 to 10 minutes, to quickly rinse the sections in water, and to differentiate at once with absolute alcohol, for the stronger the alcohol, the less the danger of extracting the basic dyes.

The term differentiation with Ehrlich's stain does not mean that by the action of alcohol the dyes are extracted in different proportions, for they are not. It means that the section is cleared of all particles of stain which are simply imbibed physically or mechanically.

Generally speaking, Ehrlich's triple stain is for the beginner somewhat fickle, because of some fault of his own, but when once mastered it is one of the quickest and most reliable stains to work with.

C. Successive application of dyes.

Under this heading are included all methods by which sections are stained first in an acid and subsequently in a basic dye or vice versa.

II. Staining methods requiring differentiation.

17. *Stroebe's method*[1] for staining axis-cylinders of nerves is a modification of Weigert's[2] acid fuchsin method for the central nervous system, as in both methods bichromate sections and a sulphuric acid dye are used with subsequent differentiations in caustic alcohol.

Celloidin sections of the central or peripheral nervous system, which has been hardened for 4 to 5 months in Müller's fluid, are treated as follows: (1) Stain in a freshly prepared, saturated, watery anilin-blue solution from 10 to 30 to 60 minutes. (2) Wash in distilled water. (3) Transfer to a dish containing absolute alcohol (quantity not stated), to which 20 to 30 drops of 1 per cent. solution of caustic potash in absolute alcohol have previously been added[3]. In the caustic alcohol the blue sections first become of a rusty red colour and red clouds are seen to leave the sections. Within a minute, when the sections have gradually become transparent and of a light-brown tint, transfer them to a large quantity of distilled water for five minutes to restore the blue colour; counter-stain for 15 to 30 minutes in a saturated watery safranin solution diluted with an equal amount of water; remove the superfluous safranin with absolute alcohol; and finally clear in xylol and mount in balsam.

I use paraffin sections of tissue hardened in Müller's fluid, and attached to slides by my albumin method (p. 373). Stain 3 to 6 hours; rinse in water; thoroughly dehydrate, before placing the sections in the caustic alcohol; take 5 drops of 1 per cent. caustic alcohol (p. 217) to 25 cc. of absolute alcohol; after sections have turned reddish, rinse them with absolute alcohol to remove the greater part of the caustic alcohol, and place the slide in distilled water.

[1] H. Stroebe: *Centralbl. f. allgem. Path. u. path. Anat.* 4, 49 (1893).

[2] Weigert: *Centralbl. f. d. med. Wissensch.* 42, 753, and 43, 772, (1882).

[3] To prepare the caustic alcohol treat 1 grm. of KOH with 100 cc. of absolute alcohol for twenty-four hours, then filter.

If there is any tendency of the blue to be too pale, the sections are placed in dilute acetic acid (2 drops of glacial acetic acid to 100 cc. of distilled water). After counter-staining in safranin, differentiate to the desired extent with clove oil, if this dye does not come out readily with alcohol. If clove oil is used take care to replace it completely with xylol or benzol before mounting. Stroebe points out that the anilin-blue solution must be freshly prepared, as otherwise failure may be the result. I find that addition of any acid, for example sulphuric acid, to the staining bath overcomes this difficulty. Stroebe's method is one of the best for the nervous system.

18 *a. Gram's method*[1]. Make a saturated solution of anilin oil in distilled water by shaking the oil vigorously with the water, and filter. The filtrate is called anilin water. To 100 cc. of this anilin water add 5 cc. of a saturated alcoholic solution of gentian-violet, or still better crystal-violet (p. 424); shake and filter; stain in this mixture for 5 minutes; rinse in water; treat with Lugol's solution (iodine 1 gram, potassium iodide 2 grams, water 300 cc.) for 5 minutes. Wash in water and differentiate in alcohol, or, if this procedure takes too long, differentiate in clove oil. Kisskalt's method is given on p. 452.

Unna[2] uses pararosanilins, and not rosanilins, because iodine has a special chemical affinity for the former. Gottstein[3] holds that the iodine has no action in this method; but that the potassium iodide loosens the union between the dye and the tissue, with the result that the subsequent treatment with alcohol or clove oil readily removes the dye. Since that time the ionic view has been developed, and we know that potassium iodide does not act as KI but might act as $K^{\cdot}+I'$. This salt, however, seems to become hydrolysed into KOH + HI, as perfectly pure KI gives a slight alkaline reaction when placed on litmus paper and wetted.

The following explanations are offered: (1) The KOH dissociates into $K^{\cdot}+OH'$ and the alkaline an-ion OH′ (p. 12), exerting its influence on the stained tissue, liberates the dye radical—the kat-ion—from its bond with the tissue. The kat-ionic dye radical once liberated meets with the an-ionic iodine radical to form the water-insoluble dye + iodine compound.—(2) The HI radical breaks up into $H^{\cdot}+I'$; the kat-ion $H^{\cdot}$ attacks the tissue which is playing the part of an an-ion; the kat-ion $H^{\cdot}$ is substituted for the dye kat-ion, and the latter unites with the iodine an-ion as above. The second explanation is supported by the following method:

18 *b. Claudius' modification of Gram's method*[4]. The necessaries are a 1 per cent. methyl-violet 6B solution (p. 424); a half-saturated watery picric acid solution (I take a ¼ per cent.); chloroform or clove oil. The method consists in staining sections for 2 minutes;

[1] Gram: *Fortschr. d. Med.* 2, 6 (1884).

[2] Unna: 'Die Rosaniline und Pararosaniline,' *Monatsh. f. prakt. Dermatol.* (1887).

[3] Gottstein: 'Über Entfärbung gefärbter Zellkerne u. Mikroorganismen durch Salzlösungen,' *Fortschr. d. Medicin*, 3 (1885).

[4] M. Claudius: *Ann. de l'Inst. Pasteur*, 11, 332 (1897).

rinsing them in water; removing the greater part of the water with filter or cigarette paper (or thin No. 1 toilet paper); acting on them with the picric acid solution for two minutes; rinsing in water, drying with cigarette paper short of complete dryness, and differentiating in chloroform or in clove oil. Alcohol is to be avoided in dehydrating the sections, because it extracts the picric acid and also decolourizes certain bacteria which normally stain by Gram's method. Anilin oil is also not to be recommended. For cover-glass preparations of bacteria Czaplewski differentiates with alcohol or anilin-xylol, washes in xylol and mounts in glycerin.

Picric acid dissociates into the an-ion $C_6H(NO_2)_3O'$ and the kat-ion $H^{\cdot}$. The latter may act according to the second explanation given under Gram's method, while the picric acid an-ion remainder forms with the gentian-violet a water-insoluble compound.

19. *Kupffer's* method of staining nerve fibrils in acid fuchsin and differentiating subsequently in alcohol, and (20) *Apáthy's* method of differentiating nerve-cells and fibres stained in methylene-blue by ammonia, also belong to this group of staining methods.

21. *The long methyl-blue-eosin method* (p. 216) is an example of the application of two dyes, followed by a differentiation with the view of staining red blood-corpuscles red and the rest of the tissue blue.

22. *Baumgarten's*[1] *method* of using fuchsin and methylene-blue. Sections of material fixed in solutions containing chrome-compounds, are stained for 24 hours in the following mixture: saturated alcoholic solution of fuchsin 1 part, water 10 parts; are rinsed in alcohol; differentiated and counter-stained in a saturated watery solution of methylene-blue for 4 or 5 minutes; still further differentiated with alcohol for 5 to 10 minutes; cleared with clove oil, and mounted in balsam. I remove the clove oil with benzene, and finally mount in neutral balsam.

The methylene-blue displaces the fuchsin from the cytoplasm, a result which cannot be procured by using either neutral or acid alcohol.

CHAPTER XIX.

Chemical Methods—Staining after Mordanting.

A mordant, using the term in a broad sense, is any chemical agent which brings about a chemical union between two other substances which by themselves have no chemical affinity for one another. Thus tissues fixed in absolute alcohol are potential acids or bases, or, as it is termed, are in the state of pseudo-acids and pseudo-bases (pp. 27 and 337). As long as this condition persists they are unable to react with colour bases and colour acids to form coloured salts, but after their pseudo-character has been changed into the real or actual acid or basic state, staining with colour acids or colour bases becomes possible.

As pointed out elsewhere, pseudo-acids are converted into real acids by the addition of bases, and pseudo-bases into real bases by

[1] Baumgarten: *Zeitsch. f. wiss. Mikr.* 1, 315 (1884).

acids, and we are therefore, according to the definition given above, justified in considering as a mordant any base or acid capable of acting on the pseudo-compounds just indicated.

In the restricted sense, however, a mordant is an agent which becomes itself included, and which forms the connecting link by holding on both to the tissue and the stain.

A mordant may be used in histology at the time of fixing tissues, or after they have been fixed; during the process of staining or after physical staining has been completed. We should therefore always keep in mind that a great many of our fixing reagents, in addition to fixing, act also as mordants, as do, for example, all chrome-compounds and many salts of the heavy metals, as will be pointed out in greater detail afterwards;—further, that many dyes, for example ordinary haematoxylin and carmin stains, contain a mordant, namely alum. Knowing what dye we are going to employ, and sufficient about its chemistry to be able to tell whether it is a colour acid or a colour base, it is possible to choose such mordants as possess affinities for the dye, trusting at the same time that the mordant will attach itself to the tissue because of the amido nature of the latter, for amido acids, according to circumstances, may act either as acids or as bases.

Mordants can be divided, according as the radical contained in them which enters into chemical combination in the process is acid or basic. The compounds formed by the union of basic mordants with colour acids are termed lakes in this country, while it would appear that this term is applied in Germany also to compounds formed between acid mordants and colour bases.

The mordants commonly used in combination with basic dyes are (1) tannin (p. 74), (2) colour acids, such as picric acid (p. 74), gallein (p. 431), and coerulein, and (3) acid salt-colours, for example yellow chrysamin, also canarin and the sulphide dyes (p. 434).

The mordants in common use with acid dyes are:

1. *The Alums*: especially potash alum, $Al_2(SO_4)_3 + K_2SO_4 + 24H_2O$, iron alum, $Fe_2(SO_4)_3 + K_2SO_4 + 24H_2O$, and chrome alum, $Cr_2(SO_4)_3 + K_2SO_4 + 24H_2O$ (the behaviour of alum is described on p. 230).

2. *The Sulphates of iron.*

The ferric sulphate, $Fe_2(SO_4)_3 + 9H_2O$, is apt to decompose into the basic salt $Fe_2(SO_4)_2(OH)_2 + 14H_2O$, and the latter into the still more basic compound $Fe_2SO_4(OH)_4 + 5H_2O$ [1].

Persulphate of iron solution, or liquor ferri tersulphatis, is the liquor ferri sulfurici oxydati of the German Pharmacopoeia. This solution is the one which Benda uses for his haematoxylin method. According to the *Pharmacopoeia of the United States*, edition VI, 1883, it is prepared as follows:

Sulphate of iron . . .	80 parts
Sulphuric acid	15 ,,
Nitric acid	(11) ,,
Distilled water	
	200

[1] *Ber. deutsch. chem. Gesellsch.* 8, 77.

Mix the sulphuric acid with 11 parts of nitric acid and 50 parts of distilled water in a capacious porcelain capsule, and having heated the mixture to the boiling point, add the sulphate of iron (one-fourth of it at a time), stirring after each addition, until effervescence ceases. Should the addition of a few drops of nitric acid cause a further evolution of red fumes, cautiously add nitric acid till red fumes cease to be evolved. Then continue the heating until the solution acquires a reddish-brown colour and is free from nitrous odour. Lastly, add enough distilled water to make the whole measure 200 parts. The dark reddish-brown liquid, almost odourless, has a specific gravity of 1·320 and is miscible with water and alcohol in all proportions without decomposition.

The iron salts commonly used as mordants are :

Ferric potassium sulphate or iron-alum, $Fe_2(SO_4)_3 + K_2SO_4 + 24H_2O$, apt to decompose into $SK_2SO_4 + 2Fe_2(SO_4)_2(OH)_2 + 16H_2O$.

Ferrous ammonium sulphate, $FeSO_4 + (NH_4)_2SO_4 + 6H_2O$, a very stable salt with these solubilities, according to Tobler—

at 0° C.	20° C.	30° C.	60° C.	75° C.
12·2	21·6	28·1	44·6	56·7

3. *The Acetates* :

(*a*) Iron acetate is formed by adding a neutral acetate to a solution of ferric chloride, $Fe(C_2H_3O_2)_3$. It readily decomposes on boiling into the basic insoluble salt

$$Fe(C_2H_3O_2)_3 + H_2O = Fe(OH)(C_2H_3O_2)_2 + C_2H_4O_2.$$

(*b*) Copper acetate, $Cu(C_2H_3O_2)_2 + H_2O$.

(*c*) Aluminium and chromium acetates.

4. *Chrome-compounds* : the chemistry of chromium trioxide and its derivatives is given on pp. 72 and 75. It is sufficient for our present purposes to remember that chromates have a tendency to become bichromates, and that the latter cannot exist in the presence of OH-ions, thus :

$$2CrO_4'' + 2H^{\cdot} = Cr_2O_7'' + H_2O$$

chromate ions + hydrogen ions = bichromate ions + water.

$$Cr_2O_7'' + 2OH' = 2CrO_4'' + H_2O$$

bichromate ion + hydroxyl ion = chromate ion + water.

Chromium trioxide, CrO_3, when dissolved in water, breaks up transitorily into the an-ion CrO_4'' and the kat-ion $2H^{\cdot}$, which, however, cannot exist together (see the formula given above), there being formed at once the bichromate-ions Cr_2O_7'' + water. These bichromate-ions coming in contact with the 'alkaline' tissue constituents are converted into the chromate-ions CrO_4.

It follows that it is quite immaterial whether chromic acid or a bichromate such as that of potassium be used, as in either case a certain amount of CrO_4 ions will be formed in the 'alkaline' tissue, the tendency towards this formation being, of course, greater in the potassium bichromate because of the existence of the $K^{\cdot} + OH'$ ions.

Chromic-ions are trivalent, and closely resemble in their properties the aluminium-ions and the ferri-ions, being isomorphic with these. It is therefore not difficult to understand why chrome-compounds act as mordants.

Suppose chromium trioxide in a watery solution, usually called 'chromic acid,' be used for mordanting, then we start with the bichromate ion Cr_2O_7'', which by the alkaline tissue becomes changed into the chromate-ion CrO_4, and this in its turn is reduced to Cr_2O_3 or chromic oxide, which latter may get changed into the chromous hydroxide $Cr(OH)_2$.

5. *Tartar emetic* or potassium antimonyl tartrate, $C_4H_4O_6K(SbO)+\frac{1}{2}H_2O$, is derived from tartaric acid, $C_4H_6O_6$ or $COOH{\cdot}(OH)HC—CH(OH){\cdot}COOH$. It is used by professional dyers in combination with tannin, and was introduced into histology by Rawitz. The tannate of antimonium oxide has great affinity for basic dyes, and the compounds formed by its union with colour bases are insoluble in an excess of tannin, while the ordinary tannates are soluble.

Instead of using potassium antimonium tartrate, the oxalate may be employed, or the antimony radical may be replaced by iron.

How the mordants fix themselves to the tissue is, in many cases, not known. It is not difficult to imagine tannin (p. 74) with its 5 hydroxyl groups fixing on to the tissue and also to basic dyes. There arises, however, a difficulty with picric acid, which contains only one salt-forming group, namely the phenol radical OH (p. 74), and therefore we are dealing perhaps in this case not with a true mordant action, but simply with the precipitation in the tissues of the insoluble picrate of a colour base, for example, of Victoria-blue, night-blue, kresyl-violet (see, however, below). What happens on dissolving tannin in water is that some of its OH groups dissociate analogously to phenol or picric acid into the kat-ion $H^{\cdot}$ + the an-ion tannin-remainder. The tissue, playing the part of a stronger kat-ion than the $H^{\cdot}$, unites with the tannin to form an insoluble tannate. But all the OH groups of tannin are not occupied in thus linking the tannin molecule to the tissue, and those which remain free on coming into contact with stronger colour bases, will exchange the hydrogen kat-ion for the colour bases. There is thus brought about an indirect union between the tissue and the dye according to this scheme

$$\text{tissue}^{\cdot}+[O'-\text{tannin}-O']+\text{colour base}^{\cdot}.$$

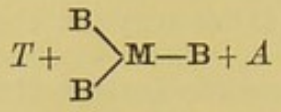
This view supposes the mordant to play the part of an an-ion between two kat-ions represented by the tissue and the colour base.

An entirely different, possible mode of action of mordants must, however, also be kept in mind, namely the formation of additive compounds, a view which would dispense with the assumption that only those molecules are active which have undergone an electrolytic dissociation. On this theory the colour-base picrate may be after all an example of a true mordant.

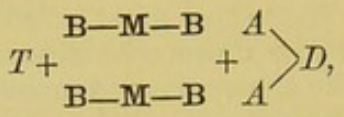
For the basic mordants enumerated above the same rule holds

good as for the acid ones, namely, that the mordant after it has united with the tissue must still possess some unsatisfied radicals capable of forming salt-like combinations.

If *M* represents a mordant, *BBB* its 3 basic radicals, and *A* the colour acid of the dye, while *T* is the tissue, then a tribasic mordant can, at the most, hold on to the tissue with two out of its three basic radicals, thus leaving the third radical free for a union with the colour acid.



If the acid radicals, *A*, of a dye, *D*, be multiple, they may all be satisfied by the mordant according to this scheme:



which represents two molecules of a mordant, *M*, holding on to the tissue with one basic radical each, while the other two basic radicals are satisfied by a dye *D* with two acid radicals, *AA*.

Liebermann and Kostanecki[1] have pointed out that only those dyes will fix on to iron oxide or alumina mordants, which contain two hydroxyl or carboxyl (OH or COOH) groups in the ortho-position to one another. The greater the number of these acid radicals, the greater will be the acidity of the dye, and the more strongly will it be held by the mordant.

Nitro- or sulpho-acid dyes cannot be used in combination with mordants, because the NO_2 or SO_3 radicals are sufficiently strong to take possession of all the available radicals of the mordant, and to force the latter to give up its hold on the tissue. It follows, therefore, that some of the salt-forming radicals of a mordant must always remain unsatisfied either as regards the tissue or the dye.

The choice of mordants in many cases is determined by the tint or shade of colour it is desired to produce; thus alums with carmin give a bright red colour, but with iron compounds, a greyish-black. There are, further, special dyes on the market called chromotropes, which are used with different mordants to produce different shades, as stated more fully on p. 393. It is customary to call dyes which stain only with one colour monogenetic, while those capable of giving different colours are called polygenetic.

In using mordants for certain acid stains, it is possible to first mordant the tissue and then to apply the stain, as, for example, in M. Heidenhain's iron-alum method; or secondly, to combine the mordant with the stain as in the ordinary haematein or carmin alums; or thirdly, to impregnate the tissue first with the dye, and apply the mordant subsequently, as in Hermann's haematoxylin-bichromate method.

[1] Liebermann and Kostanecki: *Chem. Ind.* 6, 724–6.

After having fixed an acid dye by means of a basic mordant, it is still in many cases possible to fix to the acid dye a basic one, as, for example, in Benda's method (p. 243). That an acid dye used directly may become a mordant for a basic dye was pointed out above in connexion with picric acid.

'Remontage' is a term given to the principle of remordanting; thus in Weigert's process for medullated nerves, the tissue is fixed first in the mordant bichromate, and subsequently mordanted again with copper sulphate. The same principle holds good in using alum haematoxylin after having fixed in chrome-salts or sublimate[1] (Pappenheim).

Whenever mordants are used either in the fixing or staining solution, or when they are applied separately, we must realize that the staining effect is due to the mordant, and not due to the tissue, and that we have no right to draw any conclusions as to the primary colour-affinities existing between the tissue and the dye.

In the following pages the methods have been arranged, not according to the reaction of the mordant, but according to the dyes we are dealing with. These have been arranged in the following order: Haematoxylin (p. 229), Brazilin (p. 242), Alizarin (p. 243), Carmin (p. 244), and Basic-anilin dyes (p. 249).

The impregnations with proteid substances after A. Fischer's plan are given on pp. 254–263.

A. *Haematoxylin and Haematein.*

Space forbids entering fully into a description of all the different 'haematoxylin' solutions which have been advocated from time to time. A good review is given by Paul Mayer[2], who has also worked out the conditions under which 'haematoxylin' stains[3].

Everything stated about haematoxylin and haematein applies equally well to brazilin and brazilein.

When dealing with the chemistry of haematoxylin and its derivatives (p. 436), it is pointed out that this compound is a weak acid which combines with basic oxides to form lakes. With some metals, as for example iron and copper, coloured lakes are formed. If such metals occur in the cell and if they be liberated from their organic union, then haematoxylin may be used for their detection, as will be shown later (p. 292).

When haematoxylin unites with metals it acts as an acid dye, whether the metal occur naturally in the cell or whether it be introduced into the cell during the processes of fixing or of mordanting. Haematoxylin may, in addition to being used directly as an acid dye, be also employed indirectly in the form of its oxidation-product haematein, when it will react as if it were a basic dye under

[1] The sublimate may be removed completely, however, by treatment with iodine-potassium iodide.

[2] Paul Mayer: *Zeitsch. f. wiss. Mikr.* 16, 196–211 (1899).

[3] Paul Mayer: *Mittheil. a. d. Zool. Station zu Neapel*, 10, 172 (1891).

certain conditions to be stated immediately. It has already been pointed out that haematoxylin is used in combination with alum for dyeing purposes. Alum, a term given originally to aluminium-potassium sulphate, $Al_2(SO_4)_3 + K_2SO_4 + 24H_2O$, was used by professional dyers for the aluminium sulphate it contains, and which formerly could not be obtained sufficiently pure by itself. Now it is easy to obtain pure aluminium and therefore the pure sulphate, for which reason the use of alum is discarded more and more by professional dyers and should also be laid aside by histologists.

Aluminium sulphate, $Al_2(SO_4)_3$, in a watery solution decomposes electrolytically into Al''' and SO_4'' ions, and hydrolytically into a certain number of $Al''' + 3OH' = Al(OH)_3$ or insoluble aluminium hydroxide radicals, and $2H^{\cdot} + SO_4''$ or sulphuric acid ions. In an aluminium sulphate solution the aluminium hydroxide is kept in solution by the free sulphuric acid. Should, however, the sulphuric acid by any means be taken away, or be bound by an alkali, such as may be derived from the glass bottle in which the alum solution is kept, or the ammonia of the air, then the insoluble aluminium hydroxide is thrown down[1].

Haematein does not react in any way with aluminium sulphate or its ions Al''' and SO_4'', but it combines at once with the hydroxide $Al(OH)_3$ to form the insoluble haematein lake. The histologists of old were often annoyed by finding haematoxylin solutions had deteriorated, owing to the formation of this lake. This difficulty was overcome by Ehrlich, who introduced an acid solution of haematoxylin (p. 239), in which the formation of the insoluble lake is impossible.

What happens when using for histological purposes an aluminium sulphate + haematein solution is difficult to say, but the following suggestions may be made. From what has been stated above, it follows that any factor tending to diminish the acid reaction of aluminium sulphate is apt to lead to the formation of the insoluble basic aluminium oxide, $Al(OH)_3$. There are present in the nucleus, as well as in the cell, more or less strongly basic substances: the protamins, histones, and the amido-acids (see p. 281) with which the sulphuric acid can unite. Such a union would of necessity lead to the formation of the insoluble aluminium hydroxide, with which haematoxylin forms insoluble lakes. In those cases where acid fixatives have not been employed, the large amount of free basic[2] salts present in the tissues leads always to an abundant precipitation of aluminium hydroxide and consequently to overstaining with

[1] Aluminium acetate, $Al(C_2H_3O_2)_3$, in water undergoes electrolytic dissociation into aluminium ions and acet-ions, $Al''' + C_2H_3O_2'$, but, especially on heating, is apt to form, as the result of hydrolytic changes, a compound with the hydroxyl ions of water $Al(C_2H_3O_2)_3 + nOH' = Al(C_2H_3O_2)_3{-}n(OH)_n$. This last compound, the basic aluminium acetate, is called a basic salt, as it contains, in addition to the acid ions (in this case the acet-ions $C_2H_3O_2$), also the basic hydroxyl ions OH'. Aluminium acetate, when substituted for alum in haematein solutions, does not give good results, probably because its acet-ion is too feeble to act on the tissue.

[2] Basic, in the sense of turning red litmus paper blue.

haematein, as will be known to any one who has worked with material fixed in neutral alcohol, or with fresh tissues.

The explanation I have offered is further supported by the fact that the nuclei, which contain the strongest basic radicals, namely the histones, still stain in very acid haematein-alum solutions, long after the less basic cell-plasm has ceased to show any affinity for this dye.

A. The haematoxylin and haematein staining methods. (For the haematein-alum solutions see p. 237.)

The method of using haematoxylin for the micro-chemical detection of iron is given on p. 292.

F. Boehmer, in 1865[1], has really done already everything that theoretically can be done with haematoxylin. He mordanted sections in copper sulphate and then stained them in haematoxylin solutions; he was the first to combine haematoxylin with alum solutions, and having impregnated tissues primarily with haematoxylin he produced, by after-treatment with chrome-solutions, the black chrome-lake.

Histologists are, as a rule, in the habit of using with iron and chrome-lakes haematoxylin instead of haematein. There is a certain justification for this procedure, as Paul Mayer[2] has shown that haematoxylin can be oxidized by solutions which contain iron, even beyond the stage of haematein, and later (p. 240) it will be shown that Harris uses mercuric oxide for the conversion of haematoxylin into haematein. Still M. Heidenhain after mordanting with iron-alum solutions gets the best results with old haematoxylin, which means with haematein, and I personally have substituted haematein for haematoxylin after mordanting in iron-salts.

1. **The iron-lakes.** *Benda*, in 1886[3], fixed tissues in saturated picric acid, washed them in water, imbedded in paraffin in preference to celloidin, mordanted the sections in saturated iron-alum (p. 225), washed in repeated changes of water; stained in 1 per cent. haematoxylin for ten minutes; differentiated for five minutes in 1:2,000 chromic acid; washed and mounted in the usual way in balsam.

Martin Heidenhain, 1892[4], mordanted sublimate sections in a $2\frac{1}{2}$ per cent. iron-alum solution, then stained in a 1 per cent. haematoxylin solution, and finally differentiated in the same solution which he used for mordanting, namely, the $2\frac{1}{2}$ per cent. iron-alum. This principle of dissolving a precipitate in an excess of the mordanting solution is used also by professional dyers.

Heidenhain's method being especially applicable for the demonstration of centrosomes, the following account is taken from his later paper published in 1896[5].

[1] Boehmer: *Aerztliches Intelligenzbl. f. Bayern*, 12. Jahrg.
[2] P. Mayer: *Anat. Anz.* 13, 318 (1897).
[3] Benda: *Arch. f. Anat. u. Physiologie, physiol. Abth.* 564 (1886).
[4] M. Heidenhain: 'Über Kern u. Protoplasma,' *Kölliker's Festschrift* (1892).
[5] M. Heidenhain: *Zeitsch. f. wiss. Mikr.* 13, 186–99 (1896).

By this method, in addition to the centrosomes, the following elements are also well shown, namely, the dim segments in striped muscle, the cement-substance (Kittleiste) between epithelial cells, nuclear chromatin segments, zymogen granules in the pancreas, eosinophilous granules in leucocytes, and other structures.

Paraffin sections of sublimate or sublimate-osmium material 3–6 μ thick are fixed to slides by the water method (see p. 372) and are dried on Born's warm stage; the paraffin is removed with xylol, the latter with absolute alcohol, and this with distilled water. The slide is then placed vertically for three to twelve hours in a vessel containing a $2\frac{1}{2}$ per cent. iron-alum solution; it is carefully washed with a large amount of distilled water and is then placed in a ripened haematoxylin solution, i. e. one containing haematein, for twenty-four to thirty-six hours.

The haematoxylin solution is made according to Weigert's formula: Dissolve one gram of haematoxylin in 10 cc. of alcohol and add 90 cc. of water. After one month this mixture is ready, and requires before use to be diluted with an equal bulk of water (I use a 1 in 500 haematein solution). The sections are, therefore, after mordanting stained in a half per cent. haematoxylin solution, containing haematein. This solution may be used time after time, provided it is occasionally filtered. It seems that traces of iron-alum by finding their way into the haematoxylin solution improve its staining properties (M. Heidenhain).

To differentiate sections place a large vessel containing at least one litre of ordinary tap-water beside a microscope, and determine the extent of differentiation preferably by means of a D* water immersion lens of Zeiss. When the centrosomes stand out clearly, wash the slide for 10–15 minutes in running water; dehydrate and clear in xylol (under no conditions should such oxidizing media as oils of clove, bergamot, origanum, or turpentine be used).

I find that preparations keep for at least six years quite unaltered in Price's pure neutral glycerin, a medium which, because of its lower refractive index, I prefer to neutral balsam. To stain the cytoplasm of sections, should this be necessary, Heidenhain uses a slightly acid solution of rubin, after differentiating the haematoxylin.

If the effect of the haematoxylin on the centrosomes is to be accentuated, M. Heidenhain employs the principle of preoccupation, by staining sections first in acid anilin dyes, to let the latter preoccupy all the different cell structures with the exception of the centrosomes. When now the sections are mordanted, the iron-alum is supposed to unite more firmly with the centrosomes than with the rest of the cell-plasm, because the latter has its affinities already partly satisfied by the acid anilin dyes.

To this end sections are first stained either in Bordeaux red or in anilin-blue, and subsequently in haematoxylin, in exactly the same manner as described above. *Benda's* 1893 method gives results undistinguishable from M. Heidenhain's, according to Lee, who stained consecutive sections in the same haematoxylin solution

after having mordanted them in iron-alum according to the method given above, or in ferric sulphate according to Benda, whose method is as follows: Sections are placed for 24 hours in liquor ferri sulfurici oxydati Pharm. Germ. (see p. 225), 1 part, and water 2 parts (Paul Mayer recommends a 10 per cent. solution of the ferric sulphate); they are then washed first in distilled water and subsequently with ordinary water, and are now placed in a 1 per cent. watery solution of haematoxylin till black; after washing, differentiation is brought about by treatment with 30 per cent. acetic acid, which acts quickly, or in dilute acetic acid, or in the liquor ferri sulfurici oxydati diluted 20 times with water.

Eisen[1] also greatly prefers Benda's method to Heidenhain's, and warns against putting sections into the iron-alum mordant before the alcohol has been completely removed, as otherwise a precipitate of the iron salt is formed over the sections.

Bütschli[2] mordants in iron acetate, stains in a half per cent. haematoxylin, and does not differentiate, and thus obtains a dense colouration of the cytoplasm.

2. **Chrome-lakes.** The chemistry of chrome-compounds is given on p. 75, and the action of mordants on p. 224.

The oldest method, that of Böhmer, in 1865, is of historical interest, as it established the fact that a tissue may be first impregnated with a dye such as haematoxylin and then be mordanted subsequently. On the same principle depend the methods of R. Heidenhain in 1885–6[3], Apáthy[4], and Platner[5].

R. Heidenhain in his second paper advocates fixing tissues preferably in alcohol or in picric acid; leaving them for 12 to 24 hours in a one-third per cent. solution of haematoxylin in distilled water; transferring the tissues next for the same length of time into a half per cent. potassium chromate solution, as this gives a sharper nuclear staining than the bichromate (the chromate solution should be changed several times in the course of the day), and finally washing out the chromate with water.

In all the following methods the tissues are mordanted first and stained subsequently.

C. Weigert in 1884[6] published his way of staining medullated nerves, a method which allowed enormous progress to be made in the deciphering of the brain and the cord. The principle of this method is described on p. 313, while here only the process of staining as employed by myself will be given.

Method of fixing sections to the slide. Tissues well hardened in Müller's fluid for 3 to 4 months, or for 4 to 5 days in one of Weigert's solutions (see p. 236), are imbedded in paraffin and are cut into sections

[1] Gustav Eisen: *Zeitsch. f. wiss. Mikr.* 14, 200 (1897).
[2] Bütschli: *Unters. mikr. Schäume*, Leipzig, 80 (1892).
[3] R. Heidenhain: *Arch. f. mikr. Anat.* 25, 468 (1885), and 27, 383 (1886).
[4] Apáthy: *Mitth. a. d. Zool. Stat. zu Neapel*, 7, 744 (1887); *Zeitsch. f. wiss. Mikr.* 5, 47 (1888), and also *ibid.*, 6, 170 (1889).
[5] Platner: *Arch. f. mikr. Anat.* 33, 126 (1889).
[6] Weigert: *Fortschr. d. Med.* 2 (1884), 190, and *Zeitsch. f. wiss. Mikr.* 1, 291 (1884).

10 to 20 μ in thickness. The sections are floated out on warm 5 per cent. potassium bichromate solution, albuminized slides[1] are immersed in the bichromate solution, and the sections arranged in order are placed without delay on a warm stove till dry. Simultaneous exposure to bright light fixes the sections even more firmly.

Method of staining. After removing the paraffin with xylol, and the latter with alcohol, the slides are washed gently for about half a minute in tap-water and are then transferred to Kultschitzky's acid haematoxylin, consisting of a one per cent. solution of haematoxylin in two per cent. acetic acid, made by dissolving the necessary quantity of haematoxylin in glacial acetic acid and then adding the water.— In this solution the sections are left in the incubator (35° C.) till the next day, when they appear quite black. After washing off the haematoxylin with ordinary tap-water, the slides are placed in a quarter per cent. sodium carbonate solution till they turn a deep blue colour. Then they are differentiated as follows.

The method of differentiation comprises two operations:

Treatment with (*a*) potassium permanganate and (*b*) with sulphurous acid.

(*a*) Following the plan of Pal[2], the sections are left for 10 to 30 seconds in a quarter per cent. potassium permanganate solution, which acts probably in two ways, namely, by oxidizing the compounds which the tissues had formed with the chrome salts, and thus liberating chromic acid, and perhaps, secondly, by oxidizing, along with the chromic acid, the haematoxylin and haematein into uncoloured higher oxides. This action of the permanganate, for physical reasons, will be most marked in the comparatively loose grey matter and will be delayed in the dense myelinated nerves. (For very fine fibres I find a one-tenth per cent. permanganate solution to be preferable to a quarter per cent.) It is as a rule advisable to leave the sections in the potassium permanganate solution till a difference between the grey and the white matter can just be made out, and then at once to treat the slide as follows:

(*b*) According to the fineness of the nerve fibres and the thickness of the section it is best to make from the ordinary saturated solution of sulphurous acid, which contains about 10 per cent. of HSO_3, dilute solutions containing 1, 2·5, 5 or 7·5 per cent. of the strong commercial solution and to transfer the sections from the potassium permanganate directly into the sulphurous acid, when the yellowish-brown colour of the grey matter will soon be changed into a well-differentiated white or colourless tint. Should this reaction not take place in 5 minutes, treat sections again for 5 seconds with the one-tenth or quarter per cent. permanganate solution and transfer to the sulphurous acid once more. Occasionally the best results are obtained by repeatedly moving the slides from the permanganate to the sulphurous acid, or leaving sections for 30 to 60 minutes in the sulphurous acid, when the yellow colour gradually fades owing to the sulphurous acid differentiating on its own account. The interaction between the

[1] Covered with a 1 : 150 white of egg solution, and allowed to become dry.

[2] *Zeitsch. f. wiss. Mikr.* 4, 92 (1887).

reduced permanganate and the sulphurous acid is according to the formula $MnO_2 + SO_2 = MnSO_4$. The manganese sulphate being colourless and readily soluble, is removed by washing the sections in water. The idea of using sulphurous acid directly, instead of employing Pal's decolourizing fluid (oxalic acid 1 grm., potassium sulphate 1 grm., and water 200 cc.), occurred to Bruce, when he read Pal's account in 1886, and since then he has used it for economy's sake. Many years later I fell on the same plan for an entirely different reason, namely, the Oxford water is very rich in lime-salts, and in consequence insoluble oxalate of lime crystals are deposited, if any but distilled water be employed. Although for final washings pure water should always be used, it is not necessary to do so for the preliminary rinsings.

Whenever differentiation has gone so far that the grey substance is colourless, the slides are transferred to ordinary water; are rinsed to put a stop to the action of the acid, and the sections are turned blue by adding a few drops of a 1 per cent. sodium or lithium carbonate solution to the washing water. Counterstaining with red acid dyes, such as eosin or Bordeaux red BX, is now still possible and occasionally desirable. The sections are mounted finally in neutral balsam in the usual way.

Benda in 1900[1], adopting Weigert's principle of first fixing in formaldehyde and subsequently mordanting, proceeds thus: Tissues are fixed in 10 to 25 per cent. formol (formalin of Schering) and subsequently mordanted in chromic acid, commencing with a one-third per cent. solution and gradually increasing the strength to one-half and two-thirds. After washing, the tissue is taken through paraffin, and is stained either simply in a watery solution of haematoxylin, or by the sodium alizarin sulphate method given on p. 243.

3. **Copper-lakes**: these were first used by Böhmer in 1865.

Weigert, in 1885[2], showed that better effects are obtainable when staining medullated nerve fibres by his method, if bichromate material, after imbedding in celloidin, is mordanted in an incubator for one to two days in a half-saturated solution of neutral acetate of copper. Weigert uses for staining a slightly alkaline solution of haematoxylin (haematoxylin 1 grm., alcohol 10 cc., water 90 cc., saturated lithium carbonate solution 1 cc.), while Kultschitzky[3], combining the mordanting with the fixing by placing tissues into his copper bichromate alcohol (see p. 94), subsequently stains the medullary sheaths in acid haematoxylin (haematoxylin 2 grams, 2 per cent. acetic acid 100 cc.).

Wolters[4] combines in his method for demonstrating axis-cylinders and nerve-cells by means of haematoxylin, four mordants, namely chrome, copper, vanadium, and aluminium. He proceeds thus: Tissues

[1] C. Benda: 'Über d. Bau u. einige pathol. Veränd. d. menschl. Hypophysis cerebri,' *Verh. d. physiol. Gesellsch.*, Berlin (1899–1900), February 1900, and 'Erfahrungen ü. d. Neurogliafärbung u. eine neue Färbungsmethode,' *Neurol. Centralbl.* (1900), No. 17.

[2] Weigert: *Zeitsch. f. wiss. Mikr.* 2, 399 (1885).

[3] Kultschitzky: *Anat. Anz.* 4, 203 (1889), and 5, 519 (1890).

[4] Wolters: *Zeitsch. f. wiss. Mikr.* 7, 470 (1891).

fixed in Kultschitzky's fluid (see p. 94) for 12 to 24 hours in the dark, are then transferred to 96 per cent. alcohol, also in the dark, for 12 to 24 hours and are taken through the celloidin process.

The celloidin sections, 5 to 10 μ thick, are mordanted for 24 hours in a mixture of

10 per cent. vanadium chloratum . .	2 parts
8 per cent. aluminium aceticum . .	8 „

They are washed for 10 minutes in water; stained for 24 hours in an incubator in Kultschitzky's haematoxylin (haematoxylin 2 grms., two per cent. acetic acid 100 cc.); differentiated in one-half per cent. hydrochloric acid in 80 per cent. spirit till the medullary sheaths have lost all colour; the acid is removed with 80 per cent. alcohol; and the sections are finally cleared in origanum oil, and mounted in balsam. Result:—Nerve-cells, axis-cylinders, and glia-cells are all stained deeply.

Weigert, 1895[1], for demonstrating the neuroglia gives the following directions: Fix portions of the central nervous system not thicker than 5 mm. for 4 days in 10 per cent. formol. Then treat for 4 to 5 days in an incubator or for 8 days at the ordinary temperature with a mordanting solution prepared in the following way:

Dissolve in 100 cc. of water by vigorous boiling in an enamel vessel

Chrome alum	2·5 parts
Then add ordinary acetic acid[2] . .	5 „
Lastly neutral copper acetate . . .	5 „

Wash the tissue in running water, then dehydrate in the usual way and imbed in celloidin. Sections are treated for 10 minutes in a watery solution of one-third per cent. permanganate of potash, and then are washed again.

Reduce sections in the following mixture for 2 to 4 hours:

A.	Chromogen .	5 parts	90 parts (carefully filtered)
	Formic acid .	5 „	
	H_2O . . .	100 „	
B.	10 p.c. sodium sulphite .		10 parts.

Wash sections and leave them overnight (12 hours) in a well-filtered 5 per cent. watery chromogen solution.

To stain the neuroglia, proceed thus:

Wash sections twice. Press them flat on a slide and cover with a solution of methyl-violet, made by saturating 75 per cent. alcohol with it, allowing the alcohol to cool and then filtering it.

Take of this methyl-violet solution	100 parts
Add 5 per cent. watery oxalic acid solution	5 „

Stain for some minutes and then differentiate thus:

(1) Dry sections with cigarette paper. (2) Add a saturated solution of iodine in 5 per cent. potassium iodide. (3) Dry sections with cigarette paper. (4) Differentiate thoroughly with a mixture of equal parts of xylol and anilin oil. (5) Remove the anilin oil with pure xylol. (6) Mount in Canada balsam. (7) Expose preparations for

[1] Weigert: *Abh. Senk. Ges. Frankfurt*, 19, 199 (1895).

[2] The ordinary acetic acid of the B.P. with a specific gravity of 0·1447 = 33 per cent. glacial acetic acid.

some days to diffuse daylight. Result: Nerve-cells and larger axis-cylinders are brown, while the neuroglia, fibrin and nuclei are blue.

The chromogen serves to give a yellowish-brown colour to the ground-substance and nerve-cells of the section.

Benda [1], in connexion with Weigert's neuroglia method, offers the following explanation : It is immaterial what stain is used ultimately so long as the neuroglia fibres have been properly fixed to begin with. It is necessary to use the material as fresh as possible, and to cut it into thin slices so as to allow ready access of the fixing fluid. Formaldehyde is best. Instead of Weigert's mordanting fluid pure chromic acid in successive strengths of $\frac{1}{3}$, $\frac{1}{2}$ and $\frac{2}{3}$ per cent. may be used, but it is still better to leave tissues for 2 days in an incubator in Weigert's chrome alum and copper acetate mixture and then to transfer them to a half per cent. chromic acid solution for 2 days; to wash the tissues for 1 to 2 days, and then to take them through successive strengths of alcohol and to imbed in paraffin. Weigert supposes that, in mordanting tissues, highly oxidized metallic compounds are formed which it is necessary to reduce before the staining can be done adequately, but Benda holds that Weigert's method, apart from the use of chromogen, corresponds in every particular with Pal's method of differentiating medullated nerve-fibres which have been stained in haematoxylin lakes (p. 235). In this method the essential factor is the process of oxidation due to the potassium permanganate, while the sulphite mixture only serves to remove the brown precipitated manganous salts. In this case therefore, as in Weigert's original method of differentiating with borax ferricyanide and in M. Heidenhain's process of differentiating in iron-alum, we are dealing essentially with a process of oxidation. Benda believes that the process of oxidation serves to render inactive or to remove from certain tissue constituents those metallic salts, particularly the chromates, which were used in fixing.

Those tissues, which retain the metallic salts after a certain treatment, will then be specially brought out, as the metals behave towards the stain as mordants. (Benda's alizarin sulphate method for neuroglia fibres is given on p. 244.)

B. Staining with haematein-alum lakes.

It was pointed out that Böhmer, in 1865, first advocated the use of haematoxylin in combination with alum, and that Paul Mayer in 1891 showed that the staining principle of logwood was not haematoxylin at all, but its oxidation product haematein (see p. 436). Haematein solutions may be classified according to the reaction of the solvent as 'neutral' or acid; as alcoholic (including glycerin) or watery, and, according to the relative amount of haematein and alum, as either haematein-alum or alum-haematein solutions.

Neutral solutions have a great tendency to stain diffusely, and are of special use when cytoplasmic or cell-structures are to be studied, while acid solutions (see p. 239) prevent the cytoplasmic colouration and simultaneously enhance the nuclear reaction. Neutral solutions

[1] Benda : *Neurol. Centralbl.* 17 (1900).

are apt to deteriorate, while acid solutions display much greater keeping qualities (p. 193). The addition of glycerin (and other alcohols) to haematein solutions produces a more even result, because staining does not take place so quickly. Further advantages are that, when dealing with sections, the dye does not dry so rapidly, and when staining in bulk, that penetration is ensured, and finally that swelling of certain cell elements, as for example mucin, may be prevented, at least in part.

Neutral Haematein solutions.

PAUL MAYER'S FORMULAE.[1]

	Haemalum (1891).	*Muc-haematein, watery.*	*Muc-haematein, alcoholic.*
Haematein	0·1	0·2	0·2
Glycerin		40	
Water	100	60	
Potash alum	5		
Aluminium chloride		0·1	0·1
Alcohol	(90 p.c.) 5		(70 p.c.) 100
Nitric acid			2 drops

Now Mayer leaves out the alcohol, and makes his haemalum by rubbing up the haematein in a mortar with some glycerin.

By comparing the haemalum with the muc-haematein it will be seen that the ratio between the haematein and aluminium has been inversed in these two solutions, the haemalum containing more alum, while the muc-haematein contains more haematein. Mayer has found that haemalum will not stain mucus, while muc-haematein does, and hence the name it has received. If, however, the haemalum solution is greatly diluted, hydrolysis will be greatly increased, and the aluminium hydroxide will tend to become precipitated more readily, or if the sulphuric acid derived from the alum is carefully neutralized, then again $Al(OH)_3$ is formed in larger quantities, and mucin does stain; on the other hand, if acid is added to the muc-haematein solution, mucus will not stain.

The watery solution stains mucus quickly, but it is best to use the alcoholic solution to prevent, as far as possible, swelling of the mucus. If nuclei are to be brought out, Mayer uses first paracarmin (see p. 245), and afterwards the muc-haematein. Hansen[2] converts the haematoxylin into haematein by means of potassium permanganate, and obtains a solution which, although it soon forms a considerable precipitate, may be used for years after filtering. It also stains mucus if used diluted. The method of preparing Hansen's haematoxylin is as follows:

Dissolve haematoxylin	1 grm.	A.
in absolute alcohol	10 cc.	
Dissolve potash alum	20 grms.	B.
in hot distilled water	200 cc.	

[1] Paul Mayer: *Mittheil. a. d. Zool. Stat. zu Neapel*, 10, 172 (1891); *ibid.* 12. 303 (1896).

[2] Hansen: *Zool. Anz.* 158 (1895).

Allow B to cool spontaneously, and filter on the following day.

Add the filtrate B to A; pour into a large porcelain dish 3 cc. of a solution of potassium permanganate, saturated at 15° C.; add gradually, under constant stirring, the haematoxylin-alum solution; now bring the solution to the boiling-point and boil for a half to one minute; quickly cool the solution, and when cool, filter it into a clean bottle with a well-fitting stopper.

Acid haematein solutions.

The haematoxylin solution which I have used since 1892 is a modification of Ehrlich's acid solution, which in its turn is modified from Friedländer's formula:

	Friedländer, 1882.	*Erhlich*, 1886.	*Mann*, 1892.
Haematoxylin . . .	2	2	
Haematein . . .			0·5 to 2
Absolute alcohol . .	100	100	100
Glycerin	100	100	100
Water	100	100	100
Potash alum . . .	2	excess	10
Glacial acetic acid . .		10	10

To make my solution I proceed thus: The haematein is placed in a flask, the glacial acetic acid is added, and the haematein dissolved either in the acid alone, or after the addition of 25 cc. of alcohol. After complete solution of the haematein, the glycerin is added, the remains of the glycerin washed out with 75 cc. of alcohol, and this also added. Then the mixture is thoroughly shaken. Finally the alum (or aluminium sulphate 5 grms.) is dissolved in the water by means of gentle heat, and while hot poured into the rest of the mixture. By means of a well-fitting glass stopper the access of air is prevented. A solution made in 1892, compared with another quantity 24 hours old, and prepared recently, only shows slightly less cytoplasmic staining than with the new mixture; the nuclei are brought out equally well by both solutions.

This haematein solution may be used also for staining in bulk either in the strength given above, or diluted with an equal quantity of a mixture of equal parts of alcohol, glycerin and water 300 cc., and glacial acetic acid 10 cc.

The advantages over the old formula of the 'haematoxylin' made with haematein, are that the staining solution is at once ready for use, and that we are dealing with a more uniform solution, provided the haematein is good; Ehrlich's solution made with haematoxylin gives the best results only after standing for six months, during which time the haematoxylin completely changes into haematein. Within the last year, various commercial haemateins have, however, not given good results, owing to having been, in all probability, over-oxidized. For this reason I have tried Harris's plan of oxidizing with mercuric oxide, and can thoroughly recommend his methods, which are given below, but I take the additional precaution of filtering

the oxidized solution immediately after cooling, as mercuric oxide is slightly soluble in water with a faintly alkaline reaction.

The methods of Harris for ripening haematoxylin :

(1) Ehrlich's acid haematein solution: Dissolve

Crystallized haematoxylin	1 grm.
in absolute alcohol	10 cc.
and potash or ammonia alum . . .	20 grms.
in hot water	200 cc.

Mix these two solutions, add 0·5 grm. of red or yellow mercuric oxide, bring to the boiling-point and cool quickly (filter). Finally add 10 cc. of glacial acetic acid to the above quantity.

(2) Delafield's haematein solution:

Dissolve in absolute alcohol . . .	6 cc.
crystalline haematoxylin	1 grm.
Add saturated watery solution of potash alum	100 cc.
Boil and add mercuric acid . . .	0·5 grm.

As soon as colour is of a deep purple tint cool quickly, filter, and add

methyl-alcohol	25 cc.
glycerin	25 cc.

(3) Mayer's haemacalcium solution:

A. Dissolve in 70 per cent. alcohol . . .	150 cc.
crystalline haematoxylin	0·5 grm.
aluminium chloride	0·5 grm.

When boiling add gradually to this mixture

mercuric oxide	1 grm.

As soon as the solution is of a deep purple colour, cool quickly, filter, and add

glacial acetic acid	2·5 cc.
B. Dissolve in 70 per cent. alcohol . .	150 cc.
calcium chloride	25 grms.
50 per cent. acetic acid	2·5 cc.

Before use mix equal quantities of A and B.

(4) Mayer's muc-haematein:

Dissolve in 70 per cent. alcohol . .	100 cc.
haematoxylin	0·2 grm.
aluminium chloride	0·1 grm.

Bring gradually to the boiling point, and add slowly 0·6 grm. of mercuric oxide. Cool quickly, as soon as colour of solution is a deep purple, filter and add one drop of hydrochloric acid.

I find that heating the solution is quite unnecessary if yellow mercuric hydroxide be used, which has not been allowed to become dry since the time of its preparation. A ready method of preparing mercuric oxide is to add caustic soda to a solution of corrosive sublimate, then to wash the precipitate till chlorine ions can no longer be detected with silver nitrate, and finally to preserve the hydroxide in distilled water.

Apáthy's haematein method[1]. Tissues not thicker than 5 mm. are fixed at the ordinary temperature in the following fixatives: saturated sublimate in 0·5 per cent. salt solution, or this mixture diluted with

[1] Apáthy: *Mittheil. a. d. Zool. Station zu Neapel* (1897), p. 712.

an equal bulk of absolute alcohol; sublimate-acetic acid; picro-sublimate acetic acid; picric acid; Kleinenberg's picro-sulphuric acid; Zenker's solution, or my osmium-sublimate mixture. Tissues after fixation may be preserved in 90 per cent. spirit for years. The staining solution is made thus: One grm. of haematoxylin is dissolved in pure non-alkaline 70 per cent. absolute alcohol, and is kept in a hard-glass bottle, not quite full, for six to eight weeks at a temperature of 16 to 20° C., to convert the haematoxylin to haematein.

This constitutes solution A.

Solution B consists of:

Water	1000 parts
Salicylic acid	1 ,,
Glacial acetic acid	30 ,,
Alum	90 ,,

To make up the final staining solution mix equal parts of A, B and pure glycerin.

To stain neuro-fibrils proceed thus: Leave tissues, whether small or big, for at least forty-eight hours in the staining solution; three days as a rule are not too long, but do not stain for longer. Now wash out the stain by suspending the pieces of tissue for twenty-four hours in doubly distilled water.

Differentiate the tissue for six, eight, sixteen hours or longer, with distilled water, which removes the stain from everything except the nuclei and neuro-fibrils. If left too long the fibrils will also become decolourized.

Stop the differentiation by putting tissues into tap-water containing lime for three to five hours, but if the water is too alkaline the fibrils become green and fade.

Imbed thus: Dehydrate the tissues as rapidly as possible by suspending them in a high column of pure absolute alcohol. Replace the alcohol with pure chloroform and imbed in paraffin.

Mount the sections in neutral glycerin, or in neutral balsam.

It seems to be the great ambition of some microscopists to prepare haematein solutions which will only stain nuclei; at least Paul Mayer says of my haematein mixture (p. 239) containing 2 grms. of haematein in 300 cc. of solution, that it stains all sorts of things. That is exactly what I mean it to do, for it is not the object of histological research to stain only the nuclei, a method which for morphological studies, I grant, may be useful.

The counter-staining of haematein preparations in many instances is useful, especially for sections to be examined under low magnifications. The counter-stains I generally use are (1) my methyl-blue-eosin mixture (p. 216) for five to seven minutes, or (2) a rubin and orange mixture as recommended by Squire (rubin S 1 grm., orange 6 grms., spirit 60 cc., water 240 cc.), staining for five to ten minutes, or (3) a rubin S and benzo-purpurin stain, which I saw first used at the Pathological Laboratory in Oxford, consisting of water 100, saturated watery solution of rubin S (acid fuchsin) 4, and 10 parts of the following benzo-purpurin mixture: benzo-purpurin 1 grm., absolute alcohol

20 cc., distilled water 80 cc. ; or (4) a half to 1 per cent. solution of Bismarck-brown in 25 per cent. methyl-alcohol ; or (5) a watery 1 per cent. solution of an extract of the cochineal insect, made by boiling the latter in distilled water to extract the colour, and inspissating the filtered deep-orange solution to dryness. A crystal of thymol will prevent the growth of organisms.

van Gieson's Method.

(*a*) Fix in Müller's fluid, alcohol or Zenker's solution (sublimate by itself is not to be recommended, Burchardt), (*b*) overstain the sections slightly in some neutral haematoxylin (such as Delafield's or Mayer's haemalum), (*c*) wash thoroughly in water, (*d*) differentiate for two and a half to five minutes in a half per cent. picric acid solution, 100 cc., to which about 5 cc. of one per cent. acid fuchsin have been added, (*e*) rinse in water for half a minute, (*f*) quickly take through alcohol, clear and mount in balsam (see footnote, p. 351).

Brazilin and Brazilein.

Instead of haematoxylin, brazilin may be used in exactly the same manner. It also requires time to become converted into brazilein (p. 437), but may readily be oxidized by Harris's method, given above (p. 240).

Flechsig[1] in 1889 recommended the use of brazilin under the name of extract of the Japanese red-wood, and Rawitz in his *Leitfaden*, p. 98 (1895), praises brazilin as a chromatin stain. Since then, Eisen, 1897[2], has substituted brazilin for haematoxylin in Böhmer's haematoxylin, and Heimann in 1898[3] uses brazilin instead of haematoxylin in Delafield's solution. Schaudinn[4] combines brazilin with methylene-blue ; Hickson[5] uses brazilin instead of haematoxylin in M. Heidenhain's iron-alum haematoxylin method, but finds according to Wadsworth, who is working under his direction, that the best results are obtained by using alcoholic solutions throughout the staining process, thus : 'The sections are placed in a solution of iron-alum (1 per cent. iron-alum in 70 per cent. alcohol) for one to three hours, and then are placed, after slight washing in 70 per cent. alcohol, in a $\frac{1}{2}$ per cent. solution of pure brazilin in 70 per cent. spirit.' Brazilin was found to stain, generally speaking, much more slowly than haematoxylin. Paul Mayer, in 1901[6], states that after varied experiments during several years, he found brazilin to stain similarly to haematoxylin when used in combination with alum, but not nearly as intensely as haemalum, or carm-alum, and that for nuclear staining it is dispensable. Mayer's experiences do not bear out my own, for I find brazilin to stain most intensely, if it has been converted into brazilein.

[1] Flechsig : *Arch. f. Anat. und Physiol.* 537 (1889), and *Zeitsch. f. wiss. Mikr.* 7, 71 (1890).
[2] Eisen : *Zeitsch. f. wiss. Mikr.* 14, 198 (1897).
[3] Heimann : *Virchow's Arch.* 152, 328.
[4] Schaudinn : *Anhang Abh. Akad. Berlin*, 11 (1899).
[5] Hickson : *Nature*, 62, 589, and *Quarterly Journ. of Micros. Science*, 469 (1900).
[6] Lee and Mayer : *Grundzüge d. mikr. Technik*, 225 (1901).

Alizarin (see p. 399).

Paul Ehrlich[1] in 1885 injected rabbits with alizarin S, which is a bisulphite compound of alizarin[2], to investigate the oxidizing power of different organs, but to Bernhard Rawitz[3] belongs the credit of having introduced into histology the important class of alizarin compounds. He was led to experiment with these dyes after having read works on the methods in vogue with professional dyers.

Rawitz obtained the best results with paraffin sections of material which had been fixed in Flemming's solution. Such sections should be mordanted at room-temperature for twenty-four hours, in the chromic acid mordant GAI[4] diluted with an equal quantity of distilled water. The sections are then washed in distilled water till they have become quite colourless, and are then transferred to a 2·5 per cent. suspension, in distilled water, of the paste alizarin I (Höchst). It is absolutely necessary to add to the alizarin-bath a few drops of 1 per cent. calcium chloride (Kahlbaum), as the alizarin develops its full staining power only in the presence of calcium salts. In the alizarin-bath sections are left twenty-four to forty-eight hours at a temperature of about 40° C.; they are then washed for thirty to sixty minutes in distilled water, for one to two hours in 96 per cent. alcohol; cleared in bergamot oil and mounted in balsam, or they are directly transferred from the 96 per cent. alcohol into Venetian turpentine.

Instead of alizarin I, which gives a Turkish-red tint, the 'alizarin-cyanin RRR doppelt' of the manufactory at Elberfeld may be used thus: Mordant sections (fixed in Flemming's solution) in dilute liquor ferri sulfurici oxydati (1 : 20 of water, p. 225) for twenty-four hours; wash thoroughly in distilled water; transfer to a 2·5 per cent. suspension of the dye in water after having added a few drops of 1 per cent. calcium acetate solution; stain for twenty-four hours at 40° C.; remove excess of dye by prolonged treatment with alcohol and mount in the usual way in balsam.

Benda mordants tissues fixed in formaldehyde in dilute chromic acid, and remordants sections with either a dilute solution of the liquor ferri sulfurici oxydati or in iron-alum, washes in distilled water, and stains in a very dilute watery solution of sodium alizarin sulphate, prepared thus: the alizarin salt is dissolved in 70 per cent. alcohol, and sufficient of the latter is added to distilled water to give it an amber colour: (1) Stain in this solution for twenty-four hours, when the preparations turn a reddish-brown; (2) immerse cover-glass, to which sections are attached, in distilled water, and remove superfluous water with cigarette paper; (3) stain in 0·1 per cent. watery solution of toluidin-blue, which has been heated up in a watch-glass; allow the stain to cool; (4) after fifteen minutes take the cover-glasses out of the stain; (5) rinse in 1 per cent. acetic acid; (6) dry with blotting-paper; (7) immerse in absolute alcohol; (8) differentiate in creosote

[1] Ehrlich: *Das Sauerstoffbedürfnis des Organismus*, Berlin (1885).
[2] The chemistry of the alizarin dyes is discussed in the Appendix, p. 398.
[3] Rawitz: *Anat. Anz.* 11, 294 (1896).
[4] Of the anilin dye manufactory in Höchst.

for about ten minutes, controlling under the microscope; (9) remove the creosote thoroughly with xylol, and (10) mount in balsam.

In this method sections are mordanted three times, namely, firstly in chromic acid, secondly in iron-alum for the alizarin salt, and thirdly, in sodium alizarin sulphate, which acts as a mordant for the toluidin-blue. Benda recommends both this method and also the following one for staining neuroglia fibres: (1) Prepare sections as stated above, and stain for twenty-four hours in yellow wine-coloured watery haematoxylin solution; (2) differentiate in 30 per cent. acetic acid till section is bluish-grey; (3) rinse in distilled water, and remove superfluous water with blotting-paper; (4) stain in Ehrlich's anilin-gentian violet (p. 212), or in Weigert's methyl-violet oxalic acid mixture (p. 213), or in Benda's fluid (one volume of a saturated solution of crystal-violet in 70 per cent. alcohol, one volume of a 1 per cent. solution of HCl in 70 per cent. alcohol, and two volumes anilin water[1]); (5) rinse and remove the superfluous water with filter-paper; (6) immerse in Lugol's solution (see p. 270); (7) rinse; dry with cigarette paper; (8) differentiate in anilin-xylol; (8) remove anilin completely with xylol; (10) mount in balsam.

Benda's method is easier to manage than the original method of Rawitz, as the sodium alizarin sulphate is perfectly soluble.

Notwithstanding the disparaging remarks of Bolles Lee, I hold the alizarin method to be one of the very best methods we possess.

Carmin Stains.

The best work on carmin stains is that of Paul Mayer, who has placed the making up of staining solutions on a rational basis, and I must refer the reader to his various papers for full information on this subject[2].

The chemistry of carmin is discussed on p. 439, while the principles underlying carmin staining are in every respect similar to those which have already been given for haematoxylin on p. 229. For the early history of carmin staining see p. 191.

Carminic acid was first employed by Dimmock in 1884[3], who obtained a diffuse staining, as is to be expected, for carminic acid behaves in this respect like any other acid dye, such as acid fuchsin or eosin.

Carminic acid further unites, analogously to haematoxylin, with the salts of heavy metals such as iron, it being quite immaterial whether the latter has been set free by Macallum's process (see p. 291), or has been introduced by Benda's method (see p. 231).

Carmin solutions, with the exception of alum-carmin and picro-carmin, are best used for staining tissues in bulk, but they may be used for sections, provided the tissues have been either impregnated

[1] The essential staining principle in gentian-violet and methyl-violet is the crystal-violet (p. 424).

[2] Paul Mayer: *Mittheil. aus d. Zool. Stat. zu Neapel*, 10, 480 (1892), and *Zeitsch. f. wiss. Mikr.* 16, 211 (1899).

[3] Dimmock: *American Natural.* 18, 324 (1884).

with iron salts, or that the iron has been liberated in the cells, and also for demonstrating mucus (see p. 246) and elastic fibres (see p. 447).

Picro-carmin should only be used for fresh tissues and never for paraffin sections, as for these we have better and more accurate stains [1].

The chief carmin solutions.

A. For staining in bulk.

Grenacher's formulae[2]:

(1) *Alum-carmin*: Boil for ten to twenty minutes a half to one gram of carmin in 100 cc. of a one to five per cent. solution of potash or ammonia alum. (I use a half per cent. carmin in a 2·5 per cent. potash alum solution and boil for thirty minutes.)

(2) *Alcoholic borax-carmin*: Dissolve by boiling 2·5 grams of carmin in 100 cc. of a 4 per cent. watery borax solution. Let the mixture cool and add 100 cc. of 70 per cent. alcohol. Filter on the next day [3].

P. Mayer's formulae to replace the Grenacher solutions [4]:

(1) *Carm-alum*: Dissolve carmin acid 1 gram and potash alum 10 grams in distilled water 200 cc., either at the ordinary temperature or with the aid of gentle heat; filter and add an antiseptic (thymol crystals, or 0·1 per cent. salicylic acid, or 0·5 per cent. sodium salicylate).

(2) *Para-carmin*: Dissolve at the ordinary temperature or by the aid of heat carminic acid 1 gram, aluminium chloride 0·5 gram, and calcium chloride 4 grams in 70 per cent. alcohol 100 cc. Filter during the following day.

Method of using these solutions and their advantages.

Grenacher's solutions are better than Mayer's as regards the brilliancy of staining, while they are worse in respect of their macerating action. Mayer's solutions have further a greater penetrating power. It is impossible to stain thick tissues in alum-carmin equally, but small embryos give good results.

If there be a carmin solution which does not come under the general statement made above, namely to avoid staining sections with carmin, it is Grenacher's alum-carmin. With it nuclei stain violet, while bone, muscle-fibres, and mucin stain reddish. Overstaining, with the exception of muscle, need not be feared.

Carm-alum stains the cell-plasm slightly, and if pure nuclear staining is required the tissues should be washed in a one per cent. alum solution, and the latter be carefully washed out before imbedding in paraffin. If alum fails, very dilute (1 : 1,000) hydrochloric acid may be tried.

In borax-carmin, tissues have to remain one to three days till they

[1] Hansen and his pupil Brinkmann stain first in haematoxylin and then in Weigert's picro-carmin.

[2] Grenacher: *Arch. f. mikr. Anat.* 16, 465 (1879).

[3] The first to advocate borax-carmin was Thiersch in 1865 (*Arch. f. mikr. Anat.* 1, 149 (1865)).

[4] P. Mayer: *Mittheil. aus d. Zool. Stat. zu Neapel*, 10, 489 (1891).

are coloured quite uniformly, both the nuclei and the cell-plasm being stained. To remove the carmin from the cell, leaving only the nuclei stained, it is necessary to transfer the tissues directly from the stain to 70 per cent. alcohol, to every 100 cc. of which five drops of hydrochloric acid have been added.

Soon red clouds of carmin are seen to leave the tissue, and if the latter cannot be suspended in the acid alcohol it is necessary to substitute fresh for the discoloured alcohol, and to repeat this at least five times during the first day. On the second and subsequent days the alcohol must be renewed every three or four hours, and this process of washing continued, till no more colour is extracted from the tissues.

In this acid alcohol the tissues ultimately acquire a transparent light-red colour, when the acid must be removed very thoroughly with neutral alcohol, and the tissue be treated according to the general plan laid down for paraffin imbedding on p. 167.

B. For staining mucus.

Mayer's muci-carmin (*Mittheil. aus d. Zool. Stat. du Neapel*, 12, 317 (1896)).

Carmin	1 grm.	Heat over a small flame for two minutes till mixture looks dark. Then add :
Aluminium chloride .	0·5 grm.	
Distilled water . .	2 cc.	
50 per cent. alcohol .	100 ,,	

Filter after twenty-four hours. This stock solution should as a rule be diluted five to ten times with 50 or 70 per cent. alcohol. Only the nuclei are stained if the carmin has been good [1].

Rawitz's muci-carmin (*Anat. Anz.* 15, 439 (1899)).

Carmin	1 grm.	are evaporated on a sand-bath to dryness and the residue redissolved in 50 p.c. alcohol.
Aluminium chloride .	2 grms.	
50 p.c. alcohol . .	100 cc.	

According to Mayer the effect of evaporating the solution is to drive off the hydrochloric acid, and thus to render the solution less acid. He suggests that the same end may be attained by adding either sodium carbonate or aluminium hydrate. Rawitz's method is preferable as no salts are formed.

I find that Mayer's solution stains more brightly, but that the method of Rawitz stains the mucus more intensely, and the rest scarcely at all. Both methods are good.

C. For staining fresh tissues and gum sections.

Picro-carmin is a combination which was introduced by Ranvier, according to whom it is an ammonio-picro-carminate. This, however, seems not to be the case, for Mayer holds that it is a mixture of ammonium picrate, ammonium carminate, free ammonia, a little aluminium and lime.

When one reads the literature it appears that very few people know what picro-carmin is suited for and what does not suit it. One constantly reads of complaints that paraffin sections float off in it: but

[1] I always get a slight colouration of the cell-plasm.

why people want to stain paraffin sections in picro-carmin, I do not understand, because for these we have other methods by which the different tissue elements can be stained both selectively and more precisely.

On the other hand, Mayer advocates that picro-carmin should be given up altogether. This I hold to be a mistake. There is no reagent which is capable of showing fresh tissues to greater advantage than picro-carmin, provided one is working with a good solution.

Ranvier's picro-carmin. Ranvier's directions (*Traité techn.*, 1e éd., 100) are not very definite, but even if they were definite they could not be relied upon, as a great deal depends on how much ammonia is given off. For years I obtained a good solution by adding to 1,000 cc. of a warm one per cent. solution of picric acid, 10 grms. of carmin dissolved in 50 cc. of ammonia, mixing the solutions in bright sunshine in the summer, and leaving the mixture exposed, adding from time to time distilled water to prevent drying up, and after six to eight weeks, having once more brought up the total bulk to 1,000 cc., I filtered the solution, evaporated it to 500 cc., filtered again and kept the filtrate. The picro-carmin I made last year for an unknown reason was a failure.

Weigert's method[1] of preparing picro-carmin is a very satisfactory one: Take a bottle which will hold 250 cc.; put into it 2 grms. of powdered carmin, add 4 cc. of ammonia, and leave in the well-stoppered bottle for twenty-four hours; now add 200 cc. of a saturated picric acid solution and again leave for twenty-four hours, shaking occasionally till all is dissolved that will dissolve. To make this picro-carmin stain quickly and precisely, add acetic acid in amounts just sufficient to give a slight permanent precipitate after the acid added has been thoroughly mixed with the picro-carmin solution. Again leave for one day and then filter. The carmin will show a fine precipitate which it is impossible to get rid of by filtration, but which may readily be dissolved by the addition of traces of ammonia, the latter in no way affecting the preciseness in staining which was induced by the addition of acetic acid. If this solution stains too yellow, add a little acetic acid, and if it stains too red, add traces of ammonia. Weigert states that any picro-carmin which does not stain properly can be made to do so by treatment with acetic acid.

Stöhr[2] takes 50 cc. of distilled water, adds 5 cc. of ammonia and then mixes with this ammoniacal water 1 grm. of best carmin, stirring with a glass rod. After the carmin has dissolved completely, 50 cc. of a saturated picric acid solution are added, and the mixture is allowed to stand for two days in a wide open dish and is then filtered.

Mayer's picro-magnesia carmin. All picro-carmins made with ammonia constantly give off the latter, and therefore constant solutions cannot be obtained. To overcome this difficulty Mayer has devised his picro-magnesia carmin. To prepare it proceed thus: boil for five minutes 1 grm. carmin and 0·1 grm. magnesium oxide in 50 cc. of distilled water, filter and add 3 drops of formol. This forms

[1] C. Weigert: *Virchow's Arch.* 84, 283 (1881).
[2] Stöhr: *Lehrbuch der Histologie*, 8 (1894).

the magnesium carminate solution. Then prepare[1] magnesium picrate by heating 200 cc. of a half per cent. watery solution of picric acid with 0·25 grm. of magnesium carbonate, and filtering the solution, or by dissolving in 100 cc. of distilled water 0·6 grm. of magnesium picrate.

To ten volumes of the magnesium picrate solution add 1 volume of the magnesium carminate solution. The growth of fungi is prevented by the addition of a few drops of formol.

Theoretically this solution is correct and is said to give good results with tissues stained in bulk, but I find it does not answer for fresh tissues, as the staining is too feeble.

Van Wijhe's picro-carmin[2]: Oxidized carmin (or carmein), prepared by van Wijhe's method (see p. 439), is used instead of ordinary carmin, and ammonium picrate is made by dissolving 9 grms. of picric acid in 100 cc. of 96 per cent. alcohol, then adding 15 cc. of ammonia and evaporating to dryness at 60° C.

To make the picro-carmin solution dissolve 1 grm. of ammonium picrate and 0·5 grm. of ammonium carmeate in 100 cc. of distilled water; boil this solution for fifteen minutes, bring up the bulk with distilled water, and after cooling filter off the slight precipitate.

With this solution the nuclei are apt to stain in a blotchy manner, a fault which is readily remedied by adding 5 to 10 cc. of a cold saturated picric acid solution to 100 cc. of van Wijhe's picro-carmin.

Osmo-picro-carmin. The best way of using picro-carmin is to combine it with osmium tetroxide, according to a method which I learned from Stirling many years ago, and which he believes he has seen in Ranvier's laboratory.

Take any good picro-carmin, for example that of van Wijhe, 4 parts, and 1 per cent. osmium tetroxide 1 part, and mix these just before use. The tissues are simultaneously fixed and stained. The method answers well even with strongly ammoniacal picro-carmins, to which I have not the same objection as has Mayer, because the free alkali helps to macerate or isolate epithelial cells from one another and to fix the nucleo-histones. These being basic in their character, it is quite likely that the carminic acid stains the histone radical of the nuclei.

With the blood of amphibians the osmo-picro-carmin method also gives very good results, especially if care is taken to keep the test-tube with the blood and stain moving for ten minutes to prevent the corpuscles from settling till they are fixed.

Five minutes as a rule will be found sufficient for fixing tissues in osmo-picro-carmin, and then this mixture should be replaced by ordinary picro-carmin.

The secret of obtaining good picro-carmin preparations is to know that staining will continue for months, and therefore traces of picro-carmin should always be left in the tissues, and secondly,

[1] Magnesium picrate may be obtained from Grübler and Hollborn, or Alexander Frazer, Edinburgh.

[2] J. W. van Wijhe: *Koninklijke Akademie van Wetenschappen te Amsterdam*, February, 1900.

a watery mounting medium must be used which does not set quickly, for example Farrant's solution or twenty per cent. glycerin (Stöhr). With a good picro-carmin solution it is unnecessary to employ an acid mounting medium, but frequently the addition of acetic acid improves the staining. Stöhr adds for this reason to the glycerin used in mounting as much acetic acid as will adhere to a needle.

Basic Anilin-dyes.

To make these dyes stain the cytoplasm instead of the nuclei, *Rawitz* proceeds as follows:—Paraffin sections of material fixed in Flemming's solution are placed for twenty-four hours in a 20 per cent. tannin solution made up at the room temperature; they are then thoroughly washed in distilled water and transferred to a 1 per cent. solution of tartar emetic for two to three hours at 37° C.; again thoroughly washed and stained for twenty-four hours in any of the ordinary safranin or fuchsin solutions. Now sections are either differentiated in alcohol, or after rinsing in water are transferred to 2·5 per cent. tannin for twenty-four hours. Rawitz obtained good results with the testes of salamanders, the attraction spheres and centrosomes being brought out vividly. I had an opportunity of seeing his preparations in 1894 and can testify to the value of the method.

Cox[1] demonstrates neuro-fibrils in the following way. Tissues are fixed for two to three days in

Saturated sublimate solution	30	or 15 parts
5 per cent. chloro-platinous acid (platinum chloride)	—	15 ,,
1 per cent. osmium tetroxide	10	10 ,,
Glacial acetic acid	5	5 ,,

Paraffin sections 5 μ thick are then mordanted for eight hours in 20 to 25 per cent. tannin, are washed in distilled water and are stained either with indoin- or with methylene-blue.

A. In indoin-blue, thus:

remordant in 5 per cent. tartar emetic solution for 5 to 10 minutes,
wash 10 minutes,
stain for 12 to 18 hours in 5 per cent. alum, 10 parts,
5 per cent. indoin-blue BB, 20 parts.

B. In methylene-blue, thus:

remordant in 2·5 per cent. iron-alum for 5 to 10 minutes,
wash 10 minutes,
stain 12 to 18 hours in 2 per cent. phenol solution, 15 parts,
alkaline methylene-blue, 1 to 2 parts.

The methylene-blue solution is prepared thus:

Boil for 5 minutes on a water bath,

Methylene-blue . .	1 part
Potassium carbonate .	1 ,,
Water	100 parts

[1] Cox: *Über d. fibrillären Bau d. Spinalganglienzelle*: Festschrift herausgegeben v. d. Niederländ. Psychiatr. Verein anlässlich d. 25jähr. Bestehens, 's Hertogenbosch, p. 227 (1896); *Zeitsch. f. wiss. Mikr.* 13, 499, 500 (1896).

Prepare the staining solutions immediately before use. After staining, remove the staining solution with filter-paper; differentiate in a mixture of xylol ninety parts, and absolute alcohol sixty parts; clear in xylol and mount in Canada balsam, to which cedar-wood oil may be added. Should the sections be over-stained they must be differentiated with Unna's alum-anilin mixture.

Typical examples of mordanting are, further, the bacteriological methods for staining the flagella of bacteria.

v. Ermengem uses three distinct baths, which he calls the 'bain fixateur,' 'bain sensibilisateur,' and 'bain réducteur.' These terms are bad, as the first bath containing tannin is, in reality, the mordant to which the silver of the second bath attaches itself, and the third bath, also containing tannin, produces simply a coarse precipitate with the silver salt remaining from the second bath.

(1) Bain fixateur:

2 per cent. osmium tetroxide .	1 part
10 to 25 per cent. tannin . .	2 parts

(2) Bain sensibilisateur:

0·5 per cent. silver nitrate.

(3) Bain réducteur et renforçateur:

Tannin	3 parts
Gallic acid	5 ,,
Fused potassium acetate . . .	10 ,,
Distilled water	350 ,,

Place a thin film of flagellated bacteria, for example typhoid bacilli from a young agar culture, on a perfectly clean cover-glass, allow it to dry spontaneously, fix by passing cover-glass twice through a flame; leave cover-glass at ordinary room-temperature for one hour in solution 1, or cover it with the solution 1, and heat over a Bunsen burner, till steam rises, for five minutes; wash first with distilled water and then with absolute alcohol for three to four minutes, and remove the alcohol with distilled water. Now cover the film for a few seconds with solution 2, and without washing transfer it to solution 3 for a few seconds, and back into solution 2 till the preparation turns black; wash in water, and after having allowed the film to dry spontaneously, mount in balsam.

Pitfield's method modified by Richard Muir[1].

The mordanting solution consists of:

10 per cent. watery filtered tannic acid .	10 cc.
Saturated watery corrosive sublimate . .	5 ,,
Saturated watery alum solution . . .	5 ,,
Carbol fuchsin[2]	5 ,,

After mixing these ingredients allow the precipitate which forms to deposit or centrifugalize it off. Decant the clear solution, which keeps from one to two weeks.

Stain a carefully prepared film in this solution as follows:

[1] Robert Muir and James Ritchie: *Manual of Bacteriology*, p. 116 (1899).

[2] Fuchsin 1 grm.; carbolic acid, 5 grms.; alcohol, 10 cc.; water, 100 cc.

'Pour over it as much of the mordant as the cover-glass will hold. Heat gently over a flame till steam begins to rise, allow to steam for about a minute, and then wash well in a stream of running water for about two minutes. Then dry carefully over the flame, and when thoroughly dry pour on some of this stain, which should not be more than two or three days old when used:

Saturated watery alum solution . .	10 cc.
Saturated alcoholic solution of gentian-violet	2 ,,

Heat as before, allowing to steam for about one minute, wash well in water, dry and mount in a drop of xylol-balsam.'

A method in which a mordant is combined in some unknown manner with the dye is Weigert's fuchsin + resorcin + ferric chloride stain (see p. 445).

In all the above methods mordanting was used to bring about a staining effect, and specific effects were procured by subsequent differentiation; but mordants may also be used to prevent tissues from reacting with certain reagents as they otherwise would do, as for example in Marchi's method, in which previous treatment with potassium bichromate prevents the myelin in the medullary sheaths of healthy nerves from staining with osmium tetroxide, while degenerated nerves containing oleic acid compounds are stained.

The Marchi-Algeri method.

This method is used for tracing nerve-fibres, the medullary sheaths of which have undergone a fatty degeneration. It was first published in a paper under the conjoined names of Marchi and Algeri in 1885[1]. According to Marchi[2] the method 'was studied by him' while working in Golgi's laboratory.

Marchi proceeded in this manner[3]: Tissues were left for eight days in Müller's fluid; then transferred directly to Müller's fluid two parts and 1 per cent. osmium tetroxide one part and left for five to eight days; imbedded in celloidin and mounted in xylene (not chloroform) balsam, or in pure balsam liquefied by heat.

Weigert, discussing this method[4], says he stated in 1895 that tissues might first be fixed in formaldehyde, but that according to Heilbronner formol fixation leads also to normal nerves staining; while on the other hand Luithlen and Sorgo assert that the aldehyde prevents a certain number of degenerated fibres from staining. Ciaglinski[5], when studying degeneration of the long sensory tracts in the grey substance of the spinal cord, found it necessary to first

[1] Marchi and Algeri: 'Sulle degenerazioni discendenti consecutive a lesioni della corteccia cerebrale,' *Rivista sperimentale di frenatria*, 11 (1885).

[2] Marchi: *Arch. ital. di Biol.* 17, 191 (1892).

[3] Quoted from Singer and Münzer: *Denkschr. der math.-naturw. Klasse d. k. Akad. d. Wiss. Wien*, 55.

[4] *Anat. Hefte*, Merkel u. Bonnet, 7, 1 (1897).

[5] Ciaglinski: *Neurol. Centralbl.* 773 (1896).

treat tissues with Müller's solution. He placed the cord directly into the mixture of Müller's fluid and osmium tetroxide. Pellizzi[1] recommends on the other hand to fix material for longer than eight days, preferably ten days in Müller's fluid.

Hamilton[2] first had the idea of practising Marchi's method on sections, but his method is not a Marchi method at all, for it depends on the liberation of some of the lecithin which was previously fixed by potassium bichromate. (Compare below with Haller's method.) *Langley and Anderson*[3], proceeding on the same principle, recommend us to harden tissues up to one year in Müller's solution, or in 2 per cent. potassium bichromate ; then to soak the tissues in a mixture of gum and bichromate solution ; to cut sections with the freezing microtome, and after having washed out the gum, to stain the sections in Marchi's osmium-bichromate mixture.

Busch[4] overcomes the chief difficulty in this method, namely the slight penetrating power of the osmium tetroxide, by using it in combination with sodium iodate, $NaIO_3$. The tissues, which may be 1 cm. thick, after hardening in Müller's solution are placed for six to seven days in a mixture containing osmium tetroxide 1 grm., sodium iodate 3 grms. and water 300 cc.

Starlinger[5] has specially devised an apparatus for the Marchi process. It allows of slices of uniform thickness being cut with absolute ease and with no danger of damaging the brain. It is manufactured by Reichert in Vienna[6].

Mott and Halliburton[7], having made a special study of nerve degeneration in the cat, find that after division nerves undergo no histological change during the first fifty-three hours, but after seventy-two hours the circular outline of transverse sections of normal fibres has become crinkled ; ninety-two hours after the operation the myelin sheath breaks up into short segments of irregular length, the sheath stains a blackish colour, and in some cases the whole fibre appears either completely or partially filled with a blackish material. The degenerated fatty matter, when the change is most intense, stains just like the fat cells of the surrounding adipose tissue. No further marked change occurs till the eighth day, when the degeneration shows a marked increase. From the tenth to the thirteenth day the Marchi reaction is most marked. After twenty-seven days there is still considerable evidence of the black reaction, but after forty-four days the absorption of the fatty material, which reacts with Marchi's method, is all but complete. After 106 days fine new medullated fibres make their appearance.

Mott and Halliburton compared the effects of placing nerves directly into Marchi's osmium tetroxide-potassium bichromate mixture with

[1] Pellizzi : *Arch. ital. di Biol.* (1895).

[2] Hamilton : 'On a method of demonstrating secondary degenerations of the nervous system by means of perosmic acid,' *Brain*, 20, 180 (1897).

[3] Langley and Anderson : *Journ. of Physiology*, Cambridge, 24 (1899).

[4] Busch : *Neurol. Centralbl.* 17, 476 (1898).

[5] Starlinger : *Zeitsch. f. wiss. Mikr.* 16, 179 (1899).

[6] Reichert : Bennogasse 24, Wien. The cost is £2.

[7] Mott and Halliburton : *Phil. Trans. Roy. Soc. London*, 194, 437 (1902).

the effects obtained by first treating nerves with Müller's fluid according to Marchi's original plan. They give this table:—

	Nerve-fibres.		*White matter of central nervous system.*	
	healthy.	*degenerated.*	*healthy.*	*degenerated.*
1. Marchi direct	dark greyish-green	black; adipose tissue takes the same colour	black on surface; the fluid does not penetrate well to the interior	black
2. Marchi after Müller	greyish-green, but not quite so dark	black	greenish all through	black

'In order to use the Marchi reaction for the central nervous system, it is essential to harden first in Müller's fluid, for the healthy nerve-fibres, unprotected as they are by a primitive sheath, would stain black if placed directly into Marchi's fluid[1]' (see p. 314).

I obtain the best results by injecting animals twelve to fourteen days after the lesion with 10 per cent. ordinary formol in 0·9 per cent. saline; excising the tissues after three hours; dividing the tissue into slices half a centimetre thick; placing these in 5 per cent. potassium bichromate and changing this solution twice on the first day, once on each of the succeeding days up to the fifth day; then leaving the tissues in the bichromate solution for at least two weeks; transferring them into Busch's fluid (p. 252) for three days, then renewing this fluid and leaving for five more days, always taking care to let the tissues rest separately on absorbent cotton wool; then washing very thoroughly for forty-eight hours in running water, the tissues resting again on cotton wool, and the water being led by a glass tube to the bottom of the washing-vessel; dehydrating with increasing strengths of alcohol; clearing in petroleum-ether with a boiling-point above 100° C. or in carbon disulphide, taking through the paraffin process and cutting sections about 25 μ thick.

Heller's method.

Heller's method for demonstrating medullated nerves which have been fixed in Müller's fluid depends on the principle that the lecithin-chrome compound, which does not reduce osmium tetroxide, is decomposed, and that the lecithin now liberated reduces the osmium (see chapter on protagon and lecithin, p. 313).

Heller's method[2] is as follows: Fix organs containing medullated nerves, such as skin, the tip of the tongue, or portions of the central

[1] Halliburton: 'The chemical side of nervous activity:' The Croonian Lectures (1901), London, John Ball, Sons, and Danielsson, Ltd.

[2] Heller: *Berliner Klinische Wochenschrift*, 32, 1091 (1895).

nervous system, in Müller's fluid; freeze the tissue and cut sections, wash these in water and transfer them for twenty-four to forty-eight hours to 1 per cent. osmium tetroxide at 37° C. To assist the reducing power of tissues—as Heller puts it—place the sections next in a photographic developer (pyrogallic acid 15, sodium sulphite 125, sodium nitrate 70, water 300 parts). The sections soon become quite black, and now they are differentiated in a weak potassium permanganate solution which reoxidizes the osmium which is not combined with medullated nerves or with fat. The brown colour of the reduced permanganate is removed with 1 to 2 per cent. oxalic acid.

Ford Robertson[1] has adopted the above method, but prefers to fix the tissues either in Weigert's chrome alum-copper acetate mixture (p. 236) or to mordant in this solution for a week or longer after having fixed in 10 per cent. formalin. Sections are placed for half an hour in the dark in 1 per cent. OsO_4, then in 5 per cent. pyrogallic acid for half an hour; 0·25 per cent. potassium permanganate for three to four minutes (brain sections for not more than one minute); 1 per cent. oxalic acid three to five minutes.

Author's method for class purposes. Materials are well fixed in any of the solutions of chrome salts to render the lecithin constituent of the medullated fibres insoluble. Paraffin sections of such material are fixed to mica plates or slides by my albumin method; the paraffin is removed and the sections are placed in 1 : 500 sulphuric acid in 50 per cent. alcohol to liberate the lecithin; the sections are then left for five to ten minutes in 1 per cent. OsO_4, are rinsed in water, transferred to 5 per cent. pyrogallic acid; differentiated in 0·25 per cent. potassium permanganate; cleared in 10 per cent. ordinary sulphurous acid, and mounted in neutral balsam or liquid paraffin.

CHAPTER XX.

Staining after Impregnation with Proteid and other Substances.

In connexion with mordanting may be discussed certain methods which have been introduced by Fischer, and which depend on an impregnation of the fixed tissues with solutions of albumose and similar substances.

In support of the physical explanation of staining Fischer endeavoured to impregnate granules, identical with those used in the experiments given above, with substances not acting chemically either on the granules themselves or on the dyes, being of the opinion that granules impregnated in this way would not stain, as all their mechanical affinities would be satisfied. He further made experiments with the view of altering the normal affinity for stains (chromatophily) of substances, by changing, for example, the aversion of nucleic acid for acid dyes (acidophobia), into a liking for acid dyes (acidophily) by means of impregnation with albumose solution.

It was found that inorganic salts such as sodium carbonate and

[1] Ford Robertson: *British Med. Journ.*, March (1897).

chloride, alum, potassium, nitrate, or copper sulphate in no way altered the staining power of albumose granules 'because they were not absorbed.' Notwithstanding the fact that for a whole hour granules were placed every five minutes alternately into solutions of barium chloride and sulphuric acid, the endeavour to precipitate the water-insoluble barium sulphate inside the granules was fruitless, the staining power of the granules being neither weakened nor changed.

Should, however, 'one' component of a water-insoluble salt be absorbed, then according to Fischer a precipitate is formed inside the granules; thus albumose-platino-chloride treated for three hours with 2·5 per cent. potassium bichromate, and subsequently with lead acetate, becomes partly impregnated with lead chromate and in consequence stains much less deeply.

The following seems to me a more correct explanation: before seeing Fischer's book I had made a number of experiments to precipitate the white barium sulphate in the trabeculae of the spleen, after having washed away the spleen pulp, with the view of getting distinct photographs of the spleen. I spent not only hours but days to obtain a satisfactory result, but in vain. I then attributed my failure to the fact that the barium sulphate, being an exceedingly fine precipitate, blocked at once the entrance to the intermolecular spaces, by acting on the principle of an insoluble precipitation membrane. If this explanation be true we have to assume that the barium sulphate membrane, although impermeable to one of the very constituents which formed it (probably the barium chloride), is still permeable to anilin dyes, or the granules would not stain after having been treated with sulphuric acid and barium sulphate.

It is different, however, with lead chromate, which not only forms a comparatively coarse precipitate but also one which is soluble in an excess of potassium bichromate; therefore on treating granules first with potassium bichromate and then with lead acetate, the excess of the potassium bichromate diffusing from the centre to the periphery of the large granules will dissolve the primary precipitate and make its diffusion into the granule possible.

Water-insoluble substances such as naphthalene and castor oil dissolved in absolute alcohol, or 5 per cent. sulphur dissolved in carbon disulphide, are neither absorbed by dry albumose granules nor by nucleic acid. The last observations bear out Weppen's statement that sulphur from its solutions in alcohol or in turpentine is likewise not absorbed by carbon [1].

Certain organic substances such as glycerin, urea, and cane-sugar are also said not to be absorbed by nucleic acid granules (Fischer).

Fischer believes, however, that he obtained positive proofs for his physical theory of staining, as granules either do not stain at all or only very feebly after impregnation (1) with fixing reagents, (2) with amido acids, and (3) with tannin.

The effect produced by impregnating granules with fixing reagents. Granules, after careful washing to remove all traces of the fixative which was employed to precipitate them, may for experimental

[1] Weppen: *Liebig's Annalen*, 55 (1845), 59 (1846).

purposes be impregnated either with the same fixative which was used originally or with any other fixative.

Albumose, for example, which has been precipitated with chloro-platinous acid, H_2PtCl_6, washed to remove the excess of the acid, and then finally impregnated with a new quantity of the acid, behaves in every way as if after its precipitation it had simply not been washed out at all. On the other hand, washed albumose-chromate is distinctly changed on being reimpregnated with chromic acid and potassium bichromate; I think probably because all chromates are soluble in an excess of chromic acid (p. 337).

As reimpregnation with fixatives after washing out the primary fixing reagent is in most cases equivalent to not washing out the tissues at all, it may be useful to state here how tissues stain if the fixatives be not removed.

In the first instance the question must be answered, How do stains and fixatives react on one another?

The interactions between the more commonly used fixing reagents and stains, as far as precipitation or other visible changes are affected, are indicated in the following table, in which + means an immediate or quickly appearing precipitate, while O signifies that no change was to be seen within twenty hours (Fischer).

Stain, 0·1 *per cent.*	*Chloro-platinous acid*, 1 *per cent.*	*Sublimate*, 7 *per cent.*	*Chromic acid*, 0·5 *per cent.*	*Picric acid*, 0·5 *per cent.*	*Tannin*, 2 *per cent.*	*Formaldehyde*, 4 *per cent.*	*Osmium tetroxide* 1 *per cent.*
Picric acid .	O	O	O	—	O	O	O
Acid fuchsin .	O	O	O	O	O	O	O
Light-green .	O clear but dirty violet colour	O	O	O	O	O	O
Indulin . .	O	O	+	O	O	O	O
Eosin . .	+	O	+	+	O	O	O
Basic fuchsin .	+	+	+	+	+	O	O
Methyl-green .	+	+	+	+	+	O	O
Safranin . .	+	+	+	+	+	O	O
Methylene-blue	+	+	+	+	+	O	O

Flemming's solution and potassium bichromate resemble in their action chromic acid, while Hermann's solution reacts as does its most potent constituent, namely the chloro-platinous acid.

On the strength of this table Fischer endeavours to solve the question why tissues after the use of certain fixatives do not stain readily, unless the fixative be very carefully washed out.

As regards the readiness with which tissues stain after having been fixed in different reagents, Fischer finds that reagents may be divided into three groups:

1. Indifferent fixatives which do not require washing out: alcohol, formaldehyde, acetic acid, nearly indifferent picric acid.

2. Partial colour-enemies: chromic acid, potassium bichromate, Flemming's solution, and corrosive sublimate.

3. Complete colour-enemies: chloro-platinous acid H_2PtCl_6[1], Hermann's solution, tannin, osmium tetroxide, Altmann's solution, and iodine alcohol.

The answer to the question why tissues do not stain after certain fixatives is, according to Fischer, as follows: The tissues are blocked mechanically by the fixing reagents which fill all the molecular and micellar[2] interspaces. That this view is correct, and that any chemical interpretation is out of the question, Fischer proves to his satisfaction by reasoning as follows: Albumose granules precipitated by chloro-platinous acid or tannin, fixed to cover-glasses, rinsed in water to remove the fixing reagent outside the granules, but not washed out, leaving therefore the precipitant inside the granules, do not stain on being placed into basic dyes. It is true that examined superficially they appear stained, but this is only due to a precipitate being formed on the surface of the granule, for it was shown (see table, p. 256) that both chloro-platinous acid and tannin do precipitate basic dyes. That this precipitate does not prevent the entrance of the basic dyes into the granule, and that it is not the real cause why the granules do not stain, is readily shown by the behaviour towards acid dyes of albumose-tannate granules containing free tannin. Although acid dyes are not precipitated by tannin, yet they do not stain the albumose tannate as long as it contains 'free' tannin, because the latter fills mechanically all the capillary spaces in the albumose tannate.

Fischer's view seems to me untenable for these reasons: (1) tannin (p. 74), being a more strongly acid substance than are the acid dyes, is not likely to be displaced by the latter from any compound it may be forming; (2) if tannin was held only mechanically it should diffuse out into the surrounding staining solution, according to the ordinary laws governing physical diffusion, for Fischer himself has shown in the above table (p. 256) that tannin does not interact with acid dyes. If it did diffuse out then the acid dyes ought to diffuse in, but they do not. How tenaciously tannin adheres to albumose-platino-chloride is stated on p. 260.

I have just now used the expression that tannin would not diffuse out from any compound it may be forming, and on this very point my conceptions differ completely from those of Fischer, as will be shown more fully later. It will suffice now to point out that the very process of washing out liberates in the precipitated proteid certain side-chains which play the essential part in ordinary staining with acid or basic colour-radicals. These side-chains being satisfied by the chemically strong tannin, there is no call for the chemically feeble acid dyes, and hence the albumose tannate does not stain as long as the so-called 'free' tannin is present.

I further cannot accept Fischer's explanation that basic dyes do not stain unwashed albumose chloro-platinate, because the latter is blocked mechanically by an excess of chloro-platinous acid, but believe the

[1] Called by Fischer and other histologists platinum chloride, or $PtCl_4$.

[2] See p. 36.

following view, which Fischer discards, to be the correct one: As indicated in the table (p. 256), chloro-platinous acid precipitates basic dyes, and, therefore, on placing albumose chloro-platinate granules containing an excess of chloro-platinous acid in a solution of a basic dye, the acid will tend to diffuse out, but on coming in contact with the basic dye a precipitation membrane is formed on the surface of the granules. This membrane may be coarse to begin with, but gradually it will become finer and finer till it is possible for the excess of the chloro-platinous acid to diffuse outwards, while it is impossible for the basic dye consisting of large molecules to diffuse inwards [1].

In this case the negative staining result is due to the inability of the dye-molecules to get into the albumose granule, while the negative result in the case of the albumose-tannate was due to the chemically more efficient colourless radical, the tannin, keeping out the less efficient coloured radical.

The staining results obtained by the impregnation of albumose granules with various reagents, after the primary or original fixative had been removed by washing, Fischer has given in an abridged form in the accompanying table.

Media used for a second impregnation.	*Albumose-platino-chloride, methyl-green + fuchsin, 8 minutes.*		*Albumose chromate, triacid, 4 minutes.*	
	large granules.	*small granules.*	*large granules.*	*small granules.*
Alcohol	bluish green	red	red	violet
Formaldehyde, 4 per cent.	,,	red with bluish tint	yellowish-red	red, not violet
Nitric acid, 50 per cent.	,,	,,	red	red, not neutrophil
Picric acid, 0·5 per cent.	pale bluish-green	,,	yellow and yellowish-red	violet
Chromic acid, 0·5 per cent.	colourless	,,	yellowish-red	red, no violet
Potassium bichromate, 2·5 per cent.	colourless or very pale green	,,	,,	,,
Sublimate, 3 per cent.	bluish-green	red	,,	red, with slight violet tint
'Platinum chloride,' 1 per cent.	,,	red, partly bluish-red	colourless	pale red, never violet
Osmium tetroxide, 1 per cent.	colourless	colourless	,,	colourless
Iodine alcohol	,,	,,	,,	,,
Altmann's solution	,,	pale reddish-blue	,,	pale red
Flemming's solution	,,	,,	pale yellowish-red	,,
Hermann's solution	,,	,,	colourless	,,

[1] This case is quite analogous to the precipitation membrane formed by sulphuric acid and barium sulphate (p. 255).

Alcohol, formaldehyde, nitric acid, and sublimate are indifferent, and picric acid nearly so, while the chrome-compounds, as usual, have no affinity for methyl-green. Osmium tetroxide and iodine-alcohol are especially active in preventing staining. 'Platinum chloride' hardly affects the staining power of the platino-albumose granules, while albumose chromate is distinctly changed by chromic acid and potassium bichromate. This, as has been stated above, is readily explainable, as 'platinum chloride' forms very definite compounds which are stable in an excess of the platinum salt, while chrome precipitates are readily soluble in chromic acid (p. 337), and are also readily affected by the platinum salt, as seen in the table. Fischer holds that the platinum is simply absorbed, no chemical interaction between it and the chromate taking place, and mentions in support of this view the observations of Weppen, who discovered that carbon, after having taken up one metal, is still capable of precipitating a second one. Fischer states further that after washing out the fixative used for impregnating the granules, their ordinary staining power is restored.

Nucleic acid precipitated with 10 per cent. H_2PtCl_6 is not changed in any way by formaldehyde, while 10 per cent. H_2PtCl_6 in twenty hours impregnates the granules to such an extent that the nucleic acid no longer stains with basic dyes, although its normal want of affinity for acid dyes, or acidophobia, persists.

Impregnation-Experiments with amido compounds, namely:

asparagin or amido-succinamide, $\begin{matrix} CH_2(NH_2)\cdot CCOOH \\ | \\ CH_2CONH_2 \end{matrix}$

leucin or α-amido-isocaproic acid, $(CH_3)_2\cdot CH_2\cdot CH(NH_2)CO\cdot OH$, and glycocoll or amido-acetic acid, $NH_2—CH_2—CO\cdot OH$.

All these compounds contain the basic amido group NH_2 and the acid carboxyl radical $CO\cdot OH$, in consequence of which they may act either as bases or as acids. They produce, however, as far as test-tube experiments can be relied upon, no change either in basic or in acid dyes, and may therefore as far as the dyes are concerned be regarded as indifferent. Fischer reasons further that as amido compounds occur already in the proteid molecule it is unlikely that such molecules will link on still more amido groups, and that it is practically altogether out of the question that amido compounds in any way can interact chemically with the precipitated albumose or nucleic acid granules.

Albumose-chloro-platinate impregnated for twenty hours with 1 per cent. asparagin or 0·2 per cent. leucin is in no respects altered, while 5 per cent. glycocoll renders staining somewhat less intense.

Thymus nucleic acid precipitated with 10 per cent. H_2PtCl_6 and treated with leucin or asparagin showed no changes, but 5 per cent. glycocoll acting for twenty hours prevented all basic dyes, such as fuchsin, safranin, methyl-green, gentian-violet, and Ehrlich's triacid, from staining, but methylene-blue made an exception. Acid dyes are also in no way taken up. After removing the glycocoll by

washing for half an hour in hot, or for twenty hours in cold water, the nucleic acid precipitate stains once more in all basic dyes.

Impregnation with Tannin or digallic acid,

$$C_6H_2(OH)_3—CO·O—C_6H_2(OH)_2—CO·OH.$$

Tannin is a fairly strong acid because it contains 5 hydroxyl groups, OH, and one carboxyl group, CO·OH[1]. Tannin is readily absorbed by albumose granules, but only to a small extent by nucleic acid. Again the question arises whether this reagent acts chemically on the platino-albumose and platino-nucleic acid granules. Fischer negatives the idea for these reasons: Albumose precipitated with tannin is somewhat soluble in an excess of the reagent, and disappears instantly in 0·2 per cent. caustic soda solution; in cold water it is only slightly soluble, but by strong hydrochloric acid becomes changed into unsightly masses and is also partly dissolved. Therefore whether tannin acts on the albumose-platino-chloride granules is according to Fischer readily decided by treating these granules first with tannin and subsequently with such reagents as will dissolve or erode ordinary albumose tannates. As albumose-chloro-platinate granules, after they have been acted upon by tannin, do not show any sign of solution when treated with caustic soda, it follows according to Fischer that the tannin is absorbed only mechanically; that it blocks the intermolecular and micellar spaces and thereby prevents the entrance of the dyes.

Treatment with caustics not only does not affect the shape of the granules, but by extracting the tannin restores that power of staining which the granules possessed before their impregnation with tannin.

Here again, the explanation offered above seems to me to account better for the facts than Fischer's view. It is not at all necessary to suppose that chemical interaction amounts to the formation of albumose tannate, but it may simply mean the satisfying of a number of side-chains without affecting the essential nucleus of the molecule at all.

How firmly tannin adheres to albumose-platino-chloride may be gathered from Fischer's statement that washing out in frequent changes of distilled water for twenty hours will not restore the affinity for dyes, and that even hot water acting for thirty minutes fails, but after at least two hours' treatment with hot water the staining power is restored. Yet 0·20 per cent. of caustic soda completely re-establishes the affinity for dyes in five minutes. To me this certainly looks as if sodium hydrate as the stronger (inorganic) turned out the feebler (organic) base from its union with the tannin. Sodium tannate being formed allows now of ready chemical union of the basic proteid radical with the acid colour radicals.

Nucleic acid precipitated with chloro-platinous acid does not absorb tannin nearly as readily as does albumose, but why it does not do so

[1] The hydroxyl groups in tannin act analogously to the hydroxyl groups in phenol (p. 385), and in picric acid (p. 184).

Fischer leaves unexplained. On my chemical theory basic side-chains are absent in nucleic acid as long as it is unaltered, and therefore no affinity between it and tannic acid can exist as tannin is devoid of basic side-chains.

The impregnation of granules of albumose chromate with nucleic acid makes it possible to stain the granules not only with acid dyes, but also with such basic dyes as methyl-green, which as a rule does not stain chromate preparations at all.

The impregnations with albumose are especially important, as it is possible to fix this proteid in the granules by means of ordinary fixatives, and thus to study the effect between comparatively loose and very dense compounds.

The best method of impregnation consists in treating various granules (p. 201) with 1 to 5 per cent. albumose solutions, either for twenty hours at room-temperature or for fifteen to thirty minutes in a hot solution.

Suppose (1) that a primary granular precipitate for any reason is unstainable ; (2) that this precipitate is impregnated with an albumose solution ; (3) that the whole impregnated compound is placed in a staining solution ; and it follows that by the incorporated albumose storing the colour, the compound as a whole will become coloured. It is possible by this means to completely overcome, for example, the disinclination of osmium and platinum-albumose granules to combine with acid dyes.

Similarly nucleic acid granules which do not stain normally with acid dyes at all, after impregnation with deutero-albumose will stain equally well with acid and basic dyes, because the nucleic acid radical has affinity for the basic dyes while albumose attracts the acid dyes. Staining with the Biondi modification of Ehrlich's triple stain (p. 220) for three minutes, leads to the formation of bull's-eyes according to the principles laid down above (p. 207), and Ehrlich's eosinophil mixture stains the albumose with which the nucleic acid granules are impregnated, a deep red colour.

The enormous change produced by impregnation may be gathered from Fischer's table, in which the primary colour affinity is compared to the secondary one which has been induced by the albumose-impregnation.

Thymus nucleic acid, 10 *per cent., precipitated with* (1 + 10) H_2PtCl_6.	*Biondi's mixture*, 3 *minutes.*	*Ehrlich's triacid*, 4 *minutes.*	*Ehrlich's eosinophil mixture*, 15 *minutes.*
Original colouration	pure green	pure green	colourless
After 20 hours' impregnation with 5 per cent. deutero-albumose	large granules red, small granules green	large granules yellowish-red, small granules violet or neutrophil	large granules red, small granules dirty grey, indulin-like.

Nucleic acid granules not impregnated with albumose do not stain with M. Heidenhain's iron-alum haematoxylin method, because the ammonia iron-alum $FeNH_4(SO_4)_2$, being a ferri-salt, dissociates partly

into free acid and colloidal iron oxide. The iron compound being thus an acid compound has as little affinity for the acid nucleic acid as have all other acid stains. After impregnation with albumose, nucleic acid stains, however, readily with iron-alum haematoxylin.

After washing out the albumose with hot water for several hours, the affinity for acid dyes becomes greatly diminished.

Fischer recommends the impregnation with albumose solution in all cases where during histological research for any reason it may be desirable to stain in acid dyes.

What struck me on first reading his account, was the question how much albumin may be taken up into the tissues by fixing sections on slides by either Paul Mayer's method or my own; and secondly, suppose fixatives are used which leave certain cell-constituents in solution or render them soluble, what fallacies may arise? As to the first point, experimental evidence shows that there is no danger in using my egg-white method, and the same will hold good for Mayer's method.

If it is possible to overcome the acidophobia of nucleic acid by impregnating it with albumose and then staining directly, what will be the result of precipitating the albumose inside the granules by fixing reagents?

The result, according to Fischer, is that nucleic acid + albumose granules now stain neither in acid nor in basic dyes at the ordinary temperature, 'not because the intra-granular spaces are blocked by the newly precipitated albumose,' but owing to the diminution of the mechanical affinities of the albumose-platino-chloride, 'which we must imagine stretched over the nucleic acid micellae in an unmeasurably thin layer. This layer may be regarded as sufficient to satisfy the mechanical affinities of the nucleic acid, but at the same time to display so few affinities itself, that the dyes which reach it by diffusion are not attracted sufficiently to produce colouration. We require by the use of heat to increase the energy of diffusion, and thereby to drive the stains into the impregnated and secondarily-fixed granules.'

Not only dyes but also albumose no longer enters the granules after the secondary fixation till heat is applied, which increases the power of diffusion into the albumose. 'As albumose is certainly not held in the granules by chemical forces, but only physically, by absorption, so we must without fail assume that the process of staining, which seems to occur in exactly the same manner, must also depend only on mechanical affinities.'

However much I admire the great amount of work which Fischer has done, I cannot but regret that he should have been carried away by the single idea of proving all staining to be purely physical. According to his own showing doubly impregnated granules are much denser than ordinary granules, requiring for example for the removal of dyes much more prolonged treatment with strong acids than do non-impregnated granules (p. 261). To me the simplest explanation of the phenomena above recorded, seems to be, that the application of heat causes the granules to swell, that both molecules and intermolecular spaces are dilated, and that thereby the absorption of such

large molecules, as are met with in albumose and in the anilin-dyes, is made possible. As regards albumose, heating may also have a tendency to dissociate albumose, which at ordinary temperatures I believe to be a polymer.

How a secondary impregnation with albumose, by the means of heat, of granules impregnated primarily with glycocoll or platinum chloride or albumose, can throw any light on the theory of staining I fail to see. The albumose is sucked in or driven into the granules during the application of heat, owing to a change in the physical state of the granules. As the granules cool the albumose is retained in the gradually narrowing intermolecular channels, by a purely physical process, and the granules at the ordinary temperature becoming too dense to allow the large molecules of the dye to diffuse inwards, no staining takes place. Heating the granules simply amounts to opening gates for the entrance of the dye molecules.

PART V.

CHAPTER XXI.

Impregnation Methods.

The impregnation methods aim at depositing metallic salts in certain tissues, with the view of reducing these salts subsequently into coloured compounds. This end may be attained either directly by bringing fresh tissues into contact with solutions of such salts, or indirectly after having treated tissues by some other reagent with which the metallic salt will combine. The ultimate staining effect always depends on a chemical union between the metal and some organic compound.

A. *Direct application of metallic salts.*

1. **Silver salts.** The first, according to v. Recklinghausen, to observe a precipitation of silver salts between the cells of the cornea was Flinzer, in 1854[1]. In 1856 His[2], after impregnating corneae with the silver nitrate stick, believed he had discovered extra- and intra-cellular lymph spaces. He recognized however, in 1862, that, to begin with, the deposit is always extra-cellular, and that later it may become intra-cellular, owing to a solution of the primary deposit and its transference to the cells. This phenomenon is readily observed by keeping corneae which have been stained with silver nitrate for some days in water (Ranvier). Stricker, in 1874, also noticed how living corneae with silver nitrate gave positive pictures, while dead corneae gave negative ones.

To v. Recklinghausen, however, belongs the credit of having made silver staining a general histological method in 1860[3]. He impregnated with a 0·25 to 0·5 per cent. silver nitrate, for 20 to 40 seconds, transferred the tissues directly to 0·75 per cent. sodium chloride, moved them quickly about and exposed to light. He believed the silver nitrate to be deposited in those parts which contain much water, while the more resistant tissue elements withstood it. In 1862 v. Recklinghausen described the outlines of endothelial cells in lymph-vessels, and used for their demonstration a 1 : 400 to 1 : 500 solution of silver nitrate.

With v. Recklinghausen's discoveries started a period of lively discussion as to whether the results obtained by this observer were artefacts or something reliable. Adler thought the outlines of cells to be fibrils resembling elastic fibres ; Harpeck called the appearances

[1] Flinzer : *De argenti nitrici usu et effectu praesertim in oculorum morbis sanandis*, Diss. 1854, bei Coccius gearbeitet.

[2] His : *Beiträge z. norm. u. path. Hist. d. Cornea* (Basel, 1856).

[3] v. Recklinghausen : 'Eine Methode mikrosk. hohle und solide Gebilde von einander zu scheiden,' *Virchow's Arch.* 19, 451 (1860).

artefacts; Auerbach, in 1865, explains outlines as due to a deposit of silver salt in accidental furrows in the epithelium, the silver having united with proteids and sodium chloride. This view was also adopted by Henle in 1866. Schwalbe, in 1869, said the precipitate was caused by a fine layer of albuminous material covering the surfaces of membranes. Feltz, in 1870, got similar outlines with membranes of egg-white, collodium, and also with photographic paper. Severin had similar results. As late as 1874 Adamkiewicz[1] explained the outlines of cells as lying under the cells, and as serving to connect the epithelium with the subjacent connective tissue.

This review shows that dissenters are always plentiful in histology.

In support of v. Recklinghausen the following names may be quoted:

Broneff and Eberth, in 1864, recognized that the silver is precipitated in the cement substance between the cells.

Müller, in 1867[2], combined silver nitrate with silver iodide dissolved in potassium iodide, with the view of preventing the nuclei from becoming stained, and occasionally obtained a staining of the corneal nerves.

Ranvier, in 1868[3], impregnated first with silver nitrate, then washed in distilled water, reduced the silver salt in sunlight, substituted gold for the silver salt by transferring the tissue to 1 per cent. gold chloride, stained nuclei in ammonium carminate, in which the ammonia had been neutralized with oxalic acid, and preserved the tissue in a mixture of equal parts of 5 per cent. oxalic acid and glycerin.

Legros[4] recommended hyposulphite of soda to remove the superfluous silver salt, a plan also recommended by Duval, who uses a 2 per cent. solution for a few seconds. Reich, in 1873, introduced the following very good method: Wash out the blood-vessels with a $\frac{1}{8}$ to $\frac{1}{4}$ per cent. potassium nitrate; follow up with a $\frac{1}{6}$ to $\frac{1}{4}$ per cent. silver nitrate for a few minutes; substitute lukewarm filtered gelatin for the silver solution; harden in alcohol, expose to light and examine in water or gelatin. Alférow, in 1874, used organic silver compounds, such as the acetate, citrate, picrate, but especially the lactate. The latter he employed in 1:800 watery solution, to which 10 to 15 drops of free acid had been added, so as to prevent the precipitation of all silver salts with the exception of the albuminates and chlorides.

Hoyer, in 1876, added sufficient ammonia to a silver nitrate solution to allow the precipitate which is formed at first to just disappear. This solution was then diluted to 0·5 or 0·75 per cent. strength. Only silver outlines are stained by this method.

Dekhuyzen's[5] method is a modification of those of Reich and Alférow. He washes the tissue first with an isotonic solution of potassium nitrate (1·3 in 100); then impregnates for 3 to 6 minutes

[1] Adamkiewicz: *Berl. Klin. Wochensch.* 29, 355 (1874).
[2] Carl Friedrich Müller: *Virchow's Arch.* 41, 110 (1867).
[3] Ranvier: *Journ. de l'Anat.* (1868), p. 216.
[4] Legros: *ibid.* 275.
[5] Dekhuyzen: *Anat. Anz.* (1889), p. 790.

in a 0·25 per cent. silver nitrate solution containing 3 per cent. of nitric acid; transfers the tissue to 3 per cent. nitric acid; then through 96 per cent. alcohol into clove oil, and then reduces the silver salt in bright daylight. The nuclei are readily stained with the ordinary basic anilin-dyes or with haematein alum. Fischel's mixture, used by Bergh[1], consists of 1 per cent. silver nitrate 2 parts, formic acid 1 part, and distilled water 1 part, and reduces the silver nitrate in the dark.

To attain reduction in the dark several other means have been adopted: thus v. Thanhoffer[2] states that his pupil Krauss uses a pale pink solution of potassium permanganate to 'reduce' even in the dark, and another pupil, Carl Oppitz, transfers tissues, after impregnation with silver nitrate, to 0·25 to 0·5 per cent. stannic chloride for 3 minutes, and moves them to and fro to quickly 'reduce' the silver.

Personally I use a 1:200 watery solution, to every 100 cc. of which 1 drop of nitric acid is added. The animals are injected with this solution if blood-vessels are to be studied, and the blood-vessels are kept patent after impregnation with 5 per cent. gelatin solution. After fixing the tissues in 95 per cent. spirit for 24 hours, they are imbedded in gum or dextrin, sections are cut with the freezing microtome, and the sections, kept in alcohol till a bright sunshiny day, are exposed just sufficiently long to reveal the outlines of the cells.

For the study of areolar tissue, tendon, cornea, or mesentery (after having brushed off the endothelium), I use a 5 per cent. solution of silver nitrate, allow it to act for 2 minutes, wash in a stream of distilled water, and cover preparation again with 5 per cent. nitrate solution, and expose the tissue in this solution to direct sunlight till brown. The connective-tissue cells remain uncoloured. After a thorough washing in distilled water, the preparations are placed for 1 minute in a 0·5 per cent. toluidin blue solution to bring out the nuclei, and after dehydration are mounted in balsam.

What compound the silver forms with the tissue is not known, but it is probably a proteid in combination with chlorides and carbonates. Rabl[3] states that the precipitate is certainly not metallic silver, as it is soluble in sodium hyposulphite, and believes it to be an albumin nitrate or silver oxide.

2. Gold salts. Cohnheim[4], in 1866, introduced gold staining. Tissues were treated with 0·5 per cent. gold chloride, exposed for some days to daylight in water acidulated with acetic acid, and mounted in glycerin or balsam. This primitive method, if successful, gives as good results as any of the following.

Arnold, in 1867, was the first to impregnate with an acid mixture of gold chloride. He employed a mixture of equal parts of 1 per

[1] Bergh: *Anat. Hefte*, 14, 392 (1900).
[2] v. Thanhoffer: *Das Mikroskop u. seine Anwendung* (Stuttgart, 1880).
[3] Rabl: *Sitzungsber. Akad. Wiss. Wien*, 102, 349 (1893).
[4] Cohnheim: 'Über d. Endigungen d. sensiblen Nerven in d. Hornhaut,' *Virchow's Arch.* 38, 343.

cent. acetic acid and 0·02 to 0·05 per cent. gold chloride. Spinal ganglia were left in 3 to 4 cc. of this mixture for 3 to 4 hours, and were transferred, as soon as a violet colouration was seen, to 1 per cent. acetic acid for 3 to 5 days, till they were of a fairly intense colour; then the connective-tissue sheath was removed, and the ganglia were exposed to intense light on a white surface, in glycerin, to which a few drops of strong acetic acid had been added. After 8 to 10 days the reduction was completed.—This method is good, and is the original of Apáthy's method (see p. 278).

Bastian, in 1868, dissolved 1 gram of gold chloride in 2000 cc. of water, and then added 1 drop of hydrochloric acid for every 75 cc. of the solution. Reduction was brought about in a mixture of equal parts of alcohol and formic acid, either at the ordinary temperature or, to hasten the reaction, at the temperature of an incubator. In 1869 Bastian recommended to reduce the gold in ferrous sulphate.

Hénocque, in 1870, introduced the tartaric acid method for reducing the gold. Hénocque used a nearly saturated solution of the acid in a well-stoppered bottle at the ordinary temperature, while Klein and Chrchtschonovitsch heated the solution up to 50° C. and then allowed it to cool.

Lavdowsky, in 1874, recommended Nesteroffsky's plan of reduction by means of ammonium sulphide. A drop of this reagent is put on the section, is then quickly removed with filter-paper, and the preparation is mounted in glycerin.

Loewit, in 1875, introduced the gold and formic acid method, which in principle is a modification of Bastian's method. Put small pieces of tissue, 1 to 2 mm. thick, for half a minute into formic acid 1 part and distilled water 2 parts. When the tissues have become transparent, transfer them to a second vessel containing 1 to 2 cc. of 1 per cent. gold chloride for 10 to 15 minutes till yellow. Now leave tissues for some time (3 to 6 hours) in one-third formic acid in the dark, then transfer to pure formic acid and keep in the dark till reduction is completed. Tease in distilled water, and examine in it or in glycerin.—This is a good method.

Kupffer[1], in 1876, demonstrated star-shaped cells in the liver by means of the following gold method: Sections cut with a Valentin's knife (p. 176) are rinsed in 0·6 per cent. salt solution, or still better hardened for 15 minutes in 0·05 per cent. chromic acid; are transferred to Gerlach's gold solution, consisting of gold chloride 1 part, hydrochloric acid 1 part, and water 10,000 parts; they are kept in this solution in the dark for 48 hours, till they become red or reddish-violet. Now v. Kupffer[2] proceeds according to his old method, or uses a gold solution containing formaldehyde. This latter method is based on Zsigmondy's observation that colloidal gold is precipitated by the addition of sodium chloride or of acids. Proceed thus: After killing the animal leave the liver for 1 to 2 hours in the animal; wash out the blood-vessels, and fully distend them with 1 : 10,000 chromic

[1] C. Kupffer: 'Über Sternzellen d. Leber,' *Arch. f. mikr. Anat.* 12, 353 (1876).
[2] *Arch. f. mikr. Anat.* 54, 254 (1899).

acid, and keep them distended by ligaturing the blood-vessels (or proceed according to the older method); cut sections with a Valentin's knife (or with a freezing microtome), and transfer them to a vessel containing the following gold solution: freshly prepared formaldehyde (= 40 per cent. solution), free from formic acid, 1 part, gold chloride 1 part, and water 10,000 parts. The sections must not cover one another, and the gold solution should be about 3 cm. deep. In 36 to 48 hours (occasionally in 12 hours) the star-shaped cells will appear black on a colourless or faintly pink ground. Thick sections prepared in this way may be taken through the paraffin process and be cut into thinner sections. The sections are best mounted in balsam or in acid-free glycerin, as acids dissolve the gold precipitate. By the same method the fine connective-tissue fibrils (Gitterfasern) between the liver cells will occasionally be found deeply stained, and then the star-shaped cells as a rule are only faintly coloured.

Thin, in 1876, to ensure rapid penetration of the tissues, injects animals through the arterial system with a 0·25 per cent. gold chloride solution; excises tissues and leaves them for a short time in some additional 0·25 per cent. gold solution.

Ranvier, in 1878, to stain the nerves in the cornea, proceeded thus: The cornea was excised; left for five minutes in freshly-prepared lemon juice filtered through flannel; transferred for fifteen to twenty minutes to 3 cc. of 1 per cent. gold chloride, and reduced by exposure for two to three days to direct sunlight in 25 to 30 cc. of distilled water, to which a few drops of acetic acid had been added. Muscle fibres were transferred from the gold solution to 20 per cent. formic acid, and were kept for twelve hours in the dark.

Ranvier, in 1880, used gold chloride and formic acid in combination, so as to counteract by the salt the deleterious action of the acid. Proceed thus: Mix four parts of 1 per cent. gold chloride with one part of formic acid; allow mixture to boil up and then cool it. Leave muscles in this mixture for twenty minutes, and skin for two to four hours. Reduce the gold in the dark with 20 per cent. formic acid, or in daylight in water acidulated with acetic acid. If the gold chloride and formic acid mixture be boiled too long, reduction commences to take place at once, and this should be avoided. As, however, the whole object of boiling is to give the gold a tendency to rapid reduction, it is best to let the test-tube boil up three times in the course of ten seconds, and then to cool it under the tap. Both of Ranvier's methods are good.

Böhm's method, communicated by Carrière in 1882, is as follows: Place tissues for 20 minutes in 50 per cent. formic acid till they are clear; rinse in distilled water; leave for twenty minutes in a small amount of 1 per cent. gold chloride; rinse in distilled water and transfer for twenty-four hours to a large quantity of Pritchard's reducing solution (amyl-alcohol 1 part, formic acid 1 part, water 98 parts), and leave in the dark.—Apáthy's methods are given on p. 278.

My personal experience with gold methods is, that within certain limits it is quite immaterial how one proceeds. During the last

three years I have spent a considerable time in trying to solve the laws governing gold-staining with but little success. Different samples of gold chloride were employed in 1 : 1,000 to 1 : 50 strengths; they were used by themselves and in combination with mineral and organic acids, with salts and even feeble alkalies; at low, ordinary, and high temperatures; tissues were impregnated with various electrolytes, and the electrolytes in the tissues were extracted with different strengths of glycerin; tissues living, stale, and very 'high,' were compared; after the gold bath, tissues were washed out with distilled water, or transferred directly to reducing media varying in their chemical properties, their strengths, their rapidity of action, and the temperature at which they were used. Good results are occasionally got with the most diverse methods, and methods we are relying on most will frequently fail for some unknown reason.

If any general rules may be laid down they are these: use (1) either large quantities of very dilute solutions (1 : 1,000) for 6 to 8 up to 24 hours, or (2) very small quantities of strong solutions (1 : 100 to 1 : 50) for a very short time, 20 to 60 minutes. In the former case a uniform penetration is ensured, and sections of the whole piece of tissue may be cut; but with stronger solutions the surface is over-impregnated, the centre slightly or not acted upon at all, while the intermediate zone is the best. I have obtained the best results with dilute solutions of feeble reducing agents, or with such non-reducing substances as acetic acid, which latter acts mainly by preventing bacterial putrefaction, the actual reduction of the gold being brought about by the tissues themselves.

One point is certain: If we examined the tissues at the right time we should get in many cases the desired effect, for I have seen nerves gradually make their appearance, attain a certain degree of excellence, and gradually fade away again on replacing the tissue in the reducing fluid. This last observation has already been made by Drasch[1], who explains the fact that stale tissues give better results than fresh ones, by assuming various reducing substances, probably acid in character, to be developed in the tissues as the result of post-mortem changes. The beneficial action of acids, used in conjunction with gold, according to Drasch, amounts to the production, in fresh tissues, of the very same, or at least similar, substances which occur normally after death.

It may be of interest to state that pure tryptophane (p. 324), which I obtained through the courtesy of Hopkins, is a powerful reducer of gold, platinum, and palladium. As we further know that tissues are very apt to undergo auto-digestion, it is quite possible that tryptophane in many cases determines the reduction of gold.

The fact of connective tissue staining very feebly with gold may be explained on the same ground, as tryptophane is absent in white fibrous tissue.

Gold prepared by Zsigmondy's method (p. 39) is changed by neutral salts, acid salts, or fixed alkalies, at first from red into blue

[1] Drasch: *Sitzungsb. Akad. Wiss. Wien*, 82, 171 (1881), and 88, 534 (1884); also *Abh. math.-phys. Kl., Sächs. Ges. Wiss.* 14 (1887); *Zeitsch. f. wiss. Mikr.* 4, 492 (1887).

colloidal gold, and on further addition of these reagents the gold is precipitated. Acetic acid changes the colour first into violet-red, and then into black, while ammonia produces no change. Alcohol precipitates the gold completely, and the precipitate under certain undetermined conditions remains water-soluble.

In some cases gold is converted in the tissues, first, into colloidal gold, and this by the excess of electrolytes in certain tissues becomes changed into the insoluble blue variety.

To v. Kupffer belongs the credit of having first realized the importance of Zsigmondy's work in relation to histology (see p. 267).

3. **Mercuric chloride.** Diomidoff[1] recommended for general histological fixation for the central nervous system 7 per cent. (i.e. saturated) corrosive sublimate solution, and, after remarking that corrosive sublimate is a better fixative than alcohol, incidentally states that a brain of cat left for four days in sublimate is well preserved; but that after forty-five days everywhere a fine deposit (called by the author pigment) was seen, and that after eighty days all the pyramidal cells throughout their length were crowded with the granules. The corrosive sublimate-albuminate precipitate behaved thus: Prolonged warming in distilled water dissolves the deposit; alcohol and ether do not remove the deposit, but turn the black colour into brown; strong alcoholic caustic potash and 25 per cent. acetic acid produce no effect; 25 per cent. nitric acid changes it in the course of some days; 30 per cent. potassium iodide turns it a yellowish-brown colour; Lugol's solution (water 100, potassium iodide 6, iodine 4 parts) removes it in five minutes, and distilled water at the ordinary temperature dissolves it in three to four weeks.

4. **Palladium chloride.** Schulze[2], in 1867, who recommended osmium tetroxide to Max Schultze, has also introduced palladium chloride. Tissues, 'about the size of a bean,' are placed for twenty-four hours in 15 to 30 cc. of a 1 : 1,000 palladium chloride solution, when, particularly, the muscular tissue, glands, and epithelial cells will stain while the connective tissue remains unstained. Henle and Merkel[3], in 1871, employed this salt for the study of the central nervous system, and von Thanhoffer, in 1880, especially recommends it for the corneal nerve fibre.

5. **Osmium tetroxide** is used chiefly for fixing purposes, but it also serves to stain fat (p. 306) and the medullary sheaths of nerves (pp. 313 and 314).

B. *Indirect method of applying metallic salts.*

Under this heading I have included all those methods which require a double impregnation, the reagent, which was applied firstly, acting in a certain sense as a mordant.

Landois[4], in 1865, treated tissues first with salts of lead, iron,

[1] A. Diomidoff: *Wratsch*, 24, 472-474 (1887); and *Zeitsch. f. wiss. Mikr.* 4, 499 (1887).

[2] Fr. Eilhard Schulze: *Centralbl. f. d. med. Wiss.* 13 (1867).

[3] Henle and Merkel: *Handbuch d. Nervenlehre d. Menschen* (Braunschweig, 1871).

[4] Landois: 'Die Impregnation d. Gewebe mit Schwefelmetallen,' *Centralbl. f. d. med. Wiss.* 55 (1865).

copper, platinum, and corrosive sublimate; washed them in distilled water, and then transferred them into dilute solutions of ammonium sulphide, or dilute sulphuretted hydrogen, to produce the coloured metallic sulphide compounds.

Kronthal's[1] lead sulphide method (1899) I can strongly recommend for the central nervous system. I find it best to make the lead formiate for myself instead of buying it from Merck, as does Corning[2]. Kronthal proceeds thus: (1) To a saturated solution of lead acetate pure formic acid is added drop by drop till fine needles of the less soluble lead formiate fill the whole vessel. The acetic acid, which is set free, and the mother liquor are filtered off; the lead formiate crystals are then dissolved in water till a saturated solution is obtained. The latter I find deposits a basic salt after some time, and is therefore best prepared fresh, which is readily done. (2) Equal parts of this saturated lead formiate solution and 10 per cent. formol are mixed, and fresh pieces of the central nervous system are kept in this mixture for five days. (3) The tissues are then rinsed without washing with a mixture of equal parts of 10 per cent. formol and sulphuretted hydrogen. The, to most people, disagreeable smell of the latter becomes hardly perceptible owing to the action of the formol. After a few minutes the first discoloured portion of the formol-sulphuretted hydrogen mixture is poured off and new solution is substituted, in which the preparations are left for five days. (4) Then the tissues are gradually dehydrated, imbedded in celloidin, cleared in a mixture of equal parts of crystalline phenol and xylol, and mounted in Xylene-Canada balsam.

Corning fixes tissues first in 10 per cent. formaldehyde, and then treats it according to Kronthal's procedure.

Polaillon, in 1866[3], fixed in perchloride of iron, washed the sections, and then produced an ink by transferring the sections to tannin solution till they were black. In successful preparations the nervous elements in peripheral ganglia were stained deeply, while the connective tissue remained colourless.

Leber, in 1868, first impregnated the cornea of the frog for five minutes with 0·5 to 1 per cent. solutions of a ferrous salt (for example ferrous sulphate); he carefully removed the epithelium while in this solution, washed the cornea in water, and then transferred it to 1 per cent. ferricyanide, moving the cornea about in this solution. There is produced in this way an impregnation of the ground-substance with Prussian blue, according to the reaction given on p. 290. Leber also employed a slightly ammoniacal solution of cupric hydroxide[4], $Cu(OH_2)$, for the primary impregnation of the cornea, and then applied secondly a 5 per cent. potassium ferrocyanide, $K_8(CN)_{12}Fe_2$. Analogously lead acetate and potassium bichromate give the yellow chromate of lead precipitate.

[1] Kronthal: *Neurol. Centralbl.* 18, 196 (1899); and *Zeitsch. f. wiss. Mikr.* 16, 235 (1899).
[2] Corning: *Anat. Anz.* 17, 108 (1900); and *Zeitsch. f. wiss. Mikr.* 17, 85 (1900).
[3] Polaillon: *Journ. de l'Anat. et Phys.* 3, 43 (1866).
[4] This reagent readily dissolves cellulose, and therefore should not be filtered through filter-paper (Schweizer, *Journ. Pract. Chem.* 72, 109).

Gerlach, in 1871[1], hardened cords in 1 to 2 per cent. ammonium bichromate for fifteen to twenty days; cut sections and left these in 1 : 10,000 gold chloride till they were of a pale-blue colour (about ten to twelve hours); then washed them in distilled water containing 1 : 2,000 to 1 : 3,000 hydrochloric acid, and finally transferred sections to 60 per cent. alcohol containing 1 : 1,000 hydrochloric acid. Boll, in 1873[2], stated that the shorter the time tissues were left in the ammonium bichromate mixture the better were the results. The best results were obtained after up to eight days' treatment. Alcohol should not be brought into contact with the sections. Too much 1 : 10,000 gold solution must not be used, nor should sections remain in it for more than twelve hours, this time being the best.

Golgi's methods.

Golgi, in 1873[3], gave the first account of the silver-impregnation method which now goes by his name, and which is used to bring out nervous elements. He originally fixed small pieces of the central nervous system in bichromate solutions, and then treated them with 0·5 to 1 per cent. silver nitrate, by which means nerve-cells were turned black.

Hunter[4] fixes even a whole brain or spinal cord for 1½ to 6 months in Müller's solution, then excises small pieces; rinses them in water and treats them at body-temperature for twenty-four hours in 0·75 per cent. silver nitrate, which is changed occasionally. He finally imbeds in celloidin and cuts sections.

Golgi, in 1879[5], published his corrosive sublimate-bichromate method. Portions of the cerebrum, cerebellum, and cord, 1 to 2 cm. thick, are left in Müller's solution or 2·5 per cent. potassium bichromate for fifteen to twenty days; they are then transferred for eight to ten days to 0·25 to 0·5 per cent. corrosive sublimate solution, which has to be changed daily till the brain looks as if it had been fixed in corrosive sublimate. Sections show ganglion-cells and processes with transmitted light stained black, while with reflected light the cells appear white.

The chief modifications of these two original methods are:

1. **Cox's modification** of the Golgi bichromate-sublimate method. I have used it ever since its publication in 1891. It is by far the most reliable method for young and adult brains, as blood-vessels have only a slight tendency to stain, while nerve-cells are more numerous than after other Golgi methods; and, above all, very large pieces may be fixed in this solution provided the solution is used in one-half the strength advocated by Cox. In 1892 I fixed a whole human cerebellum in it, and found the solution had penetrated well[6].

[1] Gerlach: *Stricker's Handbuch*, 678 (1871).

[2] Boll: *Arch. f. Psych. u. Nervenheilkunde* 4, 52 (1873).

[3] Golgi: 'Sulla struttura della sostanza grigia del cervello,' *Gazz. med. ital. Lomb.* Ser. 4, t. 6.

[4] Hunter: *Journ. of Anat. and Physiol.* 32, 109 (1897).

[5] Golgi: 'Un nuovo processo di tecnica microscopica,' *Rendic. R. Istituto Lombardo*, 12, 205–210 (1879).

[6] Death in this particular case was due to paraldehyde poisoning, and the

Cox's solution consists of

5 per cent. potassium bichromate . . .	10 parts
5 per cent. corrosive sublimate . . .	10 ,,
5 per cent. potassium chromate . . .	8 ,,
Water	15 to 20 parts.

I prepare it in the following way: The bichromate and the sublimate solutions are mixed together, the chromate solution is mixed with the water, and then added to the sublimate-bichromate solution. The mixture is next warmed up to the temperature of the incubator, when a slight haziness seen in the cold becomes more evident, and some mercuric chromate is thrown down. The solution may be used in this strength, or be diluted with an equal quantity of distilled water. Portions of the brain of adult animals measuring 1 cm. in thickness or entire brains of young animals, such as kittens a fortnight old, are placed on cotton wool in this solution and left in the incubator for 24 hours, when the solution is changed for some fresh solution. After a second change on the third day, the vessel, which should contain the solution to the brain in the proportion of 30 : 1, is sealed with vaseline and is left in the incubator (38° C.) for at least a month, but preferably for two months, as then a great many more axis-cylinders become impregnated.

Cox gives as his reason for adding the potassium chromate, the necessity for reducing, as far as possible, the normally acid reaction of the bichromate, as otherwise the axis-cylinders will not become impregnated, although the cells do.

Sections are cut either by free hand, or the tissues may be transferred directly from the impregnating fluid to neutral 20 per cent. dextrin, be left in this solution according to size for 1 to 3 days, and then be cut on a freezing microtome. It is best not to cut sections too thin, about 50 to 100 μ being the best thickness.

Cox converts the white sublimate impregnation into a black one by means of 5 per cent. sodium carbonate, but I prefer ammonia, as preparations appear to be more permanent if they have been treated by the latter. Sections, after they have become black, are carefully washed in water, very thoroughly dehydrated and mounted in neutral balsam, without a cover-glass, according to Golgi's plan, to allow the solvent of the balsam to evaporate as quickly as possible and thereby to render the balsam hard and to prevent deterioration of the metallic deposit.

If it be desired to use cover-glasses, one of the two following methods may be adopted:

(*a*) By means of wax fix a cover-glass by its corners temporarily to a slide; place the preparation on the cover-glass and cover it with thick balsam; add next day some more balsam if necessary, and after a week, when the balsam has become quite hard, detach the cover-glass from the slide and invert it so that the preparation is facing the slide. Taking care that the balsam does not touch the

whole brain reacted in so perfect a manner that I have never been able again to attain such good results with the human brain.

slide, fix the cover-glass first by its four corners, and subsequently along only three sides fix it to the slide with Krönig's cement (see p. 382).

(*b*) Melt solid balsam in a vessel; heat a slide till the heat can just be borne when holding it against the cheek; transfer the section to the slide, and without delay cover specimen with the liquefied balsam; hold the cover-glass for one second over the Bunsen burner to heat it, and quickly lay it on the balsam, which should still be liquid. If a warm stage is available, it will prevent the balsam from solidifying and save both time and preparations.

2. **Golgi's rapid method**[1] differs from his original slow method (p. 272) in only requiring on an average about 5 to 7 days. It comprises the following operations: Very small pieces of perfectly fresh tissue are placed in a mixture of:

2 to 2·5 per cent. potassium bichromate	4 parts
1 per cent. osmium tetroxide	1 part

In this mixture the pieces remain at least 2 days, and not more than 10 or at the most 15 days. From the third up to the seventh day some pieces are daily taken out of the osmium-bichromate mixture and transferred to 0·75 per cent. silver-nitrate solution, and left in it for 24 to 48 hours, when the impregnation of the nerve-cells will be completed. Tissues may remain in the silver bath indefinitely long, provided the solution is changed as often as it turns yellow. To obtain sections, tissues may be imbedded after thorough dehydration with absolute alcohol in either paraffin or celloidin by one of the short methods (pp. 169, 174), or the tissue may be placed in some 20 per cent. dextrin solution for 1 hour, then be transferred to the freezing microtome and be cut into thick sections.

Treat these sections as stated on p. 273 under Cox's method.

Golgi[2] now uses this mixture:

3 per cent. potassium bichromate	2 parts
1 per cent. osmium tetroxide	1 part

and remedies over-impregnation with the osmium-bichromate mixture by transferring tissues according to the amount of over-hardening from 8 hours to 10 days, and even longer, to this mixture:

2 to 3 per cent. potassium bichromate 4 to 5 per cent. copper sulphate	equal parts

and from this straight into the silver-nitrate solution[3].

To demonstrate the intra-cellular network in nerve-cells, Golgi employs Veratti's mixture, composed of:

[1] Golgi: *Arch. Ital. Biol.* 7, 15 (1886).

[2] Id.: *Cinquantenaire Soc. Biol. Paris*, Livre jubil. 514 (1899).

[3] Golgi in 1894 (*Intern. Monatssch. f. Anat. u. Physiol.* 11, 328; and *Zeitsch. f. wiss. Mikr.* 11, 389 (1894)) rejuvenated osmium-bichromate material which had remained in this mixture for weeks and months by placing it in half-saturated copper acetate solution and renewing the latter till it remained perfectly clear. Finally, the tissue was re-impregnated in the osmium-bichromate mixture for five to six days.

5 per cent. potassium bichromate . .	2 parts
0·1 per cent. chloro-platinous acid [1]. .	2 parts
1 per cent. osmium tetroxide . . .	1 to 2 parts

3. **Golgi's mixed method** is considered by him to give not only the best histological details, but also to be recommended because it allows of a more systematic research being made, provided sufficient material is available. He proceeds in this way :—A large number of pieces are put into 2·5 per cent. potassium bichromate, and, commencing with the third day and continuing up to about the thirtieth day, pieces are daily transferred to the osmium-bichromate mixture (p. 274) and treated according to his rapid method (p. 274).

4. **Ramón y Cajal's double-impregnation method** [2] is especially to be recommended for sympathetic ganglia and the spinal ganglia.

The spinal cords of chick embryos are fixed for 3 days in Golgi's osmium-bichromate solution (p. 274) ; are then transferred for 36 hours into 0·5 to 0·75 per cent. silver-nitrate solution ; put back into the osmium-bichromate mixture for 36 to 48 hours, or for the same length of time into a solution containing only 1 part of osmium to 10 parts of bichromate ; rinsed in water and replaced for 36 to 48 hours in silver-nitrate solution. Should tissues not have remained sufficiently long or too long in the first osmium-bichromate bath, it becomes necessary to repeat the impregnation for a third time.

5. **Kolossow's method** [3]. Tissues are impregnated for 1 to 7 days in a 3 to 5 per cent. potassium-bichromate solution containing 0·25 per cent. osmium tetroxide ; rinsed in distilled water ; dried with blotting-paper, and transferred for 2 to 3 days to a 2 to 3 per cent. silver-nitrate solution containing 0·25 to 0·5 per cent. osmium tetroxide. This is a good method.

6. **Strong's formaldehyde-bichromate method** [4]. This observer seems to have been the first to substitute formaldehyde for osmium tetroxide in Golgi's method. His mixture contains 2·5 to 5 per cent. formalin (formalin or formol = 40 per cent. formaldehyde) and 3·5 to 5 per cent. potassium bichromate.

Kopsch [5] also uses a freshly-prepared mixture consisting of :

3·5 per cent. potassium bichromate . .	40 parts
Pure formol	10 „

Tissues of not more than 2 cm. cubic capacity remain in this mixture, which should be moved about occasionally, for 24 hours ; they are then transferred to pure 3·5 per cent. bichromate solution, and remain here different lengths of time according to the nature of the tissue. Thus all glands (as, for example, liver and stomach for secretory ducts) remain 1 day ; the retina 1 to 2 days ; the central nervous system 3 to 6 days according to the effect desired, axis-cylinders coming out first, then nerve-cells, and lastly the neuroglia.

[1] Veratti calls chloro-platinous acid 'platinum chloride.'
[2] Ramón y Cajal : *Zeitsch. f. wiss. Mikr.* 9, 241 (1892).
[3] Kolossow : *Arch. f. mikr. Anat.* 49, 592 (1897).
[4] Strong : *Anat. Anzeiger*, 10, 494 (1895).
[5] Kopsch : *ibid.* 11, 727 (1896).

7. **Gerota's**[1] principle of fixing first in 5 to 10 per cent. pure formaldehyde for one week, and impregnating subsequently with 4 per cent. bichromate for 3–5 days, is one which may be generally applied. **Bolton**[2] fixes a whole brain of a cat for 5 weeks to 5 months in 5 per cent. formol, then excises small pieces, treats these with 1 per cent. ammonium bichromate for 5 days, and then transfers them to 0·75 silver-nitrate solution. Liver and stomach fixed in 50 per cent. formol for 24 hours, and then treated with 2·5 per cent. potassium bichromate for 1 to 3 days, renewing the bichromate thrice every day, and finally impregnating with 1 per cent. silver nitrate, gives very good results with cat material.

8. **Berkley**[3] fixes pieces of liver not thicker than 1·5 mm. while yet warm into a warm half-saturated solution of picric acid for 15 to 30 minutes; then for 48 hours at a temperature of 25° C. into a mixture of:

2 per cent. osmium tetroxide . . .	16 parts
Saturated solution of potassium bichromate	100 „

which has been exposed for several days to direct sunshine. The final silver bath is used in strengths of 0·25 to 0·75 strength for 5 days or longer. Sections are cut in celloidin. I have obtained good results with this method, but not better than with that of Kopsch (p. 275).

Method of preventing precipitation of silver chromate in the tissues.

To minimize the irregular deposit of silver chromate in the tissues, Sehrwald[4] surrounds them with a coat of gelatin. He pours 10 per cent. gelatin, which is just liquid, into a paper box, imbeds the tissues, and then places the box in the silver solution. After the impregnation is completed the gelatin is washed off with a warm saturated solution of silver chromate. I find this method gives good results provided the gelatin which comes in contact with the bichromate is not rendered insoluble by the action of light. I proceed thus: Either in the photographic dark room or in the evening with artificial light, tissues tied loosely to a piece of string are immersed three times into liquefied 10 per cent. gelatin, and as soon as the gelatin has set, they are then put into the silver bath and the latter kept in the dark room or in some dark place. It appears to me that surrounding a tissue with gelatin makes impregnation slower, although why it does so is not easy to see, as the rate of diffusion of silver nitrate through gelatin is practically the same as that through water. I allow always one day longer for the silver bath if tissues are enveloped in gelatin.

[1] Gerota: *Internat. Monatssch. f. Anat. und Physiol.* 13, 108 (1896); and *Zeitsch. f. wiss. Mikr.* 13, 314 (1896).
[2] Bolton: *Zeitsch. f. wiss. Mikr.* 15, 367 (1899).
[3] Berkley: *Anat. Anz.* 8, 772 (1893).
[4] Sehrwald: *Zeitsch. f. wiss. Mikr.* 6, 456 (1890).

Methods for rendering Golgi preparations more permanent.

It was pointed out on p. 273 that it is imperative to mount Golgi preparations without a cover-glass, to allow the balsam to become hard as rapidly as possible, but why this should be necessary is difficult to say. By some the deterioration of the preparations is attributed to moisture, by others to diffusion currents under the cover-glass, while I believe the chief cause to be the acid nature of ordinary balsam, owing to oxidation processes being at play (see p. 377), for preparations mounted in Price's No. 1, pure, neutral glycerin keep very well.

Greppin[1], in 1889, treats sections for 30 to 40 seconds with 10 per cent. hydrobromic acid, HBr, washes them very carefully in water, dehydrates, clears with clove oil, and exposes them for 10 to 15 minutes to direct sunlight, when they become of a violet colour. Sections may be counter-stained by Pal's method and should be mounted in balsam.

Obregia[2] converts the silver deposit into a gold one by the following procedure: (1) Add to 10 cc. of absolute alcohol ten drops of 1 per cent. solution of gold chloride, and expose this mixture for half an hour to diffuse daylight. (2) Leave sections in this mixture in the dark for 15 to 30 minutes according to their thickness. (3) Wash sections rapidly in 50 per cent. alcohol and then in water. (4) Remove the superfluous silver salt with 10 per cent. sodium hyposulphite solution. (5) Very carefully work out the hyposulphite. (6) Stain in carmin if thought necessary, and (7) mount in balsam with a cover-glass. If the hyposulphite is not thoroughly washed out, the preparations are sure to deteriorate quickly.

Zimmermann[3] fixes in formol-Golgi, and converts the silver deposit either into the chloride or into the sulphide, by moving about the paraffin sections fixed to slides for 10 to 15 minutes, in large quantities of a mixture of 0·75 sodium chloride 1 part and 96 per cent. alcohol 2 parts, and then exposing the sections in 75 to 96 per cent. alcohol to diffuse sunlight till the chloride has become black, which takes about half a day (direct sunlight may be used, but is apt to cause too deep a colouration); or sections are left for half to one hour in 100 cc. of absolute alcohol, to which a few drops of ammonium hydrosulphide, $H(NH_4)S$, are added. Too much ammonium sulphide or too long an exposure to its action removes all the silver, therefore caution is necessary. This method, according to my experience, is the one to be recommended most, as the interrelationship of the gland-ducts and gland-cells can be accurately determined, not only in the stomach (Zimmermann), but also in the liver, by counterstaining the silver chloride sections in thionin (toluidin-blue) or safranin and the sulphide sections in Delafield's haematoxylin.

[1] Greppin: *Arch. f. Anat.* (*und Physiol.*), Suppl. 55 (1889); and *Zeitsch. f. wiss. Mikr.* 7, 66 (1890).

[2] Obregia: *Virchow's Arch.* 122, 387 (1890); and *Zeitsch. f. wiss. Mikr.* 8, 97 (1891).

[3] Zimmermann: *Arch. f. mikr. Anat.* 52, 554 (1898).

Theory of Golgi's process.

There is a great deal of superstition about this method, as has been pointed out by Hill [1]. It is immaterial whether the tissues are kept exposed to light or whether they are left in the dark. The essential point is that both the bichromate and the silver solution penetrate rapidly. The best results he obtained by injecting potassium bichromate solutions through the aorta, after having added 1 per cent. of lactic acid to the solution to prevent a contraction of the blood-vessels. Instead of silver nitrate, $AgNO_3$, the nitrite $AgNO_2$ may be used in 0·75 per cent. strengths, after formic acid has been added to it in the proportion of 1 : 1,000. The idea of adding a reducing agent to the silver-nitrate bath originated with van Gehuchten, who later discarded this method as no real difference is produced by the addition of this acid.

Hill is of the opinion that not silver chromate is deposited in the cells and their processes, but a reduced salt (sub-salt) of silver, and that the deposit takes place in the fluid 'neuroplasm' and not in the neuro-fibrils.

A great deal has been written as to where the silver is deposited, but it will suffice for the purposes of this book if I state, that in many cases the deposit begins in the lymph spaces round the cells and that the deposit may proceed no further, as is readily determined by cutting sections sufficiently thin to enable one to trace the same cell through several sections. In this case the impregnation is faulty, for whenever complete impregnation has taken place the whole cell-body, inclusive or exclusive of the nucleus, may be stained. Golgi's and Veratti's more recent work has shown that a fine mesh-like deposit may take place on the outer surface of the cell, and that a certain system of fibrils, which I regard as lymph-channels, may be stained in the cell-plasm. Finally, I have once seen nothing but the nuclei impregnated in a long series of spinal ganglion cells of the cat. It follows therefore that the silver salt may be deposited in the most diverse situations.

As to the chemical nature of the deposit nothing is known, except that it is not simply silver chromate.

Apáthy's gold methods.

(*a*) **The direct method.** Apáthy [2] places fresh tissues in a 1 per cent. solution of 'yellow' gold chloride, aurum chloratum flavum (p. 79), for 2 to 12 hours, in the dark; transfers them to 1 per cent. formic acid, and exposes them to direct sunlight, which must have free access from all sides. After the first hour the formic acid, which now is somewhat coloured, is changed for new solution, care being taken to move the tissue about as little as possible to prevent the gold being washed out of the tissue. Mount and examine in pure glycerin or gum-syrup. This method Apáthy calls his Vorvergoldung, which means the tissues are treated with gold before they are treated with other

[1] Alexander Hill : 'The Chrome-silver Method,' &c., in *Brain* (1896), p. 1.
[2] Apáthy : *Mitth. aus d. Zool. Stat. zu Neapel*, 12, 728 (1897).

reagents. I call it the direct method, the indirect one being that given below, as in this case tissues are first brought into contact with corrosive sublimate, and subsequently with gold chloride.

(*b*) **The indirect method.** Apáthy has devised the following method for the demonstration of neuro-fibrils. It comprises a primary fixation, a secondary impregnation, and a final reduction.

1. The fixation. Tissues of invertebrates should be fixed either in a saturated solution of sublimate in 0·5 per cent. salt solution for 8 to 12 hours, or if thin membranes, for 2 to 2½ hours, or still better, for double the length of time in a mixture of equal parts of absolute alcohol and the sublimate solution just mentioned, while vertebrate material not thicker than 1 mm. is fixed for 24 hours in my osmium-sublimate mixture (p. 97), which, as I recommended, must be freshly prepared. Apáthy fixes tissues in the osmium mixture in complete darkness to prevent undue blackening. To remove the sublimate, tissues are placed for 6 to 8 hours in several changes of a watery solution of 0·5 per cent. iodine in 1 per cent. potassium iodide, and are then directly transferred to 95 per cent. or stronger alcohol, and left in it overnight. The last traces of sublimate are now removed with 0·5 per cent. iodine and 1 per cent. potassium iodide dissolved in 95 per cent. alcohol, the tissues remaining in this alcoholic iodine solution till they are yellow throughout. After having washed out carefully all traces of iodine with absolute alcohol, the tissues are imbedded in celloidin or are taken through the paraffin process. If the latter method be chosen, employ pure chloroform, or chloroform 4 parts and ethyl-ether 1 part[1]. Xylene must not be used. It is necessary to hurry the whole process up to this point as much as possible.

2. The impregnation. The paraffin sections, 10 μ thick, are fixed to slides and the paraffin is removed with chloroform, and the latter with alcohol, while the celloidin sections are taken through bergamot oil into alcohol. The sections are transferred from the alcohol to distilled water; left in it for at least 2 and not more than 6 hours; are treated with 1 per cent. formic acid for 1 minute; rinsed well in water and impregnated for 24 hours (or at least overnight) in 1 per cent. 'yellow' gold chloride (p. 79). The gold solution having been removed with filter-paper or the slide having been quickly rinsed in distilled water the sections are ready for the final reduction.

3. The reduction. Each slide is placed obliquely in a separate vessel with the sections looking lowermost to prevent the gold precipitate settling on them, and the vessel is then filled with 1 per cent. formic acid and exposed continuously for 6 to 8 hours to the direct sunlight if the temperature does not rise above 20° C., or otherwise into as bright a light as is obtainable by reflectors.

Should one so desire it, the nuclei may be stained in the usual manner and the preparation be finally mounted in balsam.

Lee[2] recommends to reduce in a weak solution of formalde-

[1] The ether is added to allow the tissues to sink in the chloroform.

[2] Lee and Mayer, *Grundzüge d. mikr. Technik*, 240 (1901).

hyde, with or without an addition of formic acid (to that normally present).

Material imbedded in paraffin keeps indefinitely, as does that in celloidin, provided the celloidin-blocks are preserved in glycerin jelly to which some thymol has been added.

v. Kupffer's gold methods.

v. Kupffer's methods of demonstrating the star-cells and the connective tissue of the liver are discussed on pp. 267 and 268.

PART VI.

CHAPTER XXII.

On the Chemistry of some Tissue Constituents.

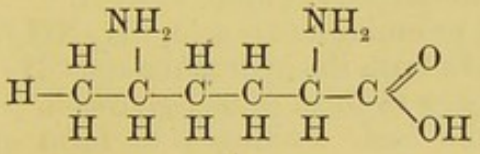
In giving a short summary of some facts bearing on the chemistry of the cell, only those points will be touched upon which seem to have a distinct bearing on the principles underlying the process of staining. Assuming that the majority of staining reactions are comparable to the process of salt-formation, if they are dependent at all on chemical affinity, the following questions naturally suggest themselves: Which tissue elements are basic, which acid in their character? Do certain tissues contain radicals which are absent in others? Can tissues act as oxidizing or reducing agents?

Bases. If we adopt the view of Kossel and his followers, we must regard as the chief basic constituents of the cell the two substances protamin and histone.

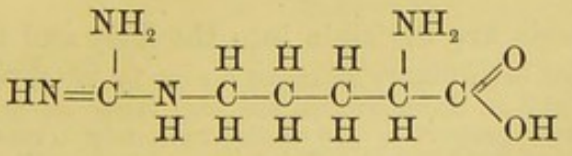
Protamin was discovered by Miescher in the milt or spermatic fluid of the salmon. It is now called salmin by Kossel, who reserves the term protamin as the generic name for that group of basic substances conforming in its behaviour to salmin. Thus from the milt of the herring clupein, from that of the sturgeon sturin, have been prepared. From the spermatozoa of the carp, ox, and boar, no protamin was obtainable but another (basic) proteid, while from the sea-urchin, Arbacia, a histone-like substance, arbacin, can be isolated.

Diamido acids. By the decomposition of protamin are formed the so-called hexone bases, namely lysin or diamido-caproic acid; arginin or guanidin-diamido-valerianic acid; lysatin, which is perhaps only a compound between lysin and arginin; and finally histidin $[C_6H_9N_3O_2]$, of unknown constitution.

Lysin



Arginin



The hexone bases are thus diamido acid compounds, forming, as shown by Kossel, definite basic nuclei called protamins, round which the less basic (or acid) mono-amido acids group themselves. (The mono-amido acids are mentioned on p. 282.)

Histone, first isolated by Kossel from the red corpuscles of goose's

blood, is characterized by the following reactions[1]: Heat precipitates it only in the presence of salts, and then never completely; nitric acid forms a precipitate in the cold which disappears on heating and reappears on cooling; ammonia forms a precipitate soluble in the smallest excess of the reagent, provided salts, and in particular ammonia salts, are absent (other alkalies are said to behave similarly); the 'alkaloidal precipitants,' namely, phosphotungstate, phosphomolybdate, sodium picrate, and the potassium ferrocyanate, require no previous addition of an acid, as in the case of proteids, and therefore histones are true bases and not 'pseudo-bases[2].' They act by displacing the potash of the ferrocyanate, becoming simultaneously insoluble.

Histone differs from all other 'proteid' substances essentially in two points, namely, its high percentage of hexone bases (see above), and the basic character of the molecule as a whole.

As examples of histone may be enumerated the nucleo-histone of the thymus (Lilienfeld), and the globin constituent of haemoglobin (Preyer and Schulz[3]).

The micro-chemical reactions are given on p. 320.

Two other basic radicals closely related to one another are cholin and neurin[4]. Of these, cholin is for micro-chemical reasons the more important, as it forms one important constituent of lecithin (see p. 312).

Cholin is trimethyl-hydroxyethyl-ammonium hydroxide

$$\left.\begin{matrix}(CH_3)_3 \\ CH_2CH_2\cdot OH\end{matrix}\right\} N\cdot OH$$

and was synthetized by Wurtz[5] from ethylene oxide C_2H_4O, trimethylamin $(CH_3)_3N$ and water. It is a strongly basic substance absorbing carbon dioxide from the air, and decomposing on being boiled with water into trimethylamin and glycol.

Acid albumins are compounds of an acid with denaturalized albumin, the latter playing the part of a base. When we, therefore, stain acid albumins with acid dyes we are staining basic compounds.

Mono-amido acids. It has to be borne in mind that these bodies may act either as bases or as acids, the basic character depending on the presence of the amidogen group, NH_2, while the acid tendencies are due to the carboxyl group, COO·H.

Glycocoll or amido-acetic acid, $CH_2(NH_2)COOH$, unites with hydrochloric acid to form the hydrochlorate, $CH_2(NH_2)COOH—HCL$; with the base copper to form copper-glycocoll, $[CH_2(NH_2)COO]_2Cu + H_2O$; and with the basic amidogen to form glycocoll-amide, $CH_2(NH_2)—CO(NH_2)$.

Amido acids are divisible into the fatty and the aromatic series.

[1] These have been kindly arranged for me by W. A. Osborne. The accounts in textbooks, e. g. in Hammarsten, are very vague because substances differing widely in their properties are indiscriminately termed histone. See also Cohnheim, *Chemistry of Proteids.* English translation (Macmillan).

[2] Kossel and Kutscher: *Zeitsch. f. phys. Chem.* 31, 210 (1900).

[3] W. Preyer: *Pflüger's Arch.* 1, 395 (1868), and *Die Blutkrystalle* (Jena, 1871); F. N. Schulz: *Zeitsch. f. physiol. Chem.* 24, 449 (1898); ibid. 25, 16 (1898).

[4] The constitution of neurin is fully discussed in Neumeister's *Lehrbuch d. physiol. Chemie*, 91–92 (Edition 1897).

[5] Wurtz: *Ann. d. Chem. u. Pharm.* Suppl. 6, 116 (1868).

The fatty amido acids contain the NH_2 group always in the α-position, and are represented by the glycocoll just mentioned, and by leucin or amido-caproic acid, $CH_3(CH_2)_3—CH(NH_2)COOH$, while the chief representatives of the aromatic series are:

Tyrosin, $C_6H_4(OH—CH_2—CH(NH_2))—COOH$, 1 : 4,

Phenyl-α-amido-propionic acid, $C_6H_5(CH_2—CH(NH_2))COOH$, and

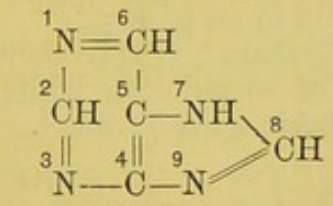
Skatol amido-acetic acids. These aromatic amido acids, according to Nencki, Hopkins and Cooper, occur normally in the cell. See also p. 324.

True acids:

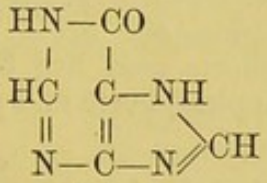
(*a*) *Nucleic acids.* [For example from salmon spermatozoa, $C_{40}H_{54}N_{14}O_{17}—2P_2O_5$.] Altmann[1] (1889), the discoverer of this group of substances, isolated the first acid from yeast. Since then many similar bodies have been discovered; and these, according to Hammarsten, may be grouped into two classes, namely (1) nucleic acids containing a non-reducing group forming laevulinic acid (Kossel), as, for example, those obtained from the spermatozoa of the salmon and from the thymus, and (2) acids which possess a pentose (guanyl) or a hexose (yeast) radical (Kossel, Bang). All nucleic acids are characterized by the presence of a comparatively large amount of phosphorus, and by the absence of sulphur; the phosphorus stands to the nitrogen in the proportion of 1 : 3, as in the thymus-, salmon-, and yeast-nucleic acids, or in the proportion of 1 : 5, as in guanyl (Kossel, Liebermann[2]). Nucleic acid is a dibasic acid.

By prolonged treatment with dilute acids or alkalies, nucleic acids are decomposed into the so-called nuclein bases or alloxure bases; for example, into hypoxanthin, xanthin, adenin, guanin, &c. All these bodies are related to uric acid, and are, as has been shown by E. Fischer, derivatives of purin, which contains a metadiazin $HC\langle^{N=CH}_{N}$, and an imidoazole ring $\begin{matrix}HC—NH\\HC—N\end{matrix}\rangle CH$.

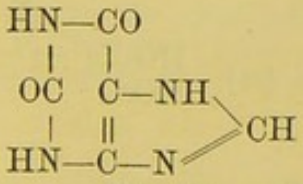
By the oxidation of purin, the substances hypoxanthin, xanthin, and uric acid are formed, while from its amidozation adenin results.



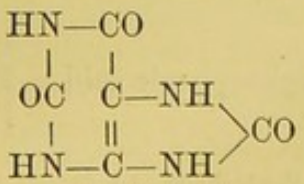
Purin.



Hypoxanthin (6-oxypurin).



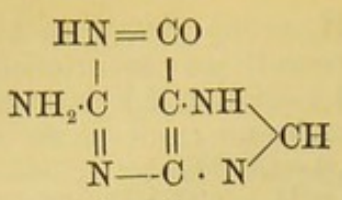
Xanthin
(2, 6-oxypurin).



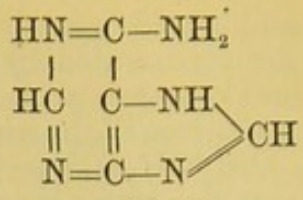
Uric Acid
(2, 6, 8-trioxypurin).

[1] Altmann: *Du Bois-Raymond's Arch., Physiol. Abtheil.* (1889), p. 524.

[2] Liebermann: *Pflüger's Arch.* 47; and *Centralbl. f. d. med. Wissensch.* 465 and 738 (1893).



Guanin
(2-amido—6-oxypurin).



Adenin
(6-amidopurin).

Nucleic acids combine with proteids in varying proportions to form nucleo-proteids or nucleins, a group of substances characteristic of all nucleated cells, and containing a large amount of iron, which latter, according to Ascoli [1], is linked on to the nucleins by means of hexa-metaphosphoric acid. With histone, as was first shown by Kossel and Lilienfeld [2], nucleo-histones are formed.

My experiments have shown that the pure base protamin (prepared from clupein sulphate with barium carbonate) unites with pure nucleic acid to form a voluminous precipitate, while it fails to give a precipitate with either pure glycocoll or tyrosin.

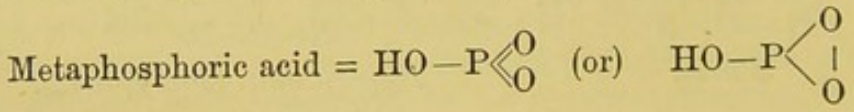
As under the action of hydrochloric acid the nuclein base hypoxanthin has been shown to absorb water and to become converted into glycocoll according to the equation $C_5H_4N_4O + 7H_2O = C_2H_5NO + 3NH_3 + CO_2 + HCOOH$, a certain relationship between this fatty amido acid and nucleic acid must exist.

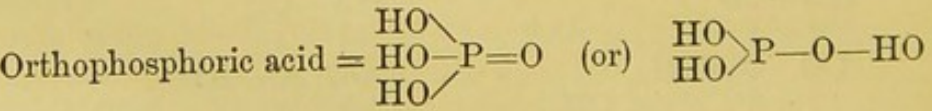
Knowing then that protamin and nucleic acid unite to form a solid body, while protamin and glycocoll do not, we have to suppose that in white fibrous tissue, from which glycocoll is obtained, some substance must exist which is intermediate in its character between nucleic acid and glycocoll, and which plays towards the basic protamin the part of an acid [3].

Nucleins behave as fairly strong acids, because of their unsatisfied affinity for bases, while white fibrous tissue, which we have to regard as a special nucleo-amido acid compound, has a neutral reaction.

(*b*) *Pyrrolidin-carboxylic acid* is referred to on p. 325.

(*c*) *Metaphosphoric acid.* Thomas Graham [4] (1833) showed that metaphosphoric acid has the power of coagulating white of egg, while orthophosphoric and pyrophosphoric acids have not.



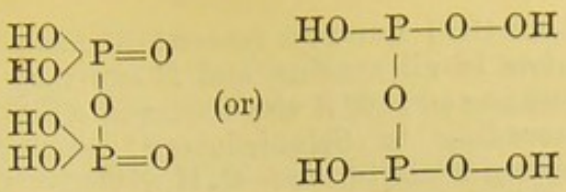


[1] Hoppe-Seyler's *Physiol. Chemie*, 28, 426 (1899).

[2] *Zeitsch. f. physiol. Chemie*, 18.

[3] To what extent aldehyde, alcohol, and especially cyanogen radicals participate in the formation of proteids is as yet quite impossible to say, and no reference will be made to these bodies. It is different, however, with the carbohydrates, to which I shall refer later.

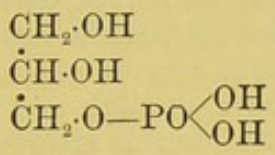
[4] Graham: *Phil. Trans.* 2, 253 (1833).

Pyrophosphoric acid =  (or)

Metaphosphoric acid combines with proteid to form pseudo-nucleins (Liebermann), substances found, for example, in the yolk of egg and in casein. Milroy [1] has pointed out that as yet it is impossible to construct a 'pseudo-nucleic' acid comparable to the true nucleic acid. The absence of the nucleic acid in pseudo-nucleins is further shown by the fact that no alloxure bases can (see p. 283) be derived from them.

As amongst true nucleins, so amongst the pseudo-compounds two groups may be distinguished, one of which contains no carbohydrate radical (casein-pseudo-nuclein) while the other does (ichthylin-pseudo-nuclein).

(*d*) *Glycero-phosphoric acid* is formed by combining orthophosphoric acid with glycerin, when one of the hydroxyl groups of the tribasic phosphoric acid becomes replaced by the glycerin remainder, thus [2]:



Glycero-phosphoric acid in combination with fatty acids, such as oleic, palmitic, and stearic acids, forms with the base cholin (trimethyl-hydroxyethyl-ammonium hydroxide), $[HO—N(CH_3)_3—C_2H_4—OH]$, substances known as lecithins (see later, p. 312).

Lecithins in their turn unite with albumins to give rise to lecith-albumins, as for example to ovovitellin [3]. In this instance the albumin acts probably as a base towards lecithin.

(*e*) *Phosphocarnic acid* ($C_{10}H_{15}N_3O_5$) was discovered by Siegfried [4], according to whom it is a normal constituent of muscle, forming with alkaline earths readily soluble compounds, which act as carriers of iron salts.

(*f*) *Chondro-sulphuric acid* ($C_{18}H_{27}NSO_{17}$) occurs in cartilage, the tunica intima of the aorta (C. Mörner), in the kidney, in human urine (K. Mörner), and the ligamentum nuchae [5] (Krawkow). The last observer has further found that its combination with mucin or mucoid forms the pathological substance 'amyloid.' This observation, as Hammarsten points out, accounts for chondro-sulphuric acid having been obtained by Oddi from livers showing amyloid degeneration.

[1] *Roy. Soc. Edinburgh*, Dec. 1896.

[2] Neumeister: *Physiol. Chem.* 91.

[3] Liebermann: *Pflüger's Arch.* 50, 25 and 55; also 55, 573. Nerking: *ibid.* 85, 330 (1901).

[4] Max Siegfried: *Du Bois-Raymond's Arch.* (1894), p. 401; see also Balke and Ide: *Zeitsch. f. physiol. Chemie*, 21 and 22.

[5] Chondro-sulphuric acid + gelatin = chondrin.

Since then Levene's researches[1] have made it very probable that it exists in all mucins, and if one may rely on micro-chemical reactions (see p. 216) it also occurs in white fibrous tissue.

According to Schmiedeberg[2] chondro-sulphuric acid has the following constitution: $C_{18}H_{27}NSO_{17}$. On decomposition it gives rise to sulphuric acid and the monobasic chondroitin ($C_{18}H_{27}NO_{14}$). The latter, when treated with dilute mineral acids, becomes hydrated and splits into acetic acid ($C_2H_4O_2$) and a gum-like body, chondrosin ($C_{12}H_{21}NO_{11}$), possessing towards Fehling's solution a stronger reducing power than grape-sugar.

Chondro-sulphuric acid when in a neutral solution is precipitated by stannous chloride, basic lead acetate, neutral ferric chloride, and also by alcohol in the presence of some neutral salt, while it is not thrown down by acetic acid, tannic acid, the ferrocyanide and acetic acid mixture, lead acetate, sublimate, or silver nitrate.

With acid solutions of albumin and gelatin, neutral solutions of chondro-sulphuric acid give a coagulum, forming the group of chondro-proteids or chondro-mucoids.

(*g*) *Mucins* resemble the last-mentioned class of substances in giving rise to reducing bodies on boiling with dilute mineral acids, as first shown in 1865 by Eichwald[3], and hence they are known as glyco-proteids. Leucin and tyrosin may be obtained from them, but not the xanthin group (see above, p. 283), and hence no proteid colour reactions are obtainable. Sulphur is always present. As compared with other proteids they contain much less carbon and nitrogen, but a great deal of oxygen because of the presence of a nitrated derivative of dextrose which its discoverer, Friedrich Müller[4], has called glycosamin. In all probability this substance exists normally as an amido-polysaccharid (see p. 297).

Mucins are strong acids probably owing to their chondro-sulphuric acid radical. They turn litmus paper red, and they are precipitable by acetic acid, in which they are more insoluble than in mineral acids. They are very readily decomposed by alkalies.

It is advisable to adopt Cohnheim's classification, by restricting the term mucin to those substances which are excreted by epithelial cells and to reserve the name mucoid for closely allied bodies which occur in various organs of the body, such as the vitreous humour, the cornea, the umbilical cord, and tendons. Cohnheim includes amongst the mucoids the chondro-mucoids above referred to under the heading of chondro-sulphuric acid.

(*h*) *Alkali albumins* are sufficiently strong acids to decompose calcium carbonate ($CaCO_3$) and thus to set free carbonic acid (Hammarsten[5]). Acid albumins do not possess this power. The probable explanation is that albumin when it becomes converted into the

[1] P. A. Levene: 'Zur Chemie d. Mucine,' *Zeitsch. f. physiol. Chem.* 31, 395 (1901).
[2] Schmiedeberg: *Arch. f. experim. Path. und Pharm.* 28.
[3] A. Eichwald: *Annal. d. Chem. u. Pharm.* 134, 177 (1865).
[4] F. Müller: 'Schleim der Respirationsorgane,' *Sitzb. d. Ges. z. Beförd. d. ges. Naturw. z. Marburg*, (1896) p. 53, (1898) p. 117.
[5] Hammarsten: *Physiol. Chem.* p. 32.

alkali salt changes its nature completely, as it gives off the ammonia bases (and also sulphur), and perhaps also because certain radicals, playing the part of pseudo-acids, are converted into true acids (see p. 25).

(*i*) *Natural albumins* are compounds consisting of both acid and basic radicals, as they contain diamido-acid compounds (protamin) and nucleic acid derivatives. In their reaction albumins are normally neutral, but may acquire a strongly acid or basic character under certain conditions, as explained in the chapter on pseudo-acids and pseudo-bases (p. 25).

C. Schmidt[1] in 1847, Mulder[2] in 1851, Danilewski[3] in 1880, but especially Sjögvist[4] in 1895, were the first to study the compounds which albumin forms with hydrochloric acid. The last named employed the van 't Hoff-Arrhenius method of studying the electrical conductivity of albumins and of their compounds with acids. Further, Paal[5] (1894) stated that albumin according to its degree of hydrolytic decomposition (peptonization) shows a gradually increasing power of absorbing acids. After Sjögvist had determined that albumin can bind 3·65 per cent. of hydrochloric acid, Cohnheim in 1896[6] found that 2·5 per cent. solutions of the following substances can combine as regards their own weight with these quantities of hydrochloric acid:

Protalbumose	4·3 per cent.
Deutero-albumose . . .	5·5 ,,
Hetero-albumose . . .	8·2 ,,
Antipeptone	16·0 ,,

With greater concentration of the albumin solutions and an excess of acid, Spiro and Pemsel[7] have obtained still higher figures; thus the acid capacity of egg-albumin and crystallized serum-albumin is respectively 5·10 and 11·23. Sjögvist finds that egg-albumin considered as a base is 1·87 times stronger than glycocoll, 3–5 times stronger than asparagin, but 74·2 times weaker than anilin.

The observations of Erb are given on p. 23.

This power of different 'proteids' to combine with varying amounts of acids is directly applicable to histological investigations, if tissues have been fixed in 'neutral' media such as alcohol. The importance of this will be shown later (p. 320).

(*k*) *Antipeptone prepared* by Kühne's method[8] behaves towards basic dyes such as toluidin-blue or methylene-blue as a strongly acid substance, as was first noticed by L. H. Huie[9] working under my

[1] C. Schmidt: 'Über d. Wesen d. Verdauungsprocesses,' *Ann. d. Chem. u. Pharm.* 61, 311 (1847).

[2] Mulder: *Versuch e. allgem. physiol. Chemie* (Braunschweig, 1851).

[3] Danilewski: *Centralbl. f. d. med. Wiss.* 929 (1880).

[4] Sjögvist: 'Physiologisch-chemische Beob. ü. Salzsäure,' *Skand. Arch. f. Physiol.* 5, 277 (1894); 6, 255 (1895).

[5] C. Paal: *Ber. d. deutsch. chem. Gesellsch.* 25, 1202 (1892); and 27, 1827 (1894).

[6] Cohnheim: *Zeitsch. f. Biol.* 33, 489–520.

[7] Spiro and Pemsel: *Zeitsch. f. phys. Chem.* 26, 233 (1898–99).

[8] Kühne: *Verhandl. d. naturhistor. Vereins zu Heidelberg*, N.F. 3.

[9] Miss L. H. Huie: *Q. J. Micros. Sc.* 39, N.S. p. 387 (1896–7); and 42, N.S. p. 203 (1899).

directions, and has also been observed subsequently by A. Fischer[1]. M. Siegfried[2] has since shown that Kühne's peptone is a mixture of albumoses, mono- and diamido acids, out of which by precipitation with ammonium sulphate in conjunction with ferri-ammonia alum (iron alum) two, chemically constant and pure, monobasic acid substances may be obtained, giving an intense biuret but no Millon's reaction. These two acids have the formulae $C_{10}N_3H_{17}O_5$ = α-antipeptone, and $C_{11}N_3H_{19}O_5$ = β-antipeptone. The second acid thus contains an additional CH_2 group.

Both acids as well as amphopeptone on being inspissated on a water bath are reconverted into albumoses.

Not only do 'proteids' act as bases, but they may also unite with bases, being, under certain conditions, acid in their character, as first shown by St. Bugarszky and Liebermann[3], and later by Spiro and Pemsel[4], who have found the capacity for bases to amount in egg-albumin to 2·45 per cent. and in serum-albumin from 3·22 to 6·14 per cent.

Generally speaking, the capacity for acids is greater than that for bases.

CHAPTER XXIII.

Detection of Tissue Constituents by Chemical Means.

Micro-chemical Reactions.

Under this heading we may distinguish two distinct classes of reactions, namely, those which we can explain and those which we cannot as yet account for from the chemical point of view. For example, we may demonstrate the presence of iron in the tissue by the same tests which chemists employ for its detection in the test-tube, or as it is termed by scholars, *in vitro*. On the other hand, we may isolate certain elements in the tissues by staining reactions, as, for example, elastic fibres by Weigert's stain, without knowing either the constitution of the colouring matter or that of the tissue.

To perform a biuret reaction or xantho-proteic test without knowing to what the reaction is due, or at least without having made strenuous efforts to understand the interrelationship of the reagents and the substance tested for, does not represent to me true chemistry, but simply a blind mechanical dabbling. It is true that many reactions are found by chance, and that it takes time to interpret them, but do not let us use such reactions without troubling as to their signifi-

[1] Fischer: *Bau, Struktur etc. d. Protopl.*

[2] Siegfried: 'Über Antipepton,' *Ber. d. deutsch. chem. Gesellsch.* 33, 2851–2858 (October, 1900).

[3] St. Bugarszky and Liebermann: 'Bindungsvermögen eiweissartiger Körper für Salzsäure, Natronlauge u. Kochsalz,' *Pflüger's Arch.* 72, 51 (1898).

[4] Spiro and Pemsel: *Zeitsch. f. physiol. Chem.* 26, 266–270 (1898–99).

cance; and yet how few histologists ask themselves: What am I doing in using a red or blue anilin dye, or haematoxylin? what is the chemical nature of these substances, and what is that of the tissues I am experimenting on?

To ascertain the presence of chemical elements in the different cell-organs is of fundamental importance, and no one has done better work in this connexion than Macallum, but there is a further development possible, namely, the recognition of complex molecules. The latter method works as yet, although it may adopt a certain systematic line of procedure, quite empirically, and it is rather chance than ingenuity which places the first clue into our hands.

(*a*) *Methods for determining the alkalinity and acidity of tissue elements.*

In the previous chapter the chief acid and basic substances have been enumerated which will react with basic and acid dyes, and the method for ascertaining the presence of free bases has already been given on p. 214. It will suffice to once more call to mind the fact that pure albumin crystals in ammonium sulphate, prepared by Hopkins's method, give a distinct basic reaction with the test of Mylius.

Of acid reactions there are several. Using any strongly basic dye, it is easy to procure a deep staining (1) of the nuclear 'chromatin' segments and of Nissl's bodies in nerve-cells, owing to the presence of nucleic acid (?) or its derivatives nuclein and nucleo-proteids; (2) of mucous and hyaline cartilage, because of the chondro-sulphuric acid they contain.

Having obtained a definite reaction with basic dyes, we can do no more than draw the legitimate conclusion that acids are present in the tissue. We know nothing as to what the nature of the acid may be (Mathews. See p. 348).

(*b*) *Test for reducing substances.*

Cross and Bevan had observed that jute fibre stains intensely blue in a ferri-ferricyanide solution, and explained their result on the solid solution theory of Witt (p. 330), but Weber[1] pointed out that the reduction of the double ferricyanide compound depended on the aldehyde group present in jute.

If equal quantities of one per cent. solutions of freshly prepared ferricyanide and ferric chloride are mixed, a pale yellowish-brown solution is obtained. To demonstrate a reducing substance in the cells, specially abundant in the cell-plasm, proceed thus:—Sections of material fixed in alcohol, according to the method stated on p. 146, are fixed to glass slides in the usual way (p. 373); the paraffin is removed with benzene, the benzene with pure ethyl-alcohol; the sections are carefully washed in water to remove all traces of the alcohol; a couple of drops of ferri-ferricyanide are placed on the sections, and covered at once with a cover-glass; a ring of paraffin, melting-point 62° C., is drawn round the cover-glass to prevent evaporation of the

[1] Weber: *Journ. Soc. Chem. Indust.* 13, 122 (1894).

iron solution, and the preparation is kept at about 30° C. for half an hour or longer, when a distinct blue colouration will be observed.

(c) *Tests for chemical compounds.*

A. Inorganic Constituents.

1. Iron.

The first observer who endeavoured to determine the presence of iron in the tissues was J. Vogel (1845), who used ammonium sulphide. Zaleski confirmed Vogel's observations by placing liver-sections for 2–30 minutes in a watery or alcoholic solution of ammonium sulphide, and subsequently in 2 per cent. alcoholic or watery hydrochloric acid, and obtained the typical greenish-black colouration. He further showed, by comparing sections cut with glass knives and clean steel instruments, that the latter may be safely used for sectioning tissues. Perls in 1867 [1] demonstrated the iron by treating tissues firstly with dilute ferrocyanide and then with hydrochloric acid [2], which decomposes the iron compounds in the tissue and thus allows of the formation of Prussian blue.

According to W. W. Fischer, ferrous and ferric salts interact according to the following formulae:

(1) $K_4FeC_6N_6 + FeCl_3 = KFe_2C_6N_6 + 3KCl$
Pot. ferrocyanide + ferric chloride = Pot. Iron Cyanides = soluble Prussian blue.

(2) $K_3FeC_6N_6 + FeCl_2 = KFe_2C_6N_6 + 2KCl.$
Pot. ferricyanide + ferrous chloride = Pot. Iron Cyanides = soluble Prussian blue.

Fe_7Cy_{18} = (3) $3KFe_2C_6N_6 + FeCl_3 = Fe_7C_{18}N_{18} + 3KCl$ = insoluble Prussian blue.
Soluble Prussian blue + ferric chloride = insoluble Prussian blue, containing no potash.

Fe_5Cy_{12} = (4) Soluble Prussian blue + ferrous chloride = $Fe_5C_{12}N_{12} + 2KCl$ = Turnbull's blue.

$FeCy_6$ = (5) Potassio-ferrous ferrocyanide $= K_2Fe + FeCy_6 = K_2Fe_2Cy_6$.

$2K_4FeCy_6 + 3H_2SO_4 = 6HCy + K_2Fe_2Cy_6 + 3K_2SO_4$

Pot. ferrous ferrocyanide on exposure to air becomes potassio-ferrous ferricyanide ($KFe \cdot FeCy_6 = KFe_2Cy_6$) which is soluble in pure water (free from salts) and has a deep blue colour. It is often called soluble Prussian blue (Forster Morley).

Although free iron is readily recognized by the above means, there are certain other iron compounds which, to use the expression of Molisch [3], are masked, which means they are not recognizable by the ordinary tests for iron. The method which Molisch used to liberate the masked iron consisted in the decomposition of the organic compounds with concentrated caustic potash. The procedure is, however, not to be recommended because of the great difficulty in obtaining iron-free potash, and also because of the deleterious action of the caustic reagent on the tissues.

Macallum finds that ammonium hydrogen-sulphide NH_4HS is able to unmask organic iron, and that it is more active in this respect than the diammonium sulphide $(NH_4)_2S$, while polysulphides are

[1] Perls: *Virchow's Arch.* 39, 42 (1867). [2] Schneider uses nitric acid.
[3] Molisch: 'Bemerk. ü. d. Nachweis von maskirtem Eisen,' *Ber. d. deutsch. bot. Gesellsch.* 11, 73 (1893).

quite inactive in liberating iron from potassium ferrocyanide, haematin, or organic compounds. To prepare the ammonium hydrogen-sulphide, take dilute ammonia having a specific gravity of 0·96, and pass through it sulphuretted hydrogen. The solution obtained in this way will keep for three weeks in well-stoppered bottles filled quite full.

Tissues fixed in alcohol may be teased with a clean goose-quill or hedgehog-spine, or sections may be cut. Cover the preparation with a freshly-prepared mixture of one drop of 50 per cent. Price's, chemically pure, glycerin and two drops of dilute ammonium hydrogen-sulphide; cover with a 16 × 22 mm. cover-glass, and place the slide in a warm oven (60° C.), when the mixture rapidly concentrates and in a few minutes passes completely under the cover-glass. Should the space under the cover-glass not be completely filled, add some more of the mixture. The solution under the cover-glass ought to remain perfectly clear; if it does not do so, but turns yellow, and if crystals of sulphur are deposited on the margin of the cover-glass, it is a sure sign of the ammonium hydrogen-sulphide having deteriorated.

Unfixed yolk of a hen's egg gives a reaction immediately, while after fixation in alcohol or by heat no effect is produced till after the lapse of some days. The yolk granules in Amphibia react in a few minutes whether they are fixed or not. Haematin requires 24 hours, masked iron 2–15 days, while haemoglobin and myohaemoglobin do not show an iron reaction even if kept for more than one year at 55° C.

It is by this reagent that Macallum in 1891 first demonstrated the presence of iron in the nuclear chromatin.

As, however, by the prolonged action of ammonium hydrogen-sulphide the tissues suffer considerably, it is best to first unmask the organic iron with acid alcohols.

For this purpose Bunge had suggested hydrochloric acid alcohol (95 per cent. alcohol = 95 volumes, and 25 per cent. hydrochloric acid = 10 volumes), but Macallum finds that both the organic and the inorganic iron are apt to be extracted by a prolonged action of this reagent, especially if the temperature rises above 20° C., and therefore the subsequent treatment of the sections with ammonium sulphide may fail to demonstrate iron in the tissues, although originally it was there.

Macallum distinguishes between inorganic and organic iron compounds as follows:—The tissues, for the purpose of extracting the inorganic iron, are subjected to the action of Bunge's fluid for 1–2 hours at a temperature of 50–60° C., or for 8–10 hours at 35° C., or longer if the temperature is kept below 20° C.

Tissues such as the spleen, containing large quantities of inorganic iron, require a longer treatment and large quantities of Bunge's fluid. If the inorganic iron is removed, ammonium sulphide ought to give no reaction after 5–10 minutes.

To unmask the organic iron, use, instead of Bunge's fluid, those of Macallum:

Sulphuric acid	4 parts		Nitric acid (1·4 sp. gr.)	3 parts	
95 per cent. alcohol	100 „	or	95 per cent. alcohol .	100	„

The acids are used in alcoholic solutions to prevent a deleterious action on the tissues, and also to minimize the extraction of the liberated iron and its diffusion from one part of the tissue into another, or from the nucleus to the cell and vice versa.

All bottles, slides, and other glass utensils must have every trace of iron removed with hydrochloric acid, distilled water, and alcohol, and the steel knife must be clean.

To demonstrate the liberated iron, Macallum uses three methods, namely, ammonium hydrogen-sulphide, ferrocyanide of potash, and haematoxylin.

The ammonium hydrogen-sulphide is the same solution as described above, and by its use the iron is demonstrated as a ferrous salt. But although the iron may be, at the moment of liberation, in the ferrous state, it quickly assumes the ferric form, and for this reason, if it is to be demonstrated as Prussian blue, a solution of ferrocyanide of potassium must be used. Macallum recommends the following procedure:—Unmask the iron with acid alcohol, then wash the sections in pure alcohol, next in distilled water, and finally place them for not more than five minutes in a freshly prepared mixture composed of equal volumes of a 1·5 per cent. ferrocyanide solution (not older than one week) and 0·5 per cent. of hydrochloric acid. The sections are again carefully washed, and may be counterstained in 1 per cent. eosin in 30 per cent. alcohol for 3 minutes, or in 1 per cent. safranin in 30 per cent. alcohol for half an hour. The sections should be carefully dehydrated, cleared in cedar-wood oil and mounted in benzene-balsam, and be kept in a dark place, as otherwise they will fade [1].

The haematoxylin method [2] is, however, the best; it depends on the principle that haematoxylin forms, with the salts of the heavy metals, deeply coloured, insoluble higher oxides (p. 436), while no colour-reactions are obtained with organic compounds, such as solutions of haemoglobin, haematin, ferrocyanides, and ferricyanides, probably because in the latter all the possible valencies are joined on to carbon atoms, thus [3]:

$$\begin{matrix} C \\ C \end{matrix}\!\!>\!\!Fe{=}Fe\!<\!\!\begin{matrix} C \\ C \end{matrix}\text{; or thus, } \begin{matrix} C \\ C \end{matrix}\!\!>\!\!Fe{-}Fe\!<\!\!\begin{matrix} C \\ C \end{matrix}$$

Inorganic iron gives the reaction at once with a half per cent. pure haematoxylin in pure distilled water, while the rest of the tissue assumes merely a yellow colour. This latter is readily extracted if sections are placed in a mixture of equal volumes of ether and absolute alcohol 'for at most an hour,' and thus minute traces of inorganic iron in the nuclei may be demonstrated.

[1] Why the sections fade is difficult to say, especially as Prussian blue is not acted upon by acids, only alkalies destroying the colour. The most probable explanation is reduction of the compound by means of the balsam, there being formed a compound analogous to $Fe_2Fe(CN)_6$ which is white in the absence of oxygen, but becomes blue with the faintest trace of this gas.

[2] A. B. Macallum: 'A new method of distinguishing between organic and inorganic compounds of iron,' *Journ. of Physiol.* 22, 92-98 (Sept. 1897).

[3] Roscoe and Schorlemmer: *Treatise on Chemistry*, 2, part 2, p. 112.

Organic compounds are first decomposed, preferably with sulphuric acid alcohol, then they are freed from acid by washing in alcohol, and finally treated with haematoxylin, of at least half per cent. strength, for a few minutes. 'Wherever iron exists in such a preparation the haematoxylin becomes blue-black or blue. Very often sections of tissue thus treated appear as if stained with Ehrlich's haematoxylin.'

Carazzi[1], to liberate the iron from its organic compounds, oxidizes paraffin sections of the eggs of Ostrea with the vapour of osmium tetroxide.

Marfori[2] has made the important observation that soluble dialysed ferric oxide, which means colloidal iron, does not give the typical black haematoxylin reaction, but when treated with dilute ammonium sulphide a black colour is obtained at once.

We have here an instance of an 'inorganic' salt of iron behaving as an 'organic' one as long as it is in the colloidal state.

2. Copper.

Boyce and Herdmann[3] have applied Macallum's tests for iron to recognize copper compounds. In a diseased condition of the American oyster certain green leucocytes were found to contain large amounts of copper. When alcohol-fixed material was treated with potassium ferrocyanide solution, a red colour was obtained at once, or if a mixture of equal parts of Macallum's ferrocyanide solution and 0·5 per cent. hydrochloric acid was employed then ammonium hydrogen-sulphide instantly gave a marked dark yellow-brown reaction. Haematoxylin gives directly a dark-blue colouration with the copper-containing leucocytes, as it also gives a direct iron reaction with liver-cells in the case of pernicious anaemia.

Whether we are dealing with iron or copper compounds is a question which must be determined by macro-chemical tests.

3. Phosphorus.

Lilienfeld and Monti (1873) treated tissues with a nitric acid solution of ammonium molybdate to convert the organic phosphorus into the yellow ammonium phospho-molybdate; they then washed the tissues in water to remove the free ammonium molybdate, and finally reduced the remaining ammonium phospho-molybdate to a brown or black lower oxide by means of pyrogallol.

Raciborski (1893) showed, however, that the Lilienfeld-Monti procedure gives a green colour with ammonium phospho-molybdate, and a brown or black colour with ammonium molybdate. He stated that the yellow colour in the tissues was simply the ordinary xantho-proteic reaction, obtainable by treating tissues with nitric acid, while the brown colour depended on the ammonium molybdate, and that the latter reaction could be diminished or quite prevented by a prolonged washing of the tissues in water. He, therefore, concluded that the ammonium molybdate is not united chemically in the tissues with the phosphorus, but that it is only imbibed mechanically.

[1] Carazzi: *Internat. Monatsch. f. Anat. und Physiol.* 14, 134 (1897).
[2] Marfori: *Arch. ital. de Biol.* 30, 181 (1898).
[3] R. Boyce and W. A. Herdmann: *Phil. Trans.*, London, 62, 34 (1897-8).

Pollacci (1894) demonstrated the presence of the ammonio-phospho-molybdate in the tissues by means of zinc chloride, which reagent produces a dark-blue or grey colour [1].

Heine (1896–7) showed that the Lilienfeld-Monti reaction was unreliable, inasmuch as histone or albumin, containing no traces of phosphorus, might still give the brown reaction, because these bodies form with a nitric acid solution of ammonium molybdate, compounds which cannot be decomposed by washing in water. The presence of ammonium molybdate is most readily demonstrated with stannous chloride, owing to the formation of the blue molybdic oxide.

Macallum (1898), whose work I have verified, gives the following account: In the first place he confirms the observations of Raciborski on pyrogallol, which in the meantime had been denied by Heine. Thus pyrogallic acid in a watery or in an ethereal solution, when added to a solution of ammonium molybdate, produces immediately a colour similar to a saturated solution of Bismarck brown, but no precipitate is formed. Pyrogallol added to a nitric acid solution of ammonium molybdate produces a brownish-black or black colour, and usually an amorphous blue-black precipitate, while if it be added to a solution of ammonium phospho-molybdate even in the presence of nitric acid, the yellow colour is converted into a green which after a lapse of two hours gradually begins to darken, till after twenty-four hours it has a faint tinge of green when examined in thin layers, the form of the crystals of ammonium phospho-molybdate being maintained.

In confirmation of Heine's observations, Macallum found that stamens of *Erythronium americanum*, treated with nitric-molybdate solution for twenty-four hours, and then washed for five months in many changes of distilled water, would still give a general blue staining with stannous chloride, indicating the presence of ammonium molybdate, and further a special green reaction with pyrogallol, showing the presence of phospho-molybdate. Animal tissues behave in exactly the same way.

As pyrogallol gives a coloured compound with both ammonium molybdate and with phospho-molybdate, Macallum endeavoured to find some substance which in the presence of nitric acid would only act on the latter compound. This requirement is fulfilled by zinc chloride (Pollacci), which gives a green colour only with the phospho compound, and none with ammonium molybdate, but as it acts only

[1] Pollacci, G.: *Atti dell' ist. botan. dell' Univ. di Pavia*, 2 Ser. 6, 15 (1900). His most recent method is as follows: Dissolve 15 grms. of ammonium molybdate crystals in 100 cc. of 'ammoniacal water.' Further mix 70 cc. of nitric acid (sp. gravity 1·18) with 30 cc. of distilled water, and then add the nitric acid solution to the ammonium molybdate solution and shake till the precipitate, formed at first, is dissolved. Allow the reaction to act on sections, then wash out thoroughly with distilled water till the latter no longer gives a blue colour reaction with stannous chloride. Now transfer the sections to a 4 per cent. solution of stannous chloride, when those elements which contain phosphorus will turn blue. As above stated, it is quite impossible to remove ammonium molybdate from the tissues by prolonged washing, and the substitution of stannous chloride for zinc chloride by Heine is a step backwards.

very slowly, owing to its feeble reducing power, the following method was devised:

Macallum's test for Phosphorus:

(1) Preferably fix tissues in alcohol, although for comparison fresh unfixed material should be used as well.

(2) Make the nitric-molybdate reagent by dissolving one part by weight of pure molybdic acid [MoO_3] in four parts of strong ammonia (sp. grav. 0·88), and add slowly fifteen parts of nitric acid (sp. grav. 1·2). The resulting faintly-yellow solution, decanted from the very slight sediment, keeps indefinitely.

(3) Treat the tissues with the nitric-molybdate reagent from ten minutes to forty-eight hours at a temperature of 35° C., when the inorganic phosphates will be affected first, then lecithin, and lastly the organic phosphorus. As the nitric-molybdate reagent only reacts with the ortho-form of phosphoric acid, all the phosphorus must be changed, somehow, into orthophosphate.

To distinguish between inorganic and organic phosphorus proceed thus:—(*a*) All inorganic phosphates, except those of iron and the nuclear elements (as explained sub *c*), may be removed with 20 per cent. acetic acid, according to Jolly's method, or, with Macallum, one may regard as inorganic all phosphorus reactions which make their appearance in the first ten minutes. (*b*) To remove lecithin, Bitto found it necessary to first treat plant-tissues with ether, then to extract the lecithin with thirty changes of boiling ethyl-alcohol, each period of extraction lasting ten minutes. Macallum uses a Soxhlet apparatus for boiling the tissues for five hours and siphoning off the condensed but still hot alcohol every six to ten minutes. This procedure is absolutely necessary with nervous tissues. (*c*) According to Milroy, nuclein compounds digested with trypsin show that about 90 per cent. of the phosphorus occurs in an organic form, while about 10 per cent. is set free as orthophosphoric acid. (*d*) Tissues very rich in orthophosphates, such as the renal tubules and the placenta, must be treated in very thin sections, as otherwise the orthophosphoric acid which is liberated, diffuses out of the section and covers the latter in the form of phospho-molybdate crystals.

(4) Reduce the phospho-molybdate to the green oxide by means of phenyl-hydrazin hydrochloride. This substance may be used in watery solutions of 1–4 per cent. strength, and should be freshly prepared. In the absence of alcohol and of caustic alkali, it produces in ammonium molybdate in the presence of nitric acid no change, while with the phospho-molybdate 'either in the presence or in the absence of ammonium molybdate or nitric acid, or of both, it gives at once the dark-green oxide of molybdenum.'

Sections which have been treated with the nitric-molybdate reagent may be transferred directly to the phenyl-hydrazin hydrochloride, or be washed first for one to two minutes in dilute nitric acid or distilled water. Within two minutes the phospho-molybdate is stained deep green, while the ammonium molybdate is coloured faintly yellow. The section may then be dehydrated, be cleared in cedar-wood oil and be mounted in balsam.

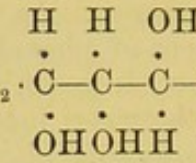
4. Sulphur.

On boiling proteids in an alkaline solution with a salt of any metal, the sulphides of which are black or dark coloured, the sulphur of the proteid is liberated and with the metal forms the deeply coloured sulphide.

My attempts so far have not been very successful in getting both sulphur reactions and relatively good histological preservation, as the liberation of the sulphur means complete destruction of the proteid molecule.

B. Organic Constituents.

1. Carbohydrates.

(*a*) **Glucose** ($C_6H_{12}O_6 + H_2O = HO \cdot CH_2 \cdot$  $\cdot CHO + H_2O$).

(1) *Fehling's test.* In plant-tissues sugars are readily demonstrated with Fehling's solution, which is prepared thus: Heat copper sulphate till it has lost its water of crystallization, then dissolve 34·64 grms. of the white dry powder in 200 cc. of distilled water; —dissolve 173 grms. of sodium potassium tartrate (Rochelle salt) in 480 cc. of caustic soda, having a specific gravity of 1·14;— mix the two solutions and dilute up to 1,000 cc. This solution is of such a strength that 10 cc. of it are reduced by 0·2 grm. of sugar. Sections placed in a hot solution of Fehling, and kept at a temperature of about 75° C., show inside the cells, if sugar be present, a yellow precipitate.

(2) *Barfoed's test for dextrose.* Barfoed's reagent (neutral acetate of copper 6·6 grms., glacial acetic acid 2·5 cc. and water 100 cc.) has been employed in a modified form by de Waele[1].

Copper acetate is more readily reduced by carbohydrates than any other salt of copper or any other acetate, as shown by the behaviour of dextrose, saccharose, lactose, maltose, and glycogen. The monosaccharid dextrose reduces at 65° C., but the di- and polysaccharids require, with increasing polymerization, higher and higher temperatures. With the compound sugars the ultimate colour-reaction becomes gradually less pronounced.

To demonstrate dextrose in the alimentary canal of the frog, de Waele gives these directions:—Introduce into the oesophagus of a frog 1 to 2 cc. of a ten per cent. watery solution of dextrose; kill the frog after three hours and fix the intestine for half an hour at 65° C. in

5 per cent. acetate of copper . . .	10 parts
2 per cent. osmium tetroxide . . .	1 part

Leave the tissue for some time in Hermann's solution and then take it

[1] de Waele: *Livre jubil. Ch. van Bambeke*, Bruxelles, 40 (1899).

through the paraffin process. Nearly all the intestinal cells covering the villi will contain numerous black granules of different sizes, which frequently become confluent into larger masses between the nuclei and the free border, and also to a less extent between the nuclei and the base of the cell. Beneath the epithelium no trace of sugar can be detected.

By control experiments, such as fixing the intestine in Flemming's solution, the absence of all fat was established; for certain fats, as is shown later (p. 306), reduce osmium tetroxide into a black compound.

(*b*) Glucosamin.

This substance was first isolated by Ledderhose[1], who boiled chitin prepared from lobsters' claws with concentrated hydrochloric acid.

d-Glucose, $C_6H_{12}O_6 = HO \cdot CH_2 \cdot \overset{H}{\overset{|}{\underset{OH}{|}}}C—\overset{H}{\overset{|}{\underset{OH}{|}}}C—\overset{OH}{\overset{|}{\underset{H}{|}}}C—\overset{H}{\overset{|}{\underset{OH}{|}}}C \cdot CHO + H_2O$

Glucosamin, $C_6H_{13}NO_5 = HO \cdot CH_2 \cdot \overset{H}{\overset{|}{\underset{OH}{|}}}C—\overset{H}{\overset{|}{\underset{OH}{|}}}C—\overset{OH}{\overset{|}{\underset{H}{|}}}C \cdot CH(NH_2)CHO$

Isoglucosamin[2], $C_6H_{13}NO_5 = HO \cdot CH_2 \cdot \overset{H}{\overset{|}{\underset{OH}{|}}}C—\overset{H}{\overset{|}{\underset{OH}{|}}}C—\overset{OH}{\overset{|}{\underset{H}{|}}}C—CO \cdot CH_2 \cdot NH_2$

Glucose penta-acetate, $C_{16}H_{22}O_{11} = C_6H_7O_6(C_2H_3O)_5$

Acetyl-glucosamin, $C_8H_{15}O_6N = C_6H_{12}O_5N(COCH_3)$

Penta-acetyl-glucosamin, $C_{16}H_{23}O_{10}N = C_6H_8O_5N(C_2H_3O)_5$.

Acetyl-glucosamin is obtained by the addition of glacial acetic acid to glucosamin dissolved in a small quantity of absolute methyl-alcohol (Breuer[3]). It is easily soluble in water and in boiling methyl-alcohol.

Penta-acetyl-glucosamin is formed by boiling glucosamin hydrochloride for a short time with sodium acetate and glacial acetic acid. It is slightly soluble in water and in cold alcohol, but very soluble in warm alcohol. It is reconverted into glucosamin hydrochloride by dilute boiling hydrochloric acid.

Ehrlich[4] in 1901 made the discovery that urine turns a more or less pronounced carmin-red colour by the addition of a few drops of the pale yellow dimethyl-paramido-benzaldehyde[5] dissolved in

[1] Ledderhose: *Ber. d. deutsch. chem. Gesellsch.* 9, 1200 (1878); *Zeitsch. f. physiol. Chem.* 2, 213 (1878–9), and 4, 139 (1880).

[2] E. Fischer: *Berichte d. deutsch. chem. Ges.* 27, 3215.

[3] Breuer: *ibid.* 31, 2198.

[4] Paul Ehrlich: 'Über d. Dimethylamidobenzaldehyde Reaction,' *Die Medic. Woche*, April, 1901, No. 15.

[5] To be obtained from J. R. Geigy and Co. in Basel.

normal hydrochloric acid. The nature of this anilin dye is discussed on p. 360. It unites with aromatic bases in a weakly acid solution to form deep-red, frequently insoluble azo-methin dye compounds; with methylene compounds such as phloroglucin and phenyl-methyl-pyrazolon it gives rise to a reddish-violet colouration, while with amido remainders only yellowish-orange tints are obtained. For these reasons Ehrlich supposed the red reaction in the urine to depend on the union of the dye with a methylene radical.

Proescher [1], on the instigation of Ehrlich, investigated the doubtful substance and obtained it in sufficient quantities to determine its formula as $C_{16}H_{24}O_6N_2$. When dimethyl-amido-benzaldehyde unites with the unknown body to form the red substance, a molecule of water may be supposed to be given off. Subtracting $C_9H_{11}N$ = dimethyl-amido-benzaldehyde $-O$ from $C_{16}H_{24}O_6N_2$ and adding two atoms of hydrogen, gives the unknown body the formula $C_7H_{15}O_6$, which approaches nearest to glucosamin $C_6H_{13}NO_5$, as it differs from the latter only in containing the formaldehyde radical OCH_2. Ehrlich left it undecided whether the body in question was formyl-glucosamin or an acetyl-derivative of the still unknown pentosamin, because neither mucin, chitin, glucosamin, nor glucosamin hydrochloride give the reaction, while certain mucinoid substances do give it. 'In a section of cartilage, for example, the perichondrium stains an intense reddish-violet, while the hyaline cartilage and the surrounding connective tissue or fat remain colourless with the exception of a few peri-vascular strands containing elastic fibres' (see p. 367).

Friedrich Müller [2] confirms Ehrlich's statement that pure mucin and mucoid substances do not give the red reaction, if their watery solutions are treated directly with the reagent or even after long treatment with boiling mineral acids. Positive results, however, are always obtained if the mucinoid substances are first rendered alkaline with a little alkali or baryta, and if they are then warmed. On adding to these alkaline solutions 2 to 5 per cent. solutions of dimethyl-amido-benzaldehyde dissolved in normal HCl, till the reaction has become acid, a red colour is obtained, especially on heating.

Mucin from sputum or the submaxillary gland, ovo-mucoid, pseudo-mucin of ovarial cysts, egg-albumin, and the cartilage of the nose, all give the red reaction after having been heated in an alkaline solution. Cartilage which had not been treated with alkali but with boiling HCl for several hours readily reduced a copper solution owing to the formation of reducing sugars, but no benzaldehyde reaction was obtained.

Animal gum prepared by Weydemann's method brought directly together with the reagent gives no colour, but if it is boiled for some time with baryta-water, if the barium is removed with H_2SO_4 and the latter with lead acetate, and if the filtrate is treated with lead acetate and ammonia, a white substance is obtained, namely Fränkel's albamin, which with the benzaldehyde gives at once an intense beautiful red. Fränkel believes his albamin to be a polymer of glucosamin.

[1] Pröscher: *Zeitsch. f. physiol. Chem.* 31, 520 (1900(1)).
[2] F. Müller: *Zeitsch. f. Biol.*, Jubelband zu Ehren von C. Voit, 468 (1901).

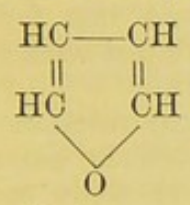
Müller has proved that free mineral acid is essential for the red colour-reaction in the following way: On the assumption that glucosamin hydrochloride does not react with Ehrlich's reagent because the hydrochloride is bound up with the amidogen radical, acetic acid was substituted for the hydrochloric acid by warming glucosamin hydrochloride with alkali or baryta-water, adding sodium acetate to prevent the formation of free HCl, and then finally adding dimethyl-amido-benzaldehyde in a solution of acetic acid. Under these conditions not a red but a bluish-green colour is obtained.

Penta-benzoyl-glucosamin does not give Ehrlich's reaction even if treated with warm KOH, by which means several benzoyl groups are separated off; but penta-acetyl-glucosamin, which itself gives negative results, gives after treatment with KOH or baryta-water a brilliant carmin colour, owing to having been split up into mono- or diacetyl-glucosamins, which latter with the dye form coloured esters.

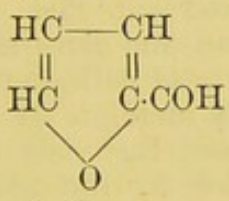
I have obtained good results histologically by fixing tissues in 0·5 per cent. KOH in 90 per cent. alcohol for 48 hours or longer at 30 to 40° C., and then transferring the tissues to a $2\frac{1}{2}$ per cent. solution of the dye in 1 per cent. HCl.

(*c*) Furfurol.

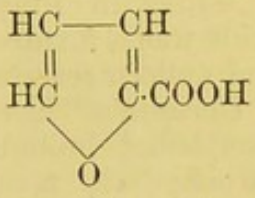
Furfurol, $C_5H_4O_2$, is a derivative of furfuran and on oxidation gives rise to pyromucic acid. Its presence is believed to indicate that the substance which is being tested either contains pentoses or substances capable of giving rise to pentoses.



furfuran

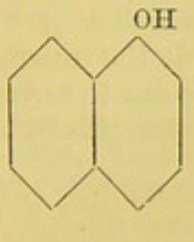


furfurol

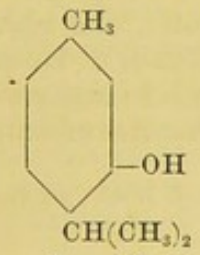


pyromucic acid

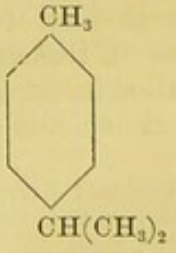
Molisch, in 1886[1], while engaged in micro-chemical research discovered 'two new sugar reactions,' one with *α*-naphthol, and the other with thymol, which is a monohydroxyl derivative of cymene:



α-naphthol



thymol



cymene

By adding to 1 cc. of a sugar solution 2 drops of an alcoholic 15 to 20 per cent. solution of *α*-naphthol; shaking the mixture; adding 1 to 2 cc. of sulphuric acid and shaking again quickly, there

[1] Molisch: *Sitzb. d. Akad. d. Wissensch. Wien*, 93, 912 (1886); *Monatshefte f. Chemie*, 7, 198 (1888).

is obtained a deep violet colouration of the fluid. On diluting the fluid with water a bluish-violet precipitate is formed, which dissolves in alcohol and ether with a yellowish and in caustic potash with a golden yellow colour, while with ammonia yellowish-brown droplets are obtained. Thymol under identical conditions gives a vermilion carmin-red colour, and on the addition of water a carmin-red precipitate.

With solutions of all sugars (except with inosit, mannit, melampyrit, and quercit), and also with inulin the reaction is obtained at once; with carbohydrates and glucosides after 2 minutes up to 30 minutes the reaction becomes more and more evident; with fresh egg-white, which with sulphuric acid alone gives a rose-red colour (Raspail's reaction), α-thymol produces at once a deep violet colour-reaction.

To apply the test micro-chemically treat a section which is not too thin with a 15 to 20 per cent. alcoholic solution of α-naphthol, then add 2 to 3 drops of sulphuric acid so as to cover the whole section, or allow the acid to flow under the cover-glass by placing a drop close to the cover-glass and then drawing the acid with a glass rod up to the margin of the cover-glass to allow it to diffuse inwards.

Seegen in 1886[1] repeated the experiments of Molisch and confirmed them, but also pointed out how peptone, pure egg-albumin, serum-albumin, and casein behaved in exactly the same manner as do sugars, and threw out the suggestion of a possible decomposition of proteid substances into sugar by means of sulphuric acid.

Mylius in 1887[2] pointed out that Pettenkofer's test for bile acids was a furfurol reaction, and gives Hugo Schiff's test with anilin and xylidin acetate as the most delicate for furfurol[3].

Udránszky[4] has on the strength of the furfurol reaction with α-naphthol brought forward the view that all proteids contain a carbohydrate radical which by the treatment with acids can be separated off as furfurol. Special attention is drawn to his laborious investigations, for although he failed in obtaining a 'class reaction' his papers deal with the interaction between a great many bodies and phenols such as α-naphthol.

Neuberg[5] discusses the α-naphthol test of Molisch-Udránszky, the resorcin test of Seliwanoff[6], the phloroglucin test of Tollens[7], and the orcin test of Cyrill Reichl[8]. He arrives at the conclusion that the so-called furfurol reaction is in many cases not due to furfurol at all, but that it depends on the presence of humus acid, $C_{18}H_{16}O_7$[9], which latter

[1] Seegen: *Centralbl. f. d. med. Wiss.* 785 and 801 (1886).
[2] Mylius: *Z. Kenntniss d. Pettenkofer'schen Gallensäurereaction.*
[3] *Ber. chem. Ges.* 20, 540.
[4] L. v. Udránszky: *Zeitsch. f. physiol. Chem.* 12, 355 to 395 (1888).
[5] Carl Neuberg: 'Über d. Farbenreactionen v. Zuckern,' *Z. f. physiol. Chem.* 31, 564 (1900–1).
[6] Seliwanoff: *Ber. d. deutsch. chem. Ges.* 20, 181.
[7] Tollens and his pupils: *Ber. d. deutsch. chem. Ges.* 22, 1046; 29, 1202; *Ann. d. Chemie*, 254, 329; 260, 304.
[8] Cyrill Reichl: *Ber. d. öster. Ges. z. Förder. d. chem. Indust.* 1, 74 (1874). See also Tollens: *Ann. d. Chem.* 260, 305.
[9] Humin substances are dark-coloured organic substances found in the soil

is readily formed from carbohydrates, as shown by Berthelot and André[1]. Neuberg believes the humus acid to form coloured condensation products with the phenols: naphthol, phloroglucin, orcin, &c.

Liebermann's reaction[2].

Dried egg-white, purified by treatment with alcohol and ether, on being boiled with strong hydrochloric acid turns deep blue or bluish-violet, and is, according to Cohnheim, also a furfurol reaction.

(*d*) Starch.

Strohmeyer in 1814 first observed that starch in combination with a watery solution of iodine gives a blue colour-reaction. Since his time many endeavours have been made to determine the nature of the reaction, but up to the present day no finality has been reached.

Mylius in 1887[3] prepared from cholalic acid an iodine compound showing a blue colour and possessing the formula $(C_{24}H_{40}O_5I)4IH$, which showed with the iodide of starch many points of analogy, both possessing for example 4I to 1IH. Thus the chemical view was strengthened.

F. W. Küster, however, in 1894[4] showed that there was no definite, constant proportion between the starch and the iodine, because the iodide of starch always contained an amount of iodine which was strictly proportional to a definite coefficient, which latter depended on the amount of iodine which was employed. He therefore denied not only all chemical but also all mechanical explanations, and adopted the view that iodide of starch was in reality a solution of iodine in starch in the sense of a solid solution according to van't Hoff's theory (see p. 330). As iodine, however, gives a blue solution with starch solutions, to support his view Küster assumed that a starch solution is not a true solution at all, but an emulsion of exceedingly minute drops of starch in an excess of water, which latter was supposed to contain traces of starch in real solution.

Mylius: Against the conception of Küster's just stated speaks a fact which was first observed by Mylius and confirmed by Spiro, namely that water is absolutely necessary for starch to turn a blue colour with iodine, for iodide of starch loses its blue colour when

and in peat, and are formed by putrefactive changes. They are obtained by acting on carbohydrates such as starch and sugar with acids and alkalies, and are acid in character. They are further formed by the action of ammonia and air on pyrogallol and protocatechuic acid. Humus acid, $C_{18}H_{16}O_7$, is formed in addition to formic acid by prolonged boiling of cane-sugar with dilute HNO_3, HCl, or H_2SO_4. The $(C_{12}H_{22}O_{11})$ sugars are first converted into $C_6H_{12}O_6$. The stronger the acid the greater is the amount of humin bodies.

[1] Berthelot and André: *Compt. Rend. de l'Acad.* 123, 567 and 625.

[2] Liebermann: *Centralbl. f. d. med. Wiss.* 371 (1887).

[3] Mylius: *Zeitsch. f. physiol. Chemie*, 11, 306.

[4] Küster: *Ann. d. Chemie*, 283; see also *Zeit. f. physik. Chemie*, 16, 156 (1894), where iodine-cholalic acid is said to be neither a chemical compound nor a solid solution, but a compound owing its existence to the formation of crystals.

dehydrated in vacuo over sulphuric acid, and starch also does not stain blue with an alcoholic solution of iodine, but becomes blue at once on the addition of water.

Spiro in his important paper on physical and physiological selection [1] has given this staining reaction his special attention. He points out that on the one hand quantitative analyses always give values varying to such a degree as to exclude the idea that a chemical compound between the iodine and the starch is formed in a constant proportion. On the other hand, the change of the brown colour of iodine into a blue tint, the fact of different starches giving different colours, and of starch solutions as well as solid starch giving the colour-reactions, all point to a chemical explanation and not to such physical ones as absorption or surface attraction.

As Spiro puts it we are forced to assume that in a solution we are dealing with a mixture of two substances acting upon one another and thereby mutually modifying their properties. According to this view, Küster's solid solution theory is untenable, and we have to imagine with Spiro that the iodine distributes itself between the two systems of water on the one hand and starch + water on the other hand in a definite manner, according to the laws governing the coefficient of distribution of a solid between two liquids (p. 329). When we are dealing with iodine and bromine the coefficient of distribution varies rapidly with their concentrations (Jakowski). In the mixture of water and watery starch, iodine becomes blue for the same reason that it becomes violet in carbon bisulphide. In either case its physical properties are altered.

It is quite immaterial whether we conceive the combination of starch, water, and iodine as a physical one—for example, as a solution —or as a chemical one for the following reasons:

Beckmann and *Strick* have shown that the change in colour which iodine undergoes in different solvents does not depend on differences in the molecular weight of the dissolved iodine, and therefore we have to take into consideration not only the changed molecular state of a substance, but also its reaction with the medium in which it is dissolved. Thus iodine distributes itself between a watery potassium iodide solution on the one hand and starch or cholalic acid solutions on the other hand in such a way, that up to a certain concentration all the iodine is held by the potassium iodide to form the molecule KI_3 [2]; but if we go on adding iodine to the potassium iodide-starch solution a point will be reached when the potassium iodide can no longer hold all the iodine, then some passes over into the starch-water compound and the blue colour is produced.

It may be that the new compound takes up iodine in definite proportions, as does cholalic acid, or it may not do so, as is the case with starch.

[1] Karl Spiro: *Über physik. u. physiol. Selection*, Habilitationsschrift (Strassburg, 1897).

[2] This compound gives off to carbon disulphide all the iodine which is not bound up as potassium iodide.

The action of solvents on substances in solution is a well-known physical fact, as evidenced for example by the birotation of grape-sugar, the molecular refraction (Gladstone), magnetic rotation (Perkin), the refractive index (Le Blanc and Rohland), the amount of affinity for substances in great concentrations (deposit of basic salts on dilution, e. g. antimony and bismuth salts). See also Brühl's and Krafft's views on p. 44.

(*e*) Glycogen.

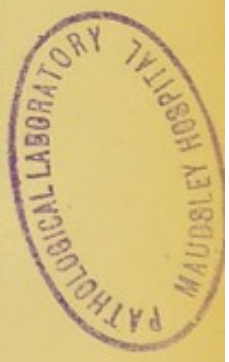
Zander[1], when applying iodine tests to chitin, arrived at the conclusion that this substance was a carbohydrate closely related to glycogen, because it gives the following reactions typical of glycogen.

Glycogen in iodine-iodide of potassium solutions turns a reddish-brown colour, which is modified in a very definite way by the following reagents:

No.	*On addition of two drops of*		*colour becomes*
1		water	weaker
2	70 per cent.	zinc chloride	disappears completely
3	*saturated*	zinc sulphate	stronger brown
4	*saturated*	alum	stronger brown
5	*saturated*	sodium acetate	violet
6	*saturated*	sodium chloride	very dark reddish-brown
7	*saturated*	ammonium chloride	dark reddish-brown

If it is desired to demonstrate glycogen in the liver quantitatively, the animal is best killed by decapitation. All anaesthetics liberate glycogen, and coal-gas is especially liable to produce a change whereby glycogen is converted into probably erythro-dextrin, for portions of a liver excised immediately after death by coal-gas, fixed in thin slices in absolute alcohol for two days and cut into sections with the free hand, show the sections to become surrounded by a deep-brown zone on placing them in dilute iodine-potassium iodide solution.

Langley states that glycogen is fixed by osmium tetroxide, and that it may be removed from sections by tryptic digestion[2]. (See de Waele's method on p. 296.)



2. Fats.

(*a*) *Fatty acids.* These acids may be divided into two distinct classes, namely into the 'normal' and into the 'acrylic' acids, with the following constitutional formulae:

$$\text{Normal acids} = C_nH_{2n}O_2 \quad = \quad C_nH_{2n+1}CO{\cdot}OH$$

$$\text{Acrylic acids} = C_nH_{2n-2}O_2 \quad = \quad C_nH_{n-1}CO{\cdot}OH.$$

Normal fatty acids are represented by the following series, in which each higher acid contains one CH_2 group more than the next lower one, thus:

[1] Enoch Zander: 'Vergleichende u. kritische Untersuchungen z. Verständniss d. Iodreaction d. Chitins,' *Pflüger's Arch.* 66, 545 (1897).

[2] J. N. Langley: *Practical Histology* (Macmillan & Co., 1901).

Formic acid.	*Acetic acid.*	*Propionic acid.*	*Butyric acid.*	*Valerianic acid.*	*Caproic acid.*
1C	2C	3C	4C	5C	6C
H	CH_3	CH_3	CH_3	CH_3	CH_3
|	|	|	|	|	|
COOH	COOH	CH_2	CH_2	CH_2	CH_2
		|	|	|	|
		COOH	CH_2	CH_2	CH_2
			|	|	|
			COOH	CH_2	CH_2
				|	|
				COOH	CH_2
					|
					COOH

The acid containing sixteen carbon atoms (C_{16}) is called palmitic, and that containing 18C, stearic acid.

Oleic acid, which is also an 18C-acid, differs from stearic acid in containing two hydrogen atoms less. It is this absence of two hydrogen atoms which leads to two carbon atoms in the carbon chain becoming joined together by a double link, a phenomenon met with in a simple form in acrylic acid, which is an unsaturated mono-carboxylic acid having the formula $H_2C{=}CH{\cdot}COOH$.

All acids having such a double link between two carbon atoms are called acids of the acrylic series, and are characterized by being very unstable compounds, because they have a great tendency to pass from the double-linked into the more stable single-linked state. This change is most readily brought about by the unsaturated carbon chain taking up two hydrogen atoms and becoming thereby saturated.

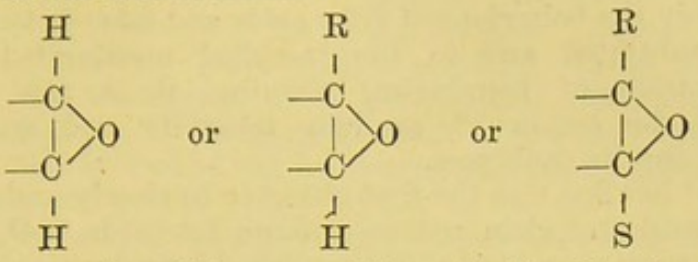
All unsaturated fatty acids, hydrocarbons, and alcohols, in short all substances containing the group $-\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}-$ and certain derivatives of this group (such as $-\overset{R}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}-$ and $-\overset{R}{\underset{|}{C}}=\overset{S}{\underset{|}{C}}-$ where R and S are monad radicals), on coming in contact with not too energetic oxidizers, take up two hydroxyl radicals, and are converted into saturated compounds according to the formulae

$$-\overset{H}{\underset{|}{C}}-\overset{H}{\underset{|}{C}}- \quad \text{or} \quad -\overset{R}{\underset{|}{C}}-\overset{S}{\underset{|}{C}}-$$
$$\qquad\quad OH\ OH \qquad\qquad\quad OH\ OH$$

This change occurs twice if two (—CH=CH—) groups, thrice if three groups are present, and so on[1].

[1] Alder Wright: *Animal and vegetable fixed oils, fat, butter, and waxes.* Griffin, London, 1893.

The first step in the conversion of (—CH=CH—) into (=CH·OH —CH·OH) consists probably in a direct combination of the unsaturated link of the carbon chain with oxygen: analogous to the union with iodine and bromine.



These compounds, while nascent, immediately absorb water; oleic acid $C_{17}H_{33}$—CO·OH, for example, combines first with oxygen to form $C_{17}H_{33}\begin{cases}=O\\-CO\cdot OH\end{cases}$; and the latter by taking up water is changed into dioxystearic acid,

$$C_{17}H_{33}\begin{cases}OH\\OH\\CO\cdot OH\end{cases}$$

By phosphorus pentachloride and similar substances, fatty acids are converted into acid chlorides:

$$C_2H_5—COOH + PCl_5 = C_2H_5—COCl + POCl_3 + HCl.$$

Acid chlorides of the fatty acids with hydroxy-compounds form ethereal salts:

$$C_2H_5—COCl + CH_3—OH = C_2H_5—CO—OCH_3 + HCl.$$

Fatty acids readily yield halogen substitution products, thus:

$C_3H_5(C_{18}H_{33}O_2)_3 + 3\,I_2 = C_3H_5(C_{18}H_{33}I_2O_2)_3$
Triolein glyceride + iodine = glyceride of di-iodostearic acid.

Fatty acids unite with alcohol to form ethereal salts or esters and water, thus:

$CH_3\cdot COOH + C_2H_5OH = CH_3CO\cdot OC_2H_5 + H_2O$
Acetic acid + ethyl-alcohol = acetic acid ethylester + water.

(*b*) **Fats:** The higher fatty acids on combining with the tribasic alcohol glycerin (glycerol[1]) form the special group of esters called fats. Thus:

$$\left.\begin{matrix}C_{16}H_{32}O_2\\C_{16}H_{32}O_2\\C_{16}H_{32}O_2\end{matrix}\right\} + \begin{matrix}CH_2\cdot OH\\|\\CH\cdot OH\\|\\CH_2\cdot OH\end{matrix} = (C_{16}H_{31}O_2)_3\begin{cases}CH_2\\|\\CH\\|\\CH_2\end{cases} + 3H_2O$$

Palmitic acid (3 molecules) + Glycerin (1 molecule) = Tripalmitin (1 molecule) + Water (2 molecules)

[1] Glycerin, being a triacid base, can combine with and neutralize three molecules of a monobasic or monocarboxylic acid forming neutral salts after the manner of the triacid bismuth hydroxide: $Bi(OH)_3 + 3HCl = BiCl_3 + 3H_2O$ (Perkin and Kipping).

Analogously oleic acid and glycerin unite to form triolein.

Fat, as it occurs in the body, is always a mixture of tripalmitin, tristearin, and triolein, of which only the latter is an unsaturated compound.

If we apply the behaviour of fatty acids and fats—belonging to the 'normal' saturated and to the 'acrylic' unsaturated series—to the explanation of histological staining, it is not difficult to understand the action of osmium tetroxide and such dyes as Sudan III and its analogues.

Altmann[1] in 1894 was the first observer to clearly understand that only oleic acid and olein reduce osmium tetroxide OsO_4 into OsO_2. In the following year *Starke*[2] distinguished between a primary reduction of OsO_4 by oleic acid, and a secondary reduction obtained by treating palmitin, stearin, and their respective acids, first with osmium tetroxide, and subsequently with alcohol.

There is no doubt that after alcohol treatment sections may show many more black granules than they did previously, but in this case the secondary reduction is due to a hydration of OsO_4 into $Os(OH_4)$ by means of the alcohol, as shown by Handwerk in 1898[3]. *Handwerk* found that the purer the stearic and palmitic acid material was, the less did it reduce osmium tetroxide, and oleic acid if frozen did not blacken osmium. To show that the negative result with the palmitin and stearin did not depend on their state of aggregation, these substances were melted on a mixture of osmium tetroxide and potassium bichromate (Marchi's solution, p. 251), when a very irregular staining resulted which Cremer suggested to Handwerk might depend on admixture with oleic acid. *My experiments* bear out Handwerk, and it is difficult to see on chemical grounds how saturated bodies such as palmitic and stearic acids, or their derivatives, can have any action on osmium tetroxide.

Daddi, in 1896[4], introduced Sudan III, a dye having the constitution given below on p. 307. It is insoluble in water, but soluble in alcohol, ether, chloroform, xylene, in bergamot, clove, and cedar-wood oils, in turpentine, anilin, and all fats. The dye obtained from Hollborn and Grübler according to Paul Mayer[5] is insoluble in glycerin, while that which Pflüger[6] used was soluble.

Daddi's method of staining: Fix tissues preferably in Müller's fluid (I prefer to fix in 10 per cent. formol, or 75 per cent. alcohol); cut sections with the free hand, or with the freezing microtome; stain sections in a saturated solution of Sudan III in spirit (?; Paul Mayer uses 70 per cent., while I use 80 per cent.); transfer sections from alcohol having the same strength as that used for dissolving

[1] R. Altmann: *Die Elementarorganismen u. ihre Beziehungen z. d. Zellen*, 116 (Leipzig, 1894).

[2] J. Starke: 'Über Fettgranula u. eine neue Eigenschaft d. Osmiumtetroxydes,' *Arch. f. Physiol.* 70 (1895).

[3] C. Handwerk: 'Beiträge z. Kenntniss v. Verhalten d. Fettkörper zu Osmiumsäure u. zu Sudan,' *Zeitsch. f. wiss. Mikr.*, 15, 177 (1898).

[4] Daddi: *Arch. ital. di Biol.* 26, 143 (1896).

[5] Lee and Mayer: *Grundzüge d. mik. Technik*, 397 (1901).

[6] *Pflüger's Arch.* 81, 379 (1900).

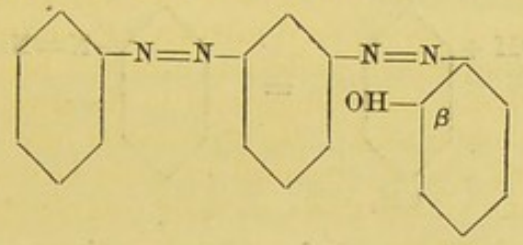
the dye, into the stain, and leave the sections in it for five to ten minutes (I prefer half an hour). Now wash out the superfluous stain with alcohol of again the same strength as that used previously. Daddi differentiates in this alcohol for the same length of time as was taken for staining the sections. Mount the sections in glycerin (Daddi; I prefer liquid paraffin). It is necessary not to use alcohols stronger than 80 per cent., as otherwise the fat is apt to be extracted. The myelin-sheath of nerves stains a pale-red colour, but this is washed out by treatment with 80 per cent. alcohol for half an hour.

Animals fed on oil coloured with Sudan III show only the adipose tissue stained of a deep orange colour.

Daddi has stated that palmitic and stearic acid crystals also stain with Sudan III, but this is wrong as shown by Rieder[1], by C. Handwerk[2], and my own experiments.

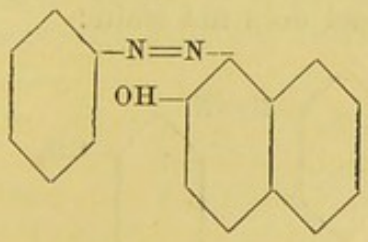
How Sudan III acts can be explained, thanks to the systematic researches of Michaelis.

Michaelis, in 1901[3], proceeded as follows: Sudan III is a tetrazo-dye, as it contains the azo-group (—N=N—) twice in its molecule (see p. 388), and it possesses further only one salt-forming group, namely, the hydroxyl-group (OH) occupying the ortho-position to the azo-group of the naphthalene nucleus.



Sudan III or azo-benzene-azo-β-naphthol[4].

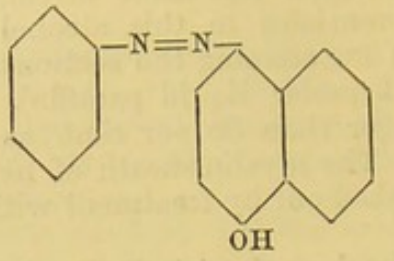
That a double azo-group was unnecessary to produce a staining of fat was proved by the behaviour of the simple azo-dye, benzene-azo-β-naphthol:



which also stains fat. Starting now with this compound, containing the hydroxyl-group in the ortho-position, the effect of having the

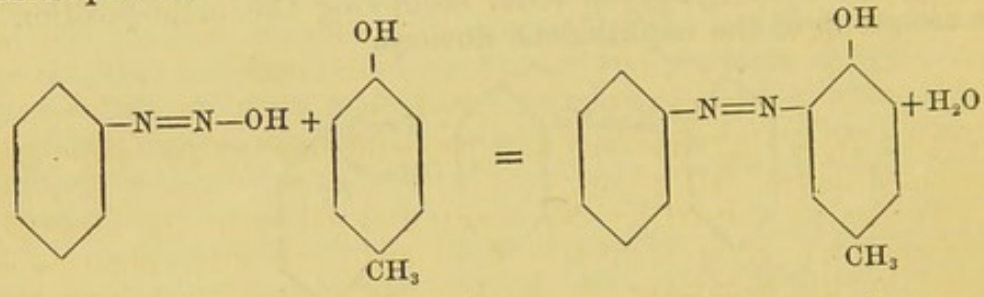
[1] H. Rieder: *Deutsch. Arch. f. klin. Med.* 59 (1898).
[2] C. Handwerk: *Zeitsch. f. wiss. Mikr.* 15, 183 (1898).
[3] Leonor Michaelis: 'Über Fett-Farbstoffe,' *Virchow's Arch.* 164, 263 (1901).
[4] This compound does not contain the hydroxyl-group OH as represented in the formula, but the oyygen attached to the naphthol radical in the β-position and the H attached to a pentavalent N, as explained later on in the text.

hydroxyl-group in the para-position was studied. Using however, benzene-azo-α-naphthol,



no staining of fat was obtained.

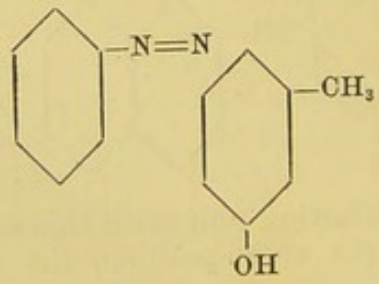
If the staining of fat depended on the hydroxyl being in the ortho-position to the azo-group, then dyes containing no naphthalene, but only a benzene nucleus, ought also to stain. Michaelis knew that an ordinary phenol has the hydroxyl-group always in the para-position to the azo-group if it be added to diazo-benzene, but that if the para-position be already occupied the OH group will take up the ortho-position. He therefore combined diazo-benzene with paracresol or toluene-phenol.



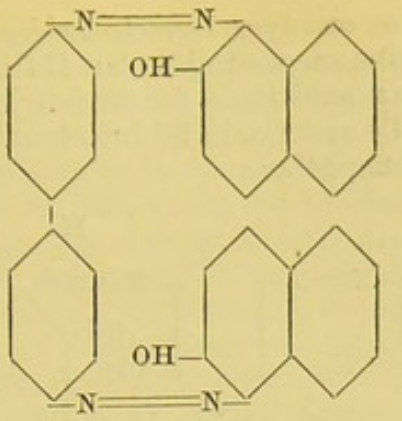
diazo-benzene + paracresol = ortho-oxyazo-dye.

This ortho-oxyazo-dye dissolved in 70 per cent. alcohol stains fat an orange-red colour.

Analogously to benzene-azo-α-naphthol (see above) the azo-dye prepared from orthocresol does not stain:

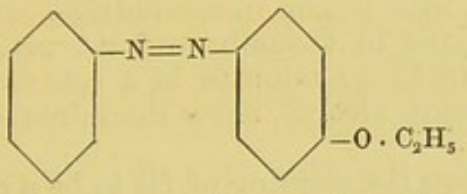


Benzidene + β-naphthol or paracresol also form azo-dyes staining fat; but because of the large size of their molecules they only diffuse with difficulty, and cannot therefore be recommended. Such a dye is diphenyl-tetrazo-di-β-naphthol.



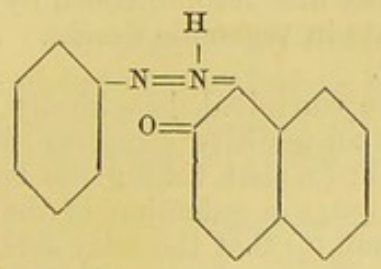
Comparable to this compound in its action is diphenyl-tetrazo-di-paracresol.

So far, Michaelis had arrived at the conception that the essential factor in staining fat was the ortho-position of the azo- and the hydroxyl groups; but then it was found that the following para-azo-dye



prepared from diazo-benzene and phenetol stained fat, although it contained no free OH group.

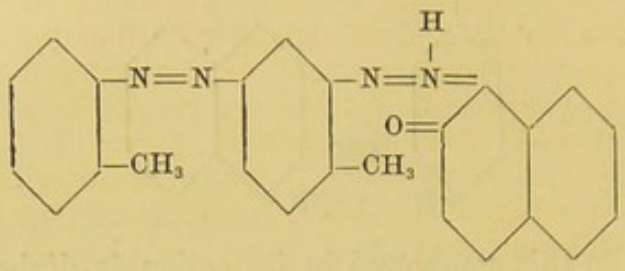
The idea of connecting the staining of fat with the absence of a salt-forming group suggested itself next, and this view was supported by the behaviour of the two above-mentioned benzene-azo α and β naphthols. The alpha-compound is an acid dye, and unites as do all phenols with alkalies, but the β-compound combines neither with acids nor alkalies, while it is readily soluble in chloroform, and to a less extent in alcohol. As the β-compound does not form salts it cannot be a phenol-like body, possessing the hydrogen-atom linked on to the oxygen-atom as in the case of hydroxyl (OH), and therefore must have the hydrogen linked on to a nitrogen-atom, thus:



(See the introduction to the azo-dyes, p. 385, and the hydrazone dyes, p. 395.)

Michaelis arrived, therefore, at the conclusion that fat will be stained by those azo-dyes which are 'indifferent,' in the sense of possessing no salt-forming groups, and he has succeeded in pointing

out along which lines azo-dyes have to be built up synthetically, if we wish to obtain stains surpassing Sudan III in their staining power. He recommends as a substitute for Sudan III, the 'Scharlach R' or fat-ponceau of Kalle and Co. in Biebreich a/Rh. This substance is azo-orthotoluol-azo-β-naphthol:



to be obtained from E. Leitz, Berlin, or Hollborn and Grübler, Leipzig, or Alexander Frazer, Teviot Place, Edinburgh.

It is insoluble in water, acids, and alkalies, slightly soluble in alcohol, very soluble in chloroform, fatty oils, and melted paraffin. It dissolves with a blue colour in concentrated sulphuric acid. Sections of material fixed in formalin and cut with a freezing microtome, stained for 15 to 30 minutes in a saturated solution of fat-ponceau in 80 per cent. alcohol, show the minutest fat-drops stained a deep-red colour.

Michaelis considers the staining of fat to be a purely physical and not a chemical process, and concludes that the physical properties of a body depend on its chemical character, because the dye molecules to be soluble in fat must have a very definite chemical constitution.

I conclude from the work of Michaelis that the union between fat-ponceau or Sudan III and oleic acid is a chemical one depending on the oxidation of the unsaturated fatty compound. Therefore the action of Sudan III and similar dyes is analogous to that of osmium tetroxide; the only difference being that azo-dyes form additive compounds with the fat without changing their colour, while osmium-tetroxide after having formed additive compounds is readily decomposed owing to the high valency of the osmium (see p. 306).

Alcannin (p. 441) was first recommended by *Dippel*[1] for the recognition of resins and fats in vegetable tissues. How this reagent acts is very difficult to say.

Rupe[2], whom I have consulted, has kindly offered the following suggestions: (1) That an additive compound is formed between the alcannin and oleic acid; (2) that, taking the two additional H-atoms into consideration (p. 304), a reduction of the fatty acid may occur, which is not likely; and (3) that the fatty acid may become oxidized at the expense of the chromophore, although this last explanation, if alcannin is really a dioxy-anthraquinone, should mean reduction of the chromophore and therefore loss of colour.

[1] Dippel: *Das Mikroskop*, 2nd edit. 271.
[2] Hans Rupe: *Chemie der natürlichen Farbstoffe*, F. Vieweg, 1900.

Cyanin or Bleu de Quinolein (p. 432), first advocated by *Certes*[1], must be used in very dilute solutions, 1 : 100,000 to 1 : 500,000, which have to be preserved in the dark. Used in these strengths only the fat granules of infusoria are stained.

Its method of action is quite obscure, although the fact of its being an iodine compound may lead to its being attracted by oleic acid; which, as shown on p. 305, has great affinities for the halogens. It is not necessary to suppose that an actual new compound is formed, but that the affinity for halogens is the factor which determines the coefficient of distribution (p. 329). To 'explain' the staining of fat by cyanin on Witt's theory of solid solutions (p. 330), is not giving an explanation at all, but amounts simply to stating a fact in a roundabout way.

K. Brandt[2] states that dilute solutions of Bismarck-brown (1 : 3—5,000) also stain fat in addition to a peculiar celluloid mucus in infusoria.

Necrosed Adipose Tissue.

Wlassak[3], in 1898, stated that fat will stain like medullated nerves by Weigert's method (p. 233), if it is fixed in Erlicky's fluid (see p. 94), because of the formation of large amounts of copper soaps.

Benda[4], in 1900, observed in material fixed in 10 per cent. formol, and subsequently in Weigert's neuroglia mordant (p. 236), that necrosed adipose tissue becomes in the copper acetate solution of a verdigris colour or as if covered with 'patina.' Fatty acids and calcium soaps give this reaction at once; oleic acid specially readily, while palmitic and stearic acids require treatment with solutions heated up to the boiling-point or a prolonged treatment with Weigert's mordant at 40° C.

Benda especially draws attention to the fact that the fatty acids form copper salts without losing their shape. It was pointed out above (p. 306) how exceedingly difficult it is to prepare pure stearic or palmitic acid, and therefore it is just possible that Benda may have had in reality the palmitic and stearic crystals contaminated by oleic acid, when working with the necrosed tissue.

(*c*) Protagon and Lecithin.

a. Their chemical constitution.

I. **Protagon** was first isolated by Liebreich[5]. It readily breaks up into the glucoside cerebrin and into lecithin, which latter on decomposition gives rise to higher fatty acids, glycero-phosphoric acid and cholin (see below).

According to Rappel, protagon is readily soluble in warm (45° C.) alcohol. It is hardly soluble in cold but fairly so in warm ether,

[1] Certes : *Zool. Anz.* 81, 208-212 ; 84, 237-288 (1881).
[2] K. Brandt : *Biol. Centralbl.* 1, 202 (1881).
[3] Wlassak : *Arch. f. Entwickelungsmechanik*, 6, 464 (1898).
[4] C. Benda : *Virchow's Archiv*, 161, 194-198 (1900).
[5] Liebreich : *Ann. d. Chemie u. Pharm.* 134, 29 (1865).

while by boiling ether it is decomposed. In cold water it is very slightly soluble, but swells up to form finally an opaque solution.

Cerebrin, according to Parcus[1], consists of three closely allied substances, namely:

(1) Phrenosin or pure cerebrin, which is completely soluble in boiling ether.

(2) Homocerebrin (kerasin), which occurs in less amount than phrenosin. It is more soluble in alcohol than phrenosin, but also soluble in boiling ether.

(3) Encephalin, which is closely related to homocerebrin. With hot water it forms a jelly.

Protagon, on being boiled with dilute sulphuric acid, gives rise to galactose (Thierfelder[2]), and to a fatty substance called cetylid by Geoghegan, which, on being melted with caustic potash, splits up into marsh gas, CH_4, and palmitic acid. Cerebrin and homocerebrin by oxidation with warm nitric acid give rise to stearic acid (Kossel and Freytag[3]).

Cerebrins are nitrogenous substances which are abundant in the medullary sheaths of nerves and in the yolk of eggs. Their mother-substance protagon is also found as Jekorin in the liver and brain (Drechsel[4]), in the spleen (Hoppe Seyler), in leucocytes (Lilienfeld), and in spermatozoa (Kossel and Freytag).

II. **Lecithin**, $C_{44}H_{90}NPO_9$, contains the following radicals: glycerin and phosphoric acid combined to form glycero-phosphoric acid:

$$\begin{array}{l} CH_2 \cdot OH \\ | \\ CH \cdot OH \\ | \\ CH_2 \cdot O{-}PO\begin{matrix} OH \\ OH \end{matrix} \end{array}$$

This in its turn is united to two molecules of a higher fatty acid, namely, to two molecules of stearic, or palmitic, or oleic acid—or there may be one stearic or one palmitic acid molecule along with one oleic acid molecule. Distearo-glycero-phosphoric acid is, for example,

$$\begin{array}{l} CH_2 \cdot O{-}C_{17}H_{35}CO \\ | \\ CH \cdot O{-}C_{17}H_{35}CO \\ | \\ CH_2 \cdot O{-}PO\begin{matrix} OH \\ OH \end{matrix} \end{array}$$

This compound unites with the ammonium base cholin

$$N\begin{matrix} (CH_3)_3 \\ -CH_2-CH_2-OH, \\ OH \end{matrix}$$

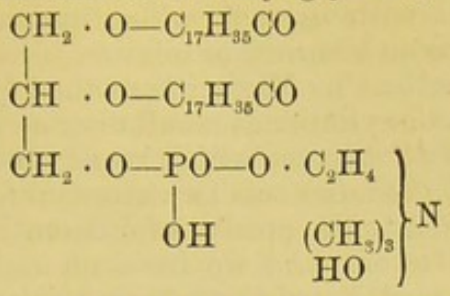
[1] Parcus: *Inaugural-Dissert.* (Leipzig, 1881); and *Journ. f. prakt. Chem.* 24, 310 (1881).

[2] Thierfelder: *Zeitsch. f. physiol. Chemie*, 14, 209 (1890).

[3] Kossel and Freytag: *Zeitsch. f. physiol. Chemie*, 17, 448 (1893).

[4] Drechsel: *Journ. f. prakt. Chemie*, 33, 425 (1886).

to form the distearin-lecithin or distearyl-glycero-phosphate of cholin :



The micro-chemical reactions of Protagon and Lecithin.

Fürst in 1896 studied the action of OsO_4 and the effect of removing it from nerves by peroxide of hydrogen. He also quotes the older literature[1].

Rudolf Wlassak[2] has thoroughly investigated the micro-chemistry of myelin or the substance of the medullary sheath of white nerves. He endeavoured to solve the questions why the medullary sheaths stain with osmium tetroxide and with the methods of Weigert (p. 233), and Marchi (p. 251).

Wlassak has investigated the action of these methods on chemically pure protagon and lecithin.

Protagon was prepared by digesting the brains of calves with alcohol at 45° C., filtering the warm extract and cooling it to 0° C. By this means protagon crystals and cholesterin are separated. The latter is extracted with ether, and the protagon crystals were repeatedly purified by recrystallization with alcohol. Of these crystals, made into a paste with alcohol, cover-glass preparations were made ; the alcohol evaporated ; the cover-glasses were rinsed in normal saline, and were then subjected to the different staining methods.

A 2 per cent. solution of osmium tetroxide and Altmann's mixture (equal parts of 2 per cent. OsO_4 and 5 per cent. $K_2Cr_2O_7$) give to the crystals a yellowish-brown colouration. Marchi's method produces a yellow colour. Osmium tetroxide gives thus negative results.

Crystals fixed for several weeks in 5 per cent. potassium bichromate, washed in water and mordanted with neutral acetate of copper and treated with Weigert's borax ferricyanide solution, give mostly dubious results: but if the crystals are fixed in Weigert's potassium bichromate and chrome alum method, a staining is obtained with haematoxylin, indistinguishable from an ordinary Weigert preparation.

To still further prove that Weigert's reaction of medullated nerves depends on the protagon radical, experiments were made with the spinal cord of the frog. The whole of the lecithin and cholesterin were removed by the following procedure :

1. Fix the cord in 80 per cent. alcohol at 0° C. for twelve hours to partially dehydrate the tissue.

2. Replace the alcohol with ether, also cooled to zero, and then extract the cord at the room-temperature for one week with many changes of ether. (Some protagon is, however, extracted.)

[1] Carl M. Fürst: *Morphol. Arb.* 6, 529 (1896).

[2] Wlassak : *Arch. f. Entwickelungsmechanik d. Org.* 6, 453 (1898).

3. Place the cord again in cooled alcohol to extract the ether; replace the alcohol with normal saline, and mordant the cord in Weigert's chrome alum-bichromate mixture.

After staining sections by Weigert's method (p. 234), the protagon is seen round the axis-cylinder as small discrete lumps and droplets.

Protagon is thus a substance which readily reduces the chromium salts into CrO_2, and the latter acts as a mordant for the haematoxylin. The resistance offered to the process of differentiation depends essentially on physical factors, and we have no right to call Weigert's method of staining medullated nerve-fibres a micro-chemical reaction.

Lecithin: Pure crystals made into cover-glass films, stained with osmium tetroxide for 10 to 12 hours, and rinsed in water show only a yellowish-brown colour. If, however, cover-glasses treated in this way are left for a longer time in water, which must be frequently changed, then there is developed gradually a pure grey tint, which approaches, where the film is thick, more or less to black. This secondary staining is seen still better if cover-glasses are treated with water containing alcohol. Wlassak points out that lecithin crystals and medullated nerves never show the intense black colour which is obtained by treating oleic acid with osmium tetroxide.

With Marchi's method, pure lecithin crystals do not stain; they only turn a yellowish-green colour, and therefore behave exactly as do medullated nerves. Lecithin thus loses its power of reducing osmium tetroxide if it is kept for some time in a solution of potassium bichromate, while olein does not.

Singer and *Münzer*[1] were the first to show that perfectly normal nerve-trunks may, and that adipose tissue always does, show a black reaction with the Marchi-Algeri method, and Wlassak adds that young developing nerves may also show a black reaction.

Wlassak in summing up states that Weigert's haematoxylin method stains the protagon; that osmium tetroxide stains fat and lecithin, while the method of Marchi only stains fat.

Halliburton and *Mott*[2] have studied the chemistry of nerve degeneration. If peripheral nerves are placed directly in Marchi's solution results are obtained which—apart from a somewhat deeper greyish-green colouration of the medullary sheaths—are indistinguishable from those seen after the ordinary process (p. 251), while the medullated fibres in the central nervous system are stained black. This difference the authors attribute to the peripheral nerves being surrounded by a primitive sheath, while the central ones are not. This explanation does not, however, take into account the fact that normal peripheral nerves do stain readily with osmium tetroxide, although the Schwann's sheath is present, nor does it account for the transverse sections of nerves fixed in Müller's fluid staining readily in osmium tetroxide if the myelin + chromium compound is broken down as in Heller's method (p. 253).

The following explanation seems more likely: As shown above

[1] Singer and Münzer: *Denkschr. der math.-naturw. Klasse d. k. Akad. d. Wiss. Wien*, 55.

[2] Halliburton and Mott: *Phil. Trans. Roy. Soc. London*, 194, 437 (1902).

only the unsatisfied acrylic series of fats, represented by olein, is capable of reducing osmium tetroxide, and it has further been pointed out (p. 312) that lecithin, usually described as a distearin compound, may also contain the oleic acid radical. From the staining effects which are obtained with a solution of osmium tetroxide, it follows either that a certain small percentage of the lecithin normally contains oleic acid, or that some oleic acid is readily formed. The latter view is based on Wlassak's observation (p. 314), that pure lecithin will stain with osmium if it be kept for some time in water.

When Halliburton and Mott find that nerves placed directly in Marchi's fluid become distinctly darker than others which have been previously treated with Müller's solution, it means one of two alternatives :

(1) In the case of the peripheral nerves the quickly diffusing potassium bichromate, by oxidizing the olein radical of the stearin-olein-lecithin, can so modify this lecithin that practically no call is made upon the osmium to part with its oxygen, and hence the osmium does not become reduced and the nerve does not appear black : or—

(2) The peripheral nerves may differ from the central nerves in possessing either less olein, or having the latter joined up to the stearin radical in some manner other than that met with in the central nervous system.

The Mott-Halliburton method of fixing peripheral nerves directly in Marchi's fluid saves a great deal of time, and amply fulfils all ordinary demands on the method, but it does not allow of a recognition of that first stage in nerve degeneration, which, long before the nerve-fibre breaks up, is characterized by a general darkening of the fibres.

In conclusion a word of warning may not be out of place : Heller's method (p. 253) shows how easy it is to break down the lecithin-chrome compound, and then subsequently to stain nerves with osmium tetroxide. Therefore in practising Marchi's method we should always be on the guard not to undo the preliminary fixation in Müller's fluid and thus to induce the appearance of degenerated fibres where none exist. I have the conviction that prolonged treatment with Busch's fluid (p. 252), especially at high temperatures, is apt to give more positive results than would the original Marchi-Algeri method. This holds good especially for that stage when fibres are commencing to degenerate ; when prolonged treatment with Müller's solution would make the fibres appear almost normal, while a short sojourn in the bichromate mixture would allow them to become stained with osmium tetroxide.

3. Proteid Substances.

Hofmeister and his school, and particularly Pick [1], distinguish in proteid substances three distinct groups, the anti-, the hemi- and the carbohydrate groups which Cohnheim [2] considers to be different derivatives of a common mother-substance.

[1] Pick : *Zeitsch. f. physiol. Chem.* 28, 219 (1899).
[2] Cohnheim : *The Chemistry of Proteids.*

The anti-group resists strongly the action of tryptic (and peptic) digestion, of acids and alkalies. It is represented by hetero-albumose, and is abundant in gelatin while absent in casein. It contains much diamido acid, much leucin and other amido fatty acids (thus probably the whole of glycocoll), and also phenyl-amido-propionic acid, but the mother radical of tryptophane and tyrosin is absent.

The hemi-group is readily acted upon by tryptic (and peptic) digestion, by acids and alkalies. It is represented by casein and protalbumose. It contains much mono-amido acid, the whole of the tyrosin and tryptophane; little diamido acid and amido fatty acids, little or no leucin; no glycocoll; as much sulphur as the original proteid, but in loose combination. It is present in all proteids with the exception of gelatin.

Both the anti- and the hemi-group contain more nitrogen and carbon but less oxygen than the original proteid.

The carbohydrate group, in addition to a carbohydrate radical, contains much oxygen, but little nitrogen and carbon. It is absent in casein.

The staining reactions of these three groups are given in a tabular form:

	Biuret R.	*Xantho-proteic R.*	*Millon's R.*	*Glyoxylic acid R.*	*Furfurol R.*	*Benzalde-hyde R.*	*Sulphur R.*
Anti-group	+	?	–	–	–	–	+
Hemi-group	+	+	+	+	–	–	+
Carbohydrate group	–	–	–	–	+	+	–

(*a*) The Biuret Reaction.

Ferdinand Rose[1], in 1833, observed how, on adding an excess of copper-sulphate solution to egg-white, or ox-serum, there was formed a greenish precipitate, which was soluble in an excess of albumin, in acetic acid, and also with a deep blue colour in ammonia. On adding caustic potash to the green copper albuminate, the latter dissolved with a violet colour; boiling this violet solution precipitated copper oxide, while the solution remained violet.

G. Wiedemann, in 1847[2], obtained biuret as one of the decomposition products of urea, and pointed out that biuret with copper sulphate and potassium hydrate forms a purple solution.

As Rose's reaction, with copper sulphate and a fixed alkali, is obtained both with biuret and with proteids, the reaction with the latter is shortly called the biuret reaction.

E. Brücke, in 1883[3], first isolated in a crystalline form the pure,

[1] Rose: 'Über d. Verbindungen d. Eiweiss mit Metalloxyden,' *Poggendorff's Ann. d. Physik u. Chem.* 28, 137 (1833).

[2] Wiedemann: *Journ. f. prakt. Chem.* 42, 255 (Oct. 1847).

[3] Brücke: *Monatsh. f. Chem.* 4, 203 (1883).

violet copper-biuret compound by adding freshly precipitated and washed copper hydroxide to an alkaline solution of biuret.

H. Schiff[1], in a very masterly manner, has solved the question as to when the biuret reaction occurs: Dry urea or carbamide $O=C\langle^{NH_2}_{NH_2}$ when heated in a crucible to 155° C. melts and then commences to boil, ammonia being given off. After a time the clear solution becomes turbid and the boiling becomes less vigorous, owing to the increased viscosity of the fluid. Whenever this stage is reached[2] a considerable part of the urea has become converted into allophanamide or biuret:

$$O=C\langle^{NH_2}_{NH_2} \;=\; HN\langle^{CO-NH_2}_{CO-NH_2}$$

urea = biuret.

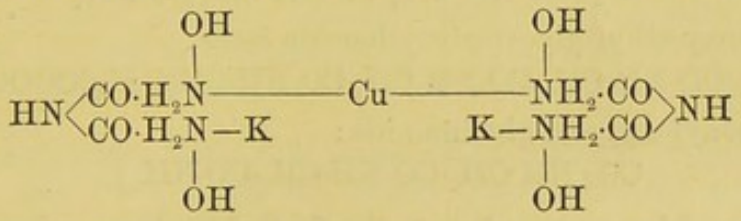
With potassium hydrate, biuret forms a potassium salt because of the displacement of H of the imin group NH, by the electrically stronger K, thus

$$HN\langle^{CO-NH_2}_{CO-NH_2} \text{ becomes } KN\langle^{CO-NH_2}_{CO-NH_2}$$

The metals silver and mercury also attach themselves usually to the imin group, but copper compounds seem to resemble rather cuprammonium compounds, as for example, the copper derivatives of many aliphatic amido acids in which the Cu is attached to the amido radical. Copper salts can unite directly with biuret, while copper hydroxide cannot, but in the presence of free caustic potash, KOH, this behaviour is reversed.

On adding copper sulphate to the potassium salt of biuret a potassium cuprammonium hydrate is formed in which the copper acts as the link between two molecules of potassium biuret.

Schiff believes the compound giving the biuret colour to have a constitution in which the nitrogen of the amido-group is pentavalent:

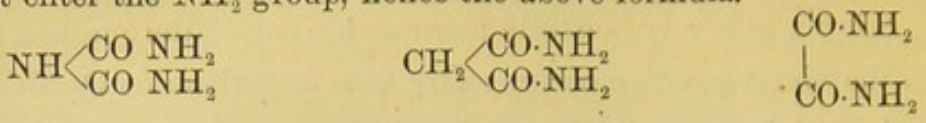


That the metallic radical is joined on to the NH_2 group is shown by the following considerations: The copper is not likely to enter the CO group, and it cannot enter the imin group NH, as the latter may be replaced in biuret by the methylene CH_2 group, as in melonamide, without the biuret reaction being interfered with: and as the NH group may be absent altogether, as in the open chain compound

[1] Schiff: *Ber. d. deutsch. chem. Gesellsch.* 29, i. 298 (1896), and *Ann. d. Chem. u. Pharm.* 299, 236 (1897).

[2] On continuing to heat cyanuric acid, $C_3H_3N_3O_3$, is formed.

oxamide in which the two carbon-atoms are directly joined, the copper must enter the NH_2 group, hence the above formula.

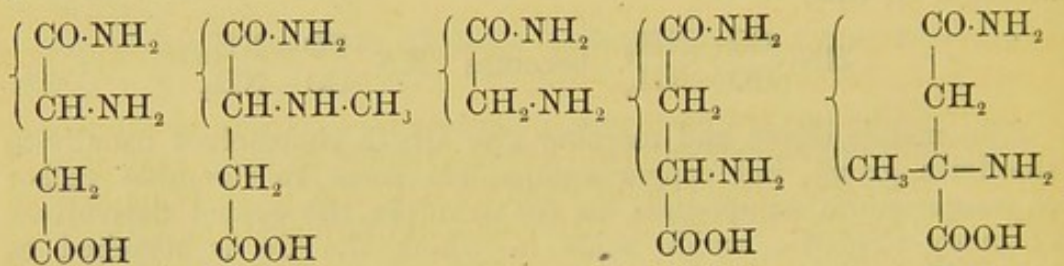


biuret malonamide oxamide.

All substances giving the biuret reaction must conform to one of the three types: of either biuret, or malonamide, or oxamide.

In malonamide and biuret two hydrogen atoms, but in oxamide only one, may be substituted.—The O of the amido-carboxyl group may be replaced by sulphur, as in thiobiuret $HN\langle^{CS\cdot NH_2}_{CO\cdot NH_2}$.

Schiff, in a later paper [1], shows that α-asparagin (onion red) and methyl-α-asparagin (red violet) belonging to the oxamide type, and glycocollamide (glycinamide or amino-acetamide); further β-asparagin and homo-asparagin (blue-violet) belonging to the malonamide type, give the biuret reaction.



α-asparagin methyl-α-asparagin glycin-amide β-aspara-gin homo-asparagin.

Emil Fischer, in confirmation of Schiff's theory, obtained a biuret reaction with the following three substances which are comparable to glycinamide [2]:

(1) carboxy-ethyl-glycyl-glycinamide:

$$C_2H_5COO\cdot NH\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot CO\cdot NH_2$$

(2) carboxy-ethyl-glycyl-glycylleucine ester:

$$C_2H_5COO\cdot NH\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot CO\cdot NH\cdot CH(C_4Hg)COOC_2H_5$$

(3) carbonyl-diglycyl-glycinamide:

$$CO\cdot[NH\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot CO\cdot NH_2]_2$$

The free acid corresponding to the first of the three substances just mentioned, namely

$$COOH\cdot NH\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot CO\cdot NH_2,$$

does not give the biuret reaction, probably owing to the presence of acid groups in the molecule. This principle cannot, however, be generalized, as α-asparagin, as shown by Schiff (see above), does give a red

[1] Schiff: *Ann. d. Chemie*, 310, 37 (1900).

[2] Fischer and Fourneau have given the term glycyl to the radical $[NH_2CH_2CO]$. Glycin or glycocoll $= (NH_2)CH_2.COOH$.

reaction. The ordinary asparagin, $COOH \cdot CH(NH_2)CH_2 \cdot CO \cdot NH_2$, which according to Schiff gives a bluish-violet colour, Fischer says reacts so little as to hardly justify the expression 'biuret.'

The diamido-aspartic acid, $NH_2 \cdot CO \cdot CH(NH_2) \cdot CH_2 \cdot CO \cdot NH_2$, gives, however, a strong biuret reaction (Fischer).

Micro-chemical Biuret Reactions.

Uric Acid.

Saint-Hilaire[1] advises the following procedure: Fix the tissue in alcohol and take it through the celloidin process. Treat sections by either of the two following processes:

(*a*) Leave sections for some hours in 5 to 10 per cent. copper sulphate solution; transfer them directly into a seething hot saturated solution of sodium bisulphite for one to two minutes to reduce the cupric salt, and to form the very insoluble cuprous urate; wash sections very carefully in distilled water, and finally place them in a solution of potassium ferrocyanide.

(*b*) Place sections in Arthaud's and Butte's reagent, rendered slightly alkaline with sodium carbonate; wash carefully, and transfer to potassium ferrocyanide.

Or fresh tissues are fixed in a hot solution of sodium bisulphate to which sufficient copper sulphate has been added to just avoid precipitation; the organ after careful washing is teased, or sections are cut and these are finally treated with potassium ferrocyanide.

The reaction depends on the formation of copper ferrocyanide $[Cu_4(CN_3)_4Fe_2]$, which has a reddish-brown colour.

[Cuprous oxide with potassium ferrocyanide produces a red colour, in addition to uric acid, also with adenin, hypoxanthin, and other bases, further with histone and dilute solutions of protamin.]

Histone.

While investigating the uric acid reactions, Saint-Hilaire noticed occasionally a marked red staining of the nuclei, which, as further researches showed, was not obtained with cuprous salts as in the case of uric acid, but with cupric salts. He arrived at the conclusion that the substance in question was histone, for reasons to be stated immediately.

Macro-chemically histone is prepared as follows: Tissues rich in nucleo-histone, for example the thymus, are extracted with water; the nucleo-histone obtained in this way is precipitated by the addition of dilute acetic acid or of calcium chloride to the watery extract; the precipitate is washed in alcohol and ether, and decomposed by dilute hydrochloric acid, which liberates the histone.

If the same procedure is adopted for micro-chemical research, then tissues extracted with water do not give the histone reaction (see below), while a feeble reaction is obtained if tissues be fixed in acetic acid. Acting with dilute hydrochloric acid on tissue fixed in acetic

[1] Constantin Saint-Hilaire: 'Über einige mikroskopische Reactionen,' *Zeitsch. f. physiol. Chem.* 26, 102–109.

acid decomposes the nucleo-histone, and the histone reaction will become more marked, provided the hydrochloric acid is not allowed to act for too long a time, as otherwise the histone is extracted. If an alcoholic instead of watery solution of hydrochloric acid be used, the nucleo-histone is also decomposed, but the histone is not extracted.

The histone or biuret reaction of Saint-Hilaire.

(1) Fix tissues in alcohol, sublimate, dilute acetic or hydrochloric acid, saturated magnesium, ammonium, or 10 per cent. copper sulphate solutions. Rapid fixation is essential to prevent the nucleo-histone and protamin passing from the nucleus into the cell-plasm. Imbed in celloidin and cut sections.

(2) Treat the sections in the presence of an alkali with a cupric (not cuprous) salt. This is most readily done by subjecting the tissues firstly to watery 0·3 per cent. cupric sulphate, and subsequently to a 0·2 per cent. watery or alcoholic solution of sodium carbonate or caustic soda.

The best plan, however, is to heat the sections in the violet, alkaline solution which is obtained by acting on a peptone solution with copper sulphate and soda, as is done in the macro-chemical biuret reaction.

The presence of histone is shown by the nuclei turning a red colour, resembling that seen in sections stained in carmin.

Histone may be extracted, after alcohol fixation, from sections (1) by boiling for 5 minutes, or heating from 35° to 60° C. for some hours, or leaving the sections in cold water for 24 to 48 hours, or (2) by boiling with dilute mineral acids for a short time.

The opposite effect, namely, fixing the histone in the nuclei, is ensured by treatment with alkalies, especially ammonia, and certain salts, such as the carbonate, hydrate, and phosphate of sodium, or ammonium and magnesium sulphates. As these reagents extract adenin and the alloxur bases, it follows that the red biuret reaction of the nuclei must be due to histone.

It was mentioned above that Saint-Hilaire, while investigating uric acid, occasionally noticed a red colour reaction with nuclei, notwithstanding the fact that cuprous salts were used. He explains this on the ground that histone and protamin are precipitated by cuprous salts, the latter in combination with ferrocyanide then giving the red colour. To me it seems more likely that, under certain conditions, uric acid is formed in the nuclei owing to the complete breaking down of nucleic acid (see p. 283).

If fresh tissues are treated first with ammonia or soda, or if they be placed directly into the alkaline peptone-copper sulphate solution, it is impossible to obtain the histone reaction; as, further, the histone is not extracted from the tissues by ammonia in the presence of salts, which are always present, it follows that it must be present in the nuclei in an undecomposed form, and also that alcohol, in fixing the tissue, must somehow decompose the nucleo-histone, or otherwise it would be impossible to obtain the histone reaction with alcohol-fixed material.

Saint-Hilaire further made use of Lilienfeld's observation that lime-water decomposes nucleo-histones, forming an insoluble lime-histone. A thymus was left for two days in limewater, and sections were treated by the methods stated above, with the result that histone could be demonstrated.

(*b*) The Xantho-proteic Reaction[1].

On adding strong nitric acid to proteid substances, a yellow colour is produced owing to the formation of nitro-substitution products with the para-hydroxyphenyl-amido-propionic acid or tyrosin, $C_6H_4(OH)CH_2 \cdot CH(NH_2)COOH$ [2], analogous to the formation of picric acid, $C_6H_2(NO_2)_3OH$ (p. 383), or its reduction compound picramic acid, $C_6H_2(NO_2)_2NH_2OH$. The yellow xantho-proteic colour by the addition of ammonia becomes orange, and by a slight excess of caustic soda reddish-brown. By the addition of nitric acid the sulphur radical in the molecule is oxidized, and therefore the sulphur reaction (see below) is not obtainable.

(*c*) Millon's Reaction.

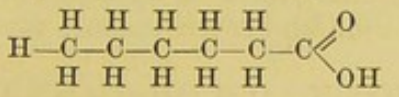
Millon in 1849[3] recommended for the detection of proteid substances a mixture of mercuric and mercurous nitrate, but there are also present in this mixture free nitric and nitrous acids. Proteids acquire in this reagent a reddish-pink colour, quickly on being heated and slowly at the ordinary temperature. *Hoffmann*[4], quite independently of Millon, advocated a mixture of mercuric nitrate and potassium nitrate for the detection of tyrosin ; and *Rudneff*[5] pointed out the connexion between the proteid and the tyrosin reactions. *Plugge*[6] obtained Millon's reaction with phenols, salicylic acid, and the decomposition products of tyrosin. *Nasse*[7] then showed that the reaction is obtained with all hydroxylated aromatic substances. *Nickel*[8] explained the reaction as a typical nitroso-reaction.

The two most recent papers are by Vaubel[9] and Nasse[10].

Vaubel prepares Millon's reagent by dissolving 1 part by weight

[1] Details as to the exact interaction are given in Cohnheim's *Chemistry of Proteids*, English translation, Macmillan & Co.

[2] Tyrosin is also called para-hydroxyphenyl-alanin, because alanin is a term given to α-amido-propionic acid $CH_3 \cdot CH(NH_2)COOH$, while leucin is α-amido-isocaproic acid $CH \cdot (CH_3)_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$, caproic acid being $C_6H_{12}O_2$ or



[3] Millon : *Compt. Rend.* 28, 40 (1849).

[4] Reinhold Hoffmann : *Ann. d. Chem. u. Pharm.* 87, 124 (1853).

[5] Rudneff : *Virchow's Arch.* 33, 71 (1865).

[6] Plugge : *Zeitsch. f. analyt. Chem.* 11, 173 (1872).

[7] Nasse : *Chem. Centralbl.* 1897, p. 487.

[8] Nickel : *Die Farbenreactionen d. Kohlenstoffverbindungen*, Berlin, 1890.

[9] Vaubel : *Zeitsch. f. angew. Chem.* (1900), p. 1125 ; abstracted in *Journ. Soc. Chem. Indust.* 20, 71 (1901), from which my account is taken.

[10] Nasse : *Pflüger's Arch.* 83, 361 (1901).

of metallic mercury in 1 part by weight of nitric acid (sp. gr. 1·4), warming the mixture gently, and then adding 2 parts by weight of distilled water. This solution contains therefore mercuric and mercurous nitrate and nitric oxide, which latter may be converted into nitrogen dioxide by the atmospheric oxygen. As these gases are essential for the reaction, Millon's reagent should not be overheated.

Vaubel obtained positive results with the following substances:

(1) $C_6H_4\overset{1}{\overline{OH}}\cdot\overset{4}{\overline{X}}$, such as tyrosin, *p*-diphenol, phenol-phthalein, and hydroquinone.

(2) $C_6H_4\overset{1}{\overline{OH}}\cdot\overset{2}{\overline{X}}$ and $C_6H_3\cdot\overset{1}{\overline{OH}}\cdot\overset{2}{\overline{X}}\cdot\overset{4}{\overline{X}}$, such as pyrocatechol, guaicol, *p*-nitro-salicylic acid.

(3) $C_6H_4\overset{1}{\overline{OH}}\cdot\overset{2}{\overline{X}}\cdot\overset{3}{\overline{X}}$ and $C_6H_4\overset{1}{\overline{OH}}\cdot\overset{2}{\overline{X}}\cdot\overset{3}{\overline{X}}\cdot\overset{4}{\overline{X}}$, such as α- and β-naphthol, 1·4 bromo and chloro-α-naphthol and bromo-β-naphthol.

(4) $C_6H_4\overset{1}{\overline{OH}}\cdot\overset{3}{\overline{X}}$, such as resorcinol and *m*-cresol.

But no reaction was obtained with—

(*a*) $C_6H_2\overset{1}{\overline{OH}}\cdot\overset{2}{\overline{X}}\cdot\overset{4}{\overline{X}}\cdot\overset{6}{\overline{X}}$, such as tribromo-phenol, dibromo-*p*-diphenol, dibromo-tyrosin, dibromo-*o*-cresol, dibromo-*p*-cresol, and dibromo-*p*-nitrophenol.

(*b*) $C_6H_3\overset{1}{\overline{OH}}\cdot\overset{3}{\overline{X}}\cdot\overset{5}{\overline{X}}$, such as orcinol and phloro-glucinol.

(*c*) Thymol (see p. 299) and pyrogallol (see p. 436).

The interaction between phenol and Millon's reagent Vaubel supposes to take place thus:

$$2C_6H_5OH + 2NO + O = 2C_6H_4OH\cdot NO + H_2O$$

$$C_6H_4OH\cdot NO + 4HgNO_3 + NO + 3C_6H_5OH$$

$$HO{-}N\equiv\left[\begin{array}{c}\cdot N\cdot O\cdot C_6H_4\cdot O\\ \cdot\qquad\qquad\cdot\\ O\text{———}Hg\end{array}\right]_4 + NO_2 + 3H_2O.$$

This compound on being heated with caustic soda solution loses mercury, and a new compound is obtained which is soluble in the caustic soda with a reddish-brown colour, and which is precipitable by acids.

$$HON(NO_2C_6H_4OHg)_4 + 9NaOH =$$

$$NaON\ll\begin{matrix}(OC_6H_4ONa)_2\\(C_6H_4ONa)_2\end{matrix} + 4Hg + 5H_2O + 2NaNO_2 + 2NaNO_3.$$

Nasse[1] lays stress on the point first mentioned by Plugge, that Millon's reagent as generally prepared will readily lead to hydroxylation of benzene derivatives, and that therefore the typical Millon's

[1] Nasse: *Pflüger's Arch.* 83, 361 (1901).

reaction may be obtained, although primarily no hydroxylated benzene compounds have been present.

For this reason Nasse prefers not to use the Millon's reagent commonly employed, as it contains nitric acid, but to substitute for it a watery solution of mercuric acetate, which is mixed immediately before use with a few drops of a 1 per cent. solution of sodium or potassium nitrite, taking special care not to add too much of the nitrite. Should the acid reaction of the mercuric acetate be not sufficient to procure the colour-reaction, then a little acetic acid must be added.

Millon's reaction is interfered with by the presence of peroxide of hydrogen, or by larger amounts of chlorides (Salkowski[1]) and alcohol (Nasse), although the latter may be added after the reaction is completed.

With the Millon-Nasse reagent ortho-compounds give a brownish-red colour, while para-compounds become bluish-red (up to pure blue), and in strong solutions rose-pink, as for example tyrosin and its derivative paracresol. Meta-compounds give only indefinite colour-reactions. Nasse strongly recommends a systematic study of the formation of aromatic compounds in plants, to determine how and when benzoic, salicylic, and *p*-oxybenzoic acids are formed.

A Specific Tyrosin Reaction.

Denigès[2] has recommended the well-known phenol-aldehyde reaction for the detection of tyrosin. *Nasse*[3], in repeating Denigès' observations, has found the following to be a very delicate test for tyrosin, as neither proteids nor peptones give the colour-reaction. Proceed thus: Add a few drops of formol solution to concentrated sulphuric acid, when on warming with tyrosin a brown-red colour is obtained, which on the addition of acetic acid becomes green.

(*d*) The Tryptophane-Glyoxylic Acid Reaction.

Adamkiewicz in 1874[4] showed that egg-white which was dried, which had its fat removed, and which was dissolved in acetic acid gave, on the addition of strong sulphuric acid at the junction of the two acids, a play of colour: red, green and violet rings being formed.

Udránszky[5] believed the reaction to be a furfurol reaction, and Hofmeister[6] interpreted the reaction as due partly to furfurol and partly to oxyphenyl radicals of an aromatic group.

Hopkins and Cole[7], however, showed the reaction to depend on glyoxylic acid, which is a common impurity in acetic acid. They

[1] Salkowski: *Virchow's Arch.* 81, 552.
[2] Denigès: *Compt. Rend.* 130, 583 (1900).
[3] Nasse: *Pflüger's Arch.* 83, 361 (1901).
[4] A. Adamkiewicz: *Pflüger's Arch.* 9, 156 (1874); and in *Ber. d. deutsch. chem. Gesell.* 8, I, 161 (1875).
[5] Udránsky: *Zeitsch. f. physiol. Chem.* 12, 395 (1888).
[6] Hofmeister: *ibid.* 24, 159 (1897).
[7] Hopkins and Cole: *Proc. Roy. Soc.* March 1901, p. 21.

also disproved the furfurol theory, as this substance gives no colour-reactions with glyoxylic acid. Pure acetic and sulphuric acids acting together on proteids char them, but if sufficient glyoxylic is present no charring occurs, and a pure violet-blue colour is developed.

Glyoxylic acid, HCO·COOH or $CH(OH)_2$·COOH, contains the aldehydic and carboxylic groups in juxtaposition. It is most readily prepared in this way: 'Place in a moderately strong solution of oxalic acid a few lumps of sodium amalgam, the amount taken of the latter being less than sufficient to neutralize the acid. When the evolution of hydrogen has ceased, the solution is poured off from the mercury and filtered.' This solution, even if greatly diluted, gives the typical Adamkiewicz reaction if it be mixed with the proteid, and if then at least one-third the volume of sulphuric acid is added.

None of the following substances give the glyoxylic reaction: glycol, glycollic aldehyde, glycollic acid, glyoxal, oxalic acid (which are typical two-carbon compounds); glycuronic acid,

$$HCO(CH{\cdot}OH)_4COOH$$

(an aldehyde acid); pyruvic acid, $CH_3{\cdot}CO{\cdot}COOH$ (a ketonic acid). Mesoxalic acid does give the reaction, but only because it loses carbon dioxide and is converted thereby into glyoxylic acid.

In a later paper Hopkins and Cole[1] describe how to isolate the proteid radical which gives the typical glyoxylic reaction. The substance in question, having the formula $C_{11}H_{12}N_2O_2$, is either indol-amino-propionic acid or skatol-amino-acetic acid.

Indol, $C_8H_7N = C_6H_4\langle^{CH}_{NH}\rangle CH.$

Amino-propionic acid $= CH_3{\cdot}CH(NH_2){\cdot}COOH.$

Indol-α-propionic acid $= C_6H_4\langle^{CH}_{NH}\rangle C{\cdot}CH(CH_3){\cdot}COOH.$

Indol-amino-propionic acid $= C_6H_4\langle^{CH}_{NH}\rangle C{\cdot}C(NH_2)(CH_3)COOH.$

Skatol (or β-methyl-indol) $C_9H_9N = C_6H_4\langle^{C(CH_3)}_{NH}\rangle CH.$

Amino-acetic acid (or glycosin) $C_2H_5NO_2 = CH_2{\cdot}(NH_2){\cdot}COOH.$

Skatol-amino-acetic acid,

$$C_{11}H_{12}N_2O_2 = C_6H_4\langle^{C{\cdot}CH_3}_{NH}\rangle C{\cdot}CH(NH_2){\cdot}COOH.$$

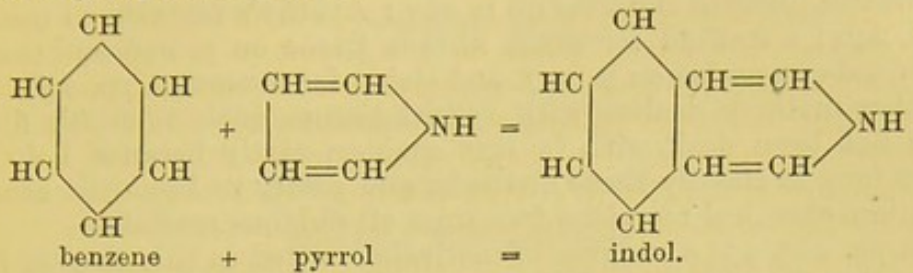
The skatol-amino-acetic acid is in all probability the much sought-after tryptophane, which is supposed to be the chief substance producing the brown and black pigments in the body.

With glyoxylic acid the tryptophane of Hopkins and Cole gives in the presence of sufficient sulphuric acid a deep indigo colour, while weak solutions show the ordinary violet-blue colour of proteid solutions. To obtain the typical tryptophane reaction, add cautiously to

[1] F. Gowland Hopkins and Sydney W. Cole: *Journ. of Physiol.* 27, 418 (1901).

a watery solution of this substance, bromine, when a rose-red colour is produced, which may be extracted with amyl-alcohol.

The indol above referred to is a condensation product of a benzene-ring and a pyrrol-ring, these two rings having, after condensation, two CH-groups in common:



For this reason indol, and also tryptophane, give the typical pyrrol reaction (see also p. 395).

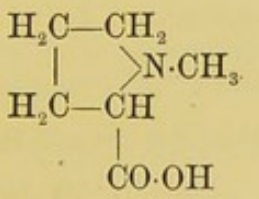
(*e*) The Pyrrol Reaction.

On steeping a piece of pine-wood in strong hydrochloric acid and alcohol, and then bringing it in contact with indol, a red colour is developed. If a pine-wood match is steeped in strong hydrochloric acid; the excess of acid washed off with water; the match dipped in a strong solution of tryptophane and then either dried or held in the steam of a water-bath,—a deep blue colour is developed (Hopkins and Cole).

Hopkins and Cole point out that in the light of their work the production of skatol and indol derivatives from proteids can no longer be looked upon as a secondary process, especially as the glyoxylic acid colour-reactions are obtained with normal proteids, and as tryptic digestion is not sufficiently violent to materially alter the constitution of the proteid molecule.

Emil Fischer's[1] discovery that pyrrolidin-carboxylic acid results from proteid hydrolysis, Hopkins and Cole believe to be fully established by their own work. The pyrrol-ring may therefore exist either as skatol or as pyrrolidin-carboxylic acid.

The N-methyl-pyrrolidin-α-carboxylic acid, according to Willstätter[2], has the formula:



(The Furfurol Reaction is given on p. 299 under the carbohydrates.)

Some special methods, usually described as 'micro-chemical,' have already been referred to when discussing the principles of staining, for example Weigert's methods for fibrin on p. 213, for neuroglia on

[1] Emil Fischer: *Z. f. physiol. Chem.* 33, 164 (1901).

[2] R. Willstätter: 'Synthese d. Hygrinsäure,' *Ber. d. deutsch. chem. Gesellsch.* 33, 1160 (1900).

p. 236; the Weigert-Stroebe method for axis-cylinders on p. 222; Ehrlich's methods for mast-cells on p. 213, and for neutrophil granules on p. 219; Carnoy's methyl-green method for nuclei on p. 215; Gram's and Claudius' methods for bacteria on p. 223; v. Kupffer's methods for nerve-fibrils on p. 224, for star-shaped cells of the liver and the connective tissue of the liver on p. 267; Apáthy's neuro-fibril method on p. 240; a method for white fibrous tissue on p. 216 and one for red blood-corpuscles on p. 217, and stains for mucus on pp. 238, 246.

Other methods dealing with special tissues, such as elastic fibres, have not been dealt with in this chapter, partly because I do not know how to classify these methods, and partly to keep this chapter on micro-chemical reactions free from all dubious reactions.

On pp. 445–451 a number of methods omitted in this chapter have been placed together under the provisional heading of micro-anatomical reactions.

The discussion of intra vitam staining has purposely been left out, as I know as yet too little about the chemical side of this most interesting aspect of histo-physiology.

The examination of fresh tissues is also not discussed.

PART VII.

CHAPTER XXIV.

The Theory of Staining.

General Introduction.

Is the staining of animal and vegetable matter by dyes a purely physical, a purely chemical, or a physico-chemical process?

These questions have been open for a long time and cannot be said to have been answered as yet in all their different aspects. There are upholders of all three views.

Whatever takes place ultimately between the tissue and the dye, it is evident that they must first of all be brought together, and this is only possible by physical means. In the chapter on the principles of staining (pp. 198–211), the physical aspects of the tissue, the dye and the solvent, and the question of time have been discussed, and I believe purely physical staining not only to be possible, but also in many cases to be mistaken for chemical.

But we have not only to reckon with tissues which as regards the staining fluid have been rendered completely insoluble and which absorb dyes as will a piece of dry chalk by imbibition, or as does carbon by adsorption, but also with material which has preserved its original characters more or less, or which, although its primary nature has been changed completely, is still able to act chemically. For this reason it is necessary to study in connexion with staining the fixatives which were used.

In addition to the physical and chemical state of the tissues we have to inquire further into the dyes and their solvents, not not only from the physical aspect as has been already done, but also from the chemical side. It is for this reason that the chapter on the chemistry of the dyes has been appended to this volume, and that solvents containing 'accentuators' have been classed together (p. 212).

The General Laws governing Osmosis.

Osmotic pressure is that force which, chemical action being excluded, causes the molecules of any substance to separate from one another, and in doing so either to pass in between the molecules of other surrounding substances 'by diffusion,'—or to attract and to store between themselves the molecules of some outside substance by 'osmosis.'

Diffusion is thus the power by which substances pass from a place of higher concentration to that of a lower one. In the case of gases the molecules diffuse into ether, the molecules of solutions

which are miscible diffuse into one another, and the molecules of solids provided they are soluble diffuse into solutions, and finally solids may diffuse into one another.

Thus common salt, when put into water, behaves as if it were a gas (van 't Hoff), the sodium chloride molecules diffusing partly as such and partly, after their electrical dissociation, as sodium kat-ions and chlorine an-ions (see pp. 11, 12).

William Henry[1] in 1803 showed that a gas will diffuse into water in direct proportion to the amount of pressure it is subjected to, or as he puts it, 'Water will take up under equal circumstances of temperature the same volume of condensed gas as of gas under ordinary pressure. But as the spaces occupied by every gas are inversely as the compressing force, it follows that water takes up of gas condensed by one, two, or more additional atmospheres, a quantity which, ordinarily compressed, would be equal to twice, thrice, and so on, the volume absorbed under the common pressure of the atmosphere.'

Dalton in 1805[2] proved that if a mixture of gases be taken, each gas is absorbed by a fluid as if the other gases were not present at all, or in other words in direct proportion to the amount of percentage or 'partial' pressure it exerts in the mixture of gases. Therefore a gas will come to a state of equilibrium whenever the pressure of the gas outside the fluid (gaseous pressure) is equal to the pressure exerted by it inside the fluid.

As the amount of action of both the gas and the fluid can be expressed in numbers we may speak of the coefficiency of action between the gas and the fluid. As, further, the phenomenon we are studying is the relative amount of absorption, we speak of the coefficiency of absorption.

Thus the absorption coefficient of oxygen for ordinary or one atmospheric pressure at 0° C. is 0·04114, and that of nitrogen at the same temperature equals 0·02035. As further 100 volumes of air contain 79·04 volumes of nitrogen and 20·96 volumes of oxygen, the percentage or partial pressure on the nitrogen is 0·7904 of an atmosphere, and that of oxygen is 0·2096. Lastly, as the solubility of a gas is proportional to its partial pressure,

0·02035	×	0·7904	=	0·016084		
absorption-coefficient of N	×	partial pressure of N	=	proportion of N dissolved in water.		
0·04114	×	0·2096	=	0·008629		
absorption-coefficient of O	×	partial pressure of O	=	proportion of O dissolved in water.		

The percentage composition of nitrogen and oxygen absorbed from the air is therefore as 0·016084 : 0·008629 or as 65·09 to 34·91.

Let us now assume that instead of dealing with gases and fluids, we have to do with two fluids *A* and *B*, which are not miscible, and that a third substance *C* is soluble in either fluid without undergoing a chemical change. On shaking up *A* and *B* with *C* the latter will

[1] Henry: *Phil. Trans.* 29, 274 (1803).
[2] Dalton: *Manchester Memoirs* (1805).

distribute itself between the two fluids in a very definite way, as was first shown, experimentally, by Berthelot and Jungfleisch[1]. Thus *C* will never dissolve only in *A* or only in *B*, but always in both *A* and *B*. Because the relative amounts of *C* in the fluids *A* and *B* can be expressed in numbers, the term 'coefficient' is again applied, and as *C* distributes itself between *A* and *B* we may speak of the coefficient of distribution.

The coefficient of distribution is independent of the relative volumes of the two solvents; for example succinic acid dissolved in a mixture of ether and water always gives the coefficient 6·0, which means that if from the mixture of ether and water equal quantities of ether and water are taken, and the amount of acid present in these is determined, the ether will always contain six times more acid than does the water. It is quite immaterial what quantities of ether or water are shaken up together with succinic acid; thus the coefficient of succinic acid is 6·0, whether the ether is to the water in the proportion of 1 : 1 or 3 : 1 or 1 : 3.

In 1887[2] van 't Hoff generalized the laws of Henry and Dalton given above, and said that if the molecules of a substance are of the same size when in the form of a gas and when in solution (or, what is the same, if molecules in equal concentration exert the same osmotic pressure in the gaseous state as when dissolved), then this substance will dissolve according to its gaseous pressure. Reversely the partial pressure exerted by the substance in the fluid is proportional to the osmotic pressure exerted by the gas in the space above the fluid.

Nernst, in 1891[3], generalized Berthelot's and van 't Hoff's laws thus: If a substance dissolves in two solutions which are only slightly miscible, and if its molecules are the same size in both fluid media, then it must possess a constant coefficient of distribution. Further, if an equilibrium has been established at a certain temperature, then the relative concentration of the substance in the two solutions must be independent of the quantity of either of the latter. Each molecule will also behave quite independently of other molecules which may be present.

The reason that a substance diffuses more readily into one of two media depends on the fact that its molecules will always travel in that direction where the least osmotic pressure is developed, and an equilibrium between the two solutions, as regards the diffusing substance, will be only established when the osmotic pressure of the substance has become the same in these two solutions.

It is possible to modify the coefficient of distribution between two fluid media by altering the nature of one of them. If, for example, to a mixture of ether and water containing succinic acid in the proportion of 6 : 1 there is added common salt, the latter will dissolve in the water, and in doing so raise the osmotic pressure of the succinic

[1] Berthelot and Jungfleisch: *Ann. de Chem. et de Phys.* (4) 26, 396 (1872); Berthelot: *ibid.*, p. 417.

[2] van 't Hoff: *Zeitsch. f. physik. Chemie*, 1, 448 (1887).

[3] Nernst: *ibid.* 8, 110 (1891).

acid, and thereby compel it to pass over into the region of less osmotic pressure, namely into the ether. If on the other hand an alkali be added to the ether-water mixture, the alkali will dissolve in the water, withdraw the succinic acid from the ether, and bind the acid by forming a salt. As a chemical change takes place in the case just mentioned we can, according to the generally accepted definition, no longer apply the term 'coefficient of distribution.'

Should, however, an alkali have no affinity for an acid, such as phenyl-glyceric acid $C_6H_3 \cdot CHOH \cdot CHOH \cdot CHOH$, then the acid is driven from the water into the ether[1].

The first to apply the physical laws just stated to the explanation of physiological phenomena, and to explain so-called vital processes of absorption, &c., on the ground of membranes being permeable only to certain radicals, while capable of withstanding the osmotic pressure of others; the first to have realized the importance of the laws governing the coefficient of distribution in relation to physiology, is Hofmeister, whose beautiful researches are so little known as to still allow some people to speculate on vitalism apart from physics and chemistry.

The preliminary account given above was necessary to explain the physical views of staining put forward by Witt, v. Georgievics, and others.

The physical aspect of staining.

O. N. Witt[2], in 1891, published his theory that staining is simply a question of solid solutions. A solid solution, according to the definition of van't Hoff[3] in 1890, is a firm homogeneous complex of several substances, the proportions of which may vary without interfering with the homogeneity of the solid, the isomorphic mixed crystals of alums, for example, being such solid solutions.

Witt thus holds that dyes dissolve in solid tissues in exactly the same way as they do in fluid media; that just as it is possible to shake out certain chemical substances from their watery solutions by means of amyl-alcohol or ether, so does the tissue under certain conditions take up the dye from its watery solution. That the dye in the tissue is in solution as is, for example, gold in ruby glass, he holds to be proved by the fact that while the solid crystals of fuchsin have a metallic green lustre, the solution of the dye in water or in the tissue has a bright red colour. He argues that a purely physical absorption ought to impart to dry fabrics or tissues not a red but a metallic green appearance. Another example given is rhodamin, which in the dry state is non-fluorescent, but shows a marked fluorescence, not only in a watery solution, but also in the 'solid' solution of the fibre, which latter is thought to play the part of a solvent.

Witt thus does not take up a purely physical standpoint, neither does he believe in a chemical explanation.

[1] Spiro: *Über physikalische und physiologische Selection*, Strassburg, 1897.
[2] O. Witt: 'Theorie d. Färbeprocesses,' *Färberzeitung* (1890–1).
[3] J. H. van't Hoff: *Zeitsch. f. physik. Chemie*, 5, 322 (1890).

Gierke[1] was the first histologist to draw attention to the fact that most staining methods can readily be explained by purely physical laws; the factors coming into play being—

(1) the form and arrangement of molecules,
(2) the relative size and shape of molecular interstices,
(3) the nature of the substance exerting attraction,
(4) the rate of diffusion,
(5) the temperature.

He also insisted on the importance of remembering that the process of fixation to a great extent determines the staining power of tissues.

Notwithstanding the fact that he supposed even mordants to be taken up by surface attraction and to adhere to the tissue by the same force, and dyes to pass into the tissue owing mainly to their power of diffusion, he definitely says: 'I do not want to deny the occurrence of chemical unions in cells. Just the very opposite,' and then goes on to point out that from a micro-chemical point of view such staining is of course essential.

Rawitz[2] is also an upholder of the physical view of staining.

G. v. Georgievics states in support of the physical theory of staining[3], that dyes are not only carried into the tissues by diffusion, but that they stain different fabrics to a different extent according to the amount of osmotic pressure they can develop in the various tissues. The lower the osmotic pressure is, to which a dye is subjected in any medium, the larger will be the number of molecules aggregating in that medium, and the deeper will be the stain. This aggregation of molecules, accompanied by a change in the molecular state of the dye radical, he holds to explain most simply why different tissues, when stained with the same dye, show varying degrees of fastness[4].

Adsorption. The dyes having once diffused into the tissues are supposed to be kept there by the adsorptive powers of the latter. The term adsorption was first used by Frankenheim[5] to express the property possessed by all solid bodies, and in particular those which are in fine subdivision (carbon or platinum black), of taking up, out of mixtures, liquid or gaseous substances, and of condensing these within themselves, or, strictly speaking, on their free surfaces (see also p. 365).

If C_s is the amount of dye in 100 grams of a tissue, and
C_w the amount of dye in 100 cc. of the dye-bath, then

[1] Gierke: 'Färberei zu mikroskopischen Zwecken,' *Zeitsch. f. wiss. Mikrosk.* 1 (1884), and 2 (1885).

[2] B. Rawitz: 'Bemerk. ü. Mikrotomschneiden u. ü. d. Färben mikroskopischer Präparate,' *Anat. Anz.* 13 (1897).

[3] G. v. Georgievics: 'Über d. Wesen d. Färbeprocesses,' *Sitzb. Akad. d. Wiss. Wien*, 103, 589 (1894). See also v. Georgievics and E. Löwy: ibid. 104, 309 (1895).

[4] A colour is said to be fast if it can withstand the action of such physical and chemical means as light, water, acids, alkalies, and soaps.

[5] Frankenheim: *Lehre von der Cohäsion*, 158, Breslau, 1835. See also Emil du Bois Raymond's *Vorlesungen ü. d. Physik d. organ. Stoffwechsels*, Berlin, 1900.

$\frac{C^s}{C_w}$ expresses the coefficient of distribution. From van 't Hoff's and Nernst's modification of Henry's law it follows further that if $\frac{\sqrt{C_w}}{C_s}$ = constant, the dye-radical adsorbed by the silk must have a molecular structure which differs from that which exists in watery media or in solutions containing sulphuric acid.

While experimenting with indigo-carmin (sulphindigotate of sodium) and silk, v. Georgievics found silk did not stain readily till sulphuric acid was added to the dye-bath. Up to a certain point, the amount of dye taken up by the fibre depended on the quantity of acid which was added, and he concluded that sulphuric acid acts in this case in exactly the same manner as does sodium chloride when cotton is dyed with congo-red. The sulphuric acid acting on the indigo-carmin, and the sodium chloride acting on the eosin, diminish the stability of these dyes in the dye-bath, or in other words increase their osmotic pressures, and thereby force them to go to a medium where they are subjected to less osmotic pressure, namely to the silk or the cotton.

To prove this point v. Georgievics extracted silk dyed in indigo-carmin, firstly with water alone, and secondly with water containing different amounts of sulphuric acid, and found that less dye was extracted by increasing the amount of acid in the water.

Beyond a certain point, however, the addition of acid to the dye-bath had the reverse effect, as now the silk took up less dye, owing to some of the sulphuric acid having entered the silk and having satisfied its adsorptive properties (v. Georgievics).

Diluting the dye-bath also changed the coefficient of distribution, as the fibres combined now with relatively more dye than when strong solutions were used, and the gradual increase in the value $\frac{\text{C}-\text{fibre}}{\text{C}-\text{dye-bath}}$ with diminishing concentrations of the stain makes it to him probable that from solutions of substantive dyes[1] the fibres take up simpler dye molecules. This observation agrees with the fact that substantive dyes stain also better in hot solutions, where big molecules are broken up into smaller ones.

Georgievics holds the fact of sulphuric acid, up to a certain degree, augmenting or facilitating the adsorption of the dye to be directly against the theory of chemical union between the fibre and the dye, as chemists must assume dye radicals in the fibre and in the bath to be in the same chemical state. He calls $\frac{\sqrt{C_w}}{C_s}$ = constant the first stone in the edifice of the theory of staining, and believes $\frac{\sqrt[x]{\text{C dye-bath}}}{\text{C fibre}}$ to represent the action of most, if not of all substantive stains, x indicating the degree of affinity of a dye for the fibre. Although the results which v. Georgievics obtained are in support

[1] Dyes which stain directly (p. 195).

of Witt's 'solid solution theory,' he objects to having the notions of colouration and solution mixed up with one another.

To me the results obtained by v. Georgievics are quite explainable by the chemical theory, and really support the latter.

It is not at all to be wondered at that fibres absorb relatively more stain from dilute solutions, because the greater the dilution the greater will also be the electrical dissociation of the dye, and correspondingly greater its chemical potency, according to the view of Arrhenius (pp. 11–13). To attribute the ready staining after the addition of sulphuric acid to an increase in the osmotic pressure of the dye in the staining fluid is a one-sided way of explaining facts, for the acid does not only act on the solvent and thereby cause an increase in the osmotic pressure of the dye, but it also acts on the sulphonic acid dyes in such a way as probably to render them more basic (see under Gillet's view, p. 362), and also on the tissue (see under Knecht, p. 362, and my criticism of Fischer's theory on p. 336).

That water containing sulphuric acid (an acid bath) extracts less acid dye than does pure water is again readily explainable on chemical grounds, for the mineral acid of the bath converts the pseudo-basic compounds of the silk into real bases, and these by forming insoluble salts with the acid dye radical prevent the extraction of the dye by the water moiety of the acid bath.

Another point emphasized by v. Georgievics, is that sulphuric acid being stronger than indigo-sulphonic acid should satisfy the tissue-demands, and should be unfavourable to staining, instead of which it actually favours the adsorption of indigo-carmin by silk.

To answer this question the following points have to be considered :

(1) How is it known that sulphuric acid does not actually satisfy the demands of the silk, at least in part ; and that the degree of colour, which we see in the fibres, is not merely that amount of colour-acid which is deposited in the fibre according to the law of mass action, which governs both the sulphuric and the indigo-sulphonic acids ?

(2) What are the absolute and the relative amounts of electrical dissociation of the indigo-sulphate (a salt) and of sulphuric acid (*a*) in pure water, (*b*) in the presence of one another, and also (*c*) in the presence of a tissue capable of reacting chemically ? All these questions will have to be answered before any attempt is made to explain the staining of silk by sulphindigotate of sodium.

Gnehm and Roetheli state the chief points on which v. Georgievics bases his theory of physical staining to be as follows :—

(1) The co-efficient of distribution between fibre and dye-bath is constant for indigo-carmin and methylene-blue (as shown above).

(2) No molecular proportion exists between the dye and the fibre.

(3) Substances dye with indifferent substances.

(4) Many dyes sublime from the fibres.

(5) The products of staining exhibit the original properties of the components as especially pointed out by Hwass and v. Perger[1].

Gnehm's and Roetheli's views are given on p. 356.

Spiro[2], adopting the views of v. Georgievics, has also arrived at the conclusion that staining depends on the principle of the coefficient of distribution. He bases his observations mainly on the researches of Hofmeister[3] and those of his pupils.

Spiro holds that chemical staining does not explain why dyes are not taken up completely from very dilute solutions. He points out that we cannot assume the hypothetical gelatin + dye compound to be soluble, as gelatin will take up relatively more dye from weak solutions than strong ones. Thus from different dilutions of methyl-violet, according to Linnemann, the following amounts expressed in percentages are taken up:

Dilution of dye.			
0·00125	=	36·7	units taken up in 3 + 24 hours by gelatin.
0·00250	=	28·3	
0·05	=	21	
0·01	=	47	

To prove his view to be correct, Spiro reasons thus: If the above law holds good then it ought to be possible to reverse the effect, and to remove the dye by washing. So he left gelatin in running water for some weeks and succeeded, provided the gelatin plates did not liquefy.

That dyes get into tissues in proportions different to those they are in in the solutions; that different tissues will take up different amounts of the same dye; that the same gelatin plate will absorb different dyes in different amounts,—all these facts are explainable according to the laws governing the coefficient of distribution, but this does not exclude the possibility of chemical union taking place simultaneously.

If Spiro succeeds in removing the dye only after washing for several weeks, till in some cases the gelatin actually turns liquid, then I hold that chemical union did take place; and that the acids and salts in the washing water, as well as bacterial agencies, were at work in breaking up the gelatin + dye compound.

A. Fischer[4] among histologists has made the greatest effort to exclude all chemical explanations of staining, and in doing so has, to my mind, established some very important facts supporting the chemico-physical view.

Fischer explains all staining by adsorption (p. 331). The purely physical results of his work have been given in full when discuss-

[1] I was unable to procure these papers as well as Gnehm's and Roetheli's original paper, and have, therefore, relied on the abstract in the *Journal of the Soc. Chem. Industry*, 17, 660 and 756 (1898). The original papers of Gnehm and Roetheli are in *Zeitsch. f. angew. Chemie* (1898), pp. 482 and 501.

[2] Spiro: *Über physikalische u. physiologische Selection*, Habilitationsschrift, Strassburg, 1897.

[3] Hofmeister: *Arch. f. experim. Pathol. u. Pharmak.* 28, 210 (1891).

[4] Fischer: *Fixirung, Färbung und Bau des Protoplasmas*, 1899.

ing physical staining on pp. 198–211, and now I shall record his view as to the chemical side of the question.

Fischer believes a chemical explanation of staining to be quite superfluous (l. c., p. 140), and in support of this view reasons as follows: Although, as shown by previous observers[1], nuclein compounds have an affinity for basic dyes—are basophil—while albumin compounds stain more readily with acid dyes—being acidophil, these results cannot be regarded as chemical phenomena, because all basic dyes (0·1 per cent. fuchsin, safranin, methyl-green, and gentian-violet) precipitate not only nucleic acid, but also the basic albumin and casein, while acid dyes do not precipitate albumin. Fischer did not take into account the amido-nature of albumin (p. 282), nor the fact that normal egg-white is basic in reaction towards litmus. Mathew's explanation given below (p. 348) is the correct one. It is further not at all necessary that two substances which interact must form a precipitate; thus, after a preliminary coagulation, egg-white forms with acetic or pyro- or ortho-phosphoric acids perfectly clear, soluble compounds (see also the addendum, pp. 452–459).

Fischer further holds that histologists are only dealing with proteids in a state of such absolute insolubility as to exclude all chemical interaction (l. c., p. 79). Such a view is, however, quite untenable, for not only is a conversion of pseudo-compounds into true acids and bases possible after alcohol fixation (p. 349), not only may cell-secretions such as eleidin granules and granules of mast-cells disappear on being brought into contact with pure water (p. 142), but albuminates of mercury are exceedingly susceptible to the presence of salts (p. 109), and are also readily broken down by iodine treatment, &c.

While determining the primary and secondary chromatophily of granules and tissues (p. 261), Fischer noticed that nucleic acid was, as he terms it, acidophobe, which is not unnatural, because two strong acids do not unite to form salts. Yet he speaks (l. c., p. 102) of nucleic acid as possessing 'a curious as yet unexplainable nature,' and then goes on to show that in reality nucleic acid stains readily with acid dyes, and that it does not behave as an acid.

To bring about this result he impregnates (l. c., p. 168) chloro-platinous-nucleic acid granules (p. 201) with 1 to 5 per cent. albumose, allowing the latter to diffuse inwards either for twenty hours at the room-temperature, or for a $\frac{1}{4}$ to $\frac{1}{2}$ hour by using hot solutions. Having impregnated nucleic acid with albumose, the granules stain equally well in acid and in basic dyes. That the nucleic acid radical of the granule stains with basic dyes, while the albumose unites with acid dyes, I do not consider 'curious,' as does the author.

In addition to impregnation experiments Fischer got thymus-nucleic acid to stain readily by adding 1 cc. of strong sulphuric acid to 8 cc. of 1 per cent. acid fuchsin solution (l. c., pp. 104–106). Similarly yeast-nucleic acid stained readily on adding twelve drops of 5 per cent. sulphuric acid during the course of ten minutes to 9 cc. of 1 per cent. acid fuchsin. This addition of acids and sodium

[1] See later, pp. 343–345.

sulphate (see p. 363) to the dye-baths is a common practice in the dyeing of textile goods.

Fischer believes, as no staining is produced by treating the granules first for ten minutes with 1 : 10 sulphuric acid, and subsequently with a neutral bath of acid fuchsin, that all chemical action of the sulphuric acid on the granules is excluded. I am not sure of this, for if both the sulphuric acid and the dye act simultaneously it is well conceivable that the sulphuric acid, acting under a certain tension, may loosen the affinity between the chloro-platinous radical and the nucleic acid radical, and thus call forth in the nucleic acid its tendency to form alloxuric bases (p. 283). I have precipitated nucleic acid with 1 : 10 sulphuric acid and centrifugalized the precipitate ten times with distilled water to remove the sulphuric acid, and then found it quite easy to stain the sulphuric acid precipitate with 1 : 1,000 of acid dyes; but we have no right to call the precipitate 'nucleic acid.' We further have to keep in mind that acid fuchsin may act as a basic dye (see p. 365).

On adding 1 cc. of 1 per cent. alum to 1 per cent. of eosin, a thick orange-coloured precipitate is formed which, according to Fischer, is eosin. He supposes the eosin to separate out owing to its becoming supersaturated by the addition of alum. I have pointed out on p. 202 that this view is erroneous. The precipitate consists essentially of the free colour acid tetrabrom-fluorescein.

In this eosin-alum solution, chloro-platinous-nucleic acid granules stain readily, not because, as Fischer assumes, the nucleic acid has an affinity for the acid eosin, but because the nucleic acid unites with the basic alum, which in its turn links on to the acid dye radical. Using eosin-alum thus amounts to employing a mordant, and using virtually a basic dye.

Yet another very important point Fischer believes that he has established against the chemical theory, namely, that mercuric chloride granules do not react towards staining reagents (see above, p. 256) like those of platinum chloride, 'although it is beyond doubt that corrosive sublimate at the time of coagulation unites with albumin in exactly the same manner as does the platinum salt' (l. c., p. 103).

Fischer is under the erroneous impression, as are most histologists, that 'platinum chloride' of commerce is $PtCl_4$, while as a matter of fact it is chloro-platinous acid H_2PtCl_6. Sublimate on dissociating electrolytically forms $HgO + 2HCl$, while the hydrogen salt of platinum gives rise to $H_2 + PtCl_6$. In the one case we have HgO, in the other case $PtCl_6$ joining the albumin. In the latter compound the affinities of the platinum for acids are satisfied partly by the six chlorine atoms, and partly by kat-ion groups of the albumin to which it joined, while in the sublimate compound the kat-ion HgO is joined on to an-ion radicals of the albumin. That the chlorine radical of sublimate does not enter the proteid coagulum or precipitate is a fact which was established by Rose long ago (p. 58).

Fischer, however, may really have used platinum tetrachloride, $PtCl_4$, in which case all I said for the compound H_2PtCl_6 will also hold good for this salt, as in a watery solution it decomposes into

$H_2^{\cdot} + PtCl_4O''$ analogous to gold chloride (p. 79), and the an-ion $PtCl_4O$ containing the acid oxygen radical, by satisfying the basic demands of the tissue, makes a union with colour acids impossible.

The reason why tissues fixed in osmium tetroxide do not stain in acid dyes is that OsO_4, although it does not dissociate electrolytically, yet joins the amido an-ion of the albumin, thus forming an additive compound. All oxy-compounds having a strongly acid tendency, osmium-material does not stain, because the osmium tetroxide has already satisfied the acid demands of the proteids.

But there is yet another explanation possible. As Lilienfeld (p. 345) and Mathews (p. 349) have shown, egg-white fixed in alcohol has no affinity for either acid or basic dyes, while the use of alcohol at once establishes an affinity for acid dyes. In the chapter on pseudo-acids and pseudo-bases (p. 26), it was pointed out that these compounds, by the addition of bases or acids, become real acids or bases.

When acetic acid, $CH_3 \cdot COOH$, is added to watery alcohol, it breaks up electrolytically into the positive kat-ion $H^{\cdot}$ and the negative an-ion $CH_3 \cdot COO'$. These ions acting on the colloidal, chemically inactive, pseudo-acid pseudo-basic proteid convert it into an electrolyte (pp. 25, 47). Proteid, changed in this manner, can readily interact with the ions derived from the salt which we employ as a dye, and in consequence chemical union between the kat-ions of the tissue and the an-ions of the dye (or the an-ions of the tissue with the kat-ions of the dye) can readily take place, provided the tissue and the dye are brought into contact with one another in a common solvent, i. e. in a fluid which allows of electrical dissociation of both the proteid and the dye.

The same reasoning was applied when discussing the effect of accentuators (p. 212).

In further support of his physical view of staining, Fischer adduces the inability of tissues to stain if the fixatives of classes 2 and 3 (p. 257) be not removed by careful washing. But to me it appears improbable that 'platinum chloride,' chromic acid, &c., prevent staining of the tissues by a physical blocking of the intermolecular channels.

With the exception of osmium tetroxide, fixatives which do prevent staining are electrolytes, and their ions being stronger radicals than those resulting from the dissociation of dye molecules, it is only natural that the stronger groups should satisfy any tendency the tissue may have for kat-ions or an-ions. It must also be borne in mind that many coagula produced by electrolytes in the presence of the latter will tend to dissociate only in infinitesimal quantities, and that a coagulum without a tendency to ion-formation, be it salt formation, oxidation, or reduction, cannot act chemically.

That prolonged fixation, in many instances, diminishes the staining capacity of a tissue is a further proof that tissues once fixed, are not unalterable. Thus egg-white precipitated by 10 per cent. chromic acid forms at first a dense coagulum, but the latter dissolves in twenty-four hours almost completely if left in the chromic acid solution.

If coagulants continue to act on tissues, the latter must be in an unstable equilibrium, and the removal of substances acting as electrolytes must therefore leave the tissues in such a condition as to allow dyes which are electrolytes to act on them. The firmer and more permanent the union between the precipitating agent and the proteid is, the less tendency will the latter exhibit to become stained. Platinum precipitates are more permanent than sublimate ones, and they consequently stain less, especially with acid dyes. Certain osmium compounds are even more stable than platinum ones.

The washing out of tissues becomes necessary, therefore, to remove the strong electrolytes CrO_3, $HgCl_2$, H_2PtCl_6, and others which are employed for fixation.

If my contention be correct, that proteid coagula are capable of acting as electrolytes after the removal of the precipitating agent, they will be induced to do so even more readily on being brought into contact with strong electrolytes, provided these differ chemically from those which were used to coagulate the proteid.

Why Fischer, in trying to solve the question whether staining is physical or chemical, made efforts to bring the staining power of acid dyes up to that of basic ones, I fail to see. A fair experiment would have been to take the dyes as they are, and not to have introduced new factors, in the belief that they only act by increasing the saturation of dyes. Thus addition of sulphuric acid to dye-baths or alum to eosin baths, according to v. Georgievics, has only the effect of rendering the dyes supersaturated, and Fischer accepting this as a fact fell into error, as pointed out above (pp. 202, 365).

It is generally believed by those who use stains that a dye cannot dissociate till it is broken up by some strong chemical agency, a view which becomes unnecessary if we adopt the Arrhenius explanation of electrical dissociation (chap. ii).

Of the existence of pseudo-compounds we were made aware by Hantzsch and his pupils who studied anilin dyes[1], and we ought to keep in mind that a conversion of these pseudo-compounds into real ones can be brought about by adding acids or alkalies to our staining reagents (pp. 212, 213).

Where factors are so exceedingly complicated, it is rashness to pin one's faith to one preconceived notion, and to explain all observations in the light of one theory.

Yet in doing so we may help those who do not share our views, and thus indirectly bring knowledge a step nearer truth.

Bearing in mind the amido-acid nature of proteids, it cannot be taken as evidence against the upholders of the chemical theory of staining, that tissues will unite with both acid and basic dyes, and yet this has continually been done by Fischer. The most important observations in his whole book I believe to be the following:

By nucleins, 'the basic dyes are absorbed at once, while with the

[1] In addition to the views stated on p. 26, further quite recent papers dealing with pseudo-acids and pseudo-bases will be found in the *Berichte d. deutsch. chem. Ges.* (1902). Space prevents a discussion of these papers at present.

acid stains there is a delay, in about the proportions that methyl-green will have stained already intensely, when acid fuchsin only just shows the faintest indication of staining. In the course of 10 minutes, however, this difference disappears in material which was fixed in indifferent reagents' (l. c., p. 94). Again (on p. 98, l. c.), when staining sections of the kidney of a mouse dead from anthrax and fixed in sublimate, he states: 'The acid dyes diffusing through the sections stain at first only the cytoplasm, and several seconds later the nuclei, which ultimately are also stained as intensely as the cytoplasm.' On p. 101, l. c., it is stated that thymus-nucleic acid precipitated with chloro-platinous acid does not stain in 2 per cent. solution of acid dyes, even after boiling for one hour in the dye.

These observations Fischer has not connected together. The two first statements he uses to prove the absence of any real difference in the absorptive power of nucleins with regard to acid and basic dyes.

Fischer believes the temporary aversion for acid dyes shown by nucleins, and for basic dyes shown by albumins, to account for sections staining differentially in a mixture of an acid and a basic dye. The basic dye unites with the nuclei because of their temporary aversion for the acid dye, and the latter unites with the albumin because of the temporary aversion of albumin for basic dyes.

To me, Fischer's observations have this significance. Each particle, either kat-ion or an-ion, has an aversion for ions of its own kind or those of the same sign; thus the kat-ion $H^{\cdot}$ will not only repel other $H^{\cdot}$ ions, but also, for example, those of potassium, $K^{\cdot}$. On the other hand, kat-ions will readily unite with an-ions.

The mutual aversion of ions of the same sign makes them separate from one another, causes them to diffuse, while ions with opposite electrical charges are drawn towards one another even if they do not unite.

Having converted pseudo-basic albumins by the addition of an acid into real bases, these will show an affinity for acid dyes, hence the staining of material fixed in acid alcohol (p. 349).

Combining nucleic acid with the basic albumin or forming nucleins, means dealing with a potential acid or basic compound, in fact a salt which, however, does not dissociate completely when it is acted upon by either acids or bases.

Nucleins, according to the amount of either the acid or the basic constituent, stain red or blue (Malfatti, p. 344). When, therefore, Fischer observes nucleins to stain readily with basic dyes, and only after some delay with acid dyes, it means that the basophil an-ion nucleic acid has its basic tendencies incompletely satisfied by the kat-ion albumin radical, and that for this reason it readily absorbs some more kat-ions, namely the colour base. Conversely, if the nucleic acid is not sufficient to satisfy the demands of the kat-ion albumin, as is the case in those compounds which contain only a little nucleic acid, then some more an-ions are attracted, namely the colour acid an-ions.

What is so remarkable in Fischer's observation, which I have

repeatedly confirmed, is that the aversion between the two an-ions, namely the nucleic acid and the colour acid, should be so great as to interfere with the normal rate of diffusion, but this is actually the case in nucleins where the diffusion is temporarily, and in nucleic acid where it is permanently, interfered with.

Laurent[1] was the first to realize that methylene-blue eosinate, when boiled (see p. 442), is in a dissociated condition; that the tissue does not require to break up this compound, and that therefore chemical energy is not needed.

I hold that all dyes split up electrolytically, or hydrolytically, according to the doctrine of Arrhenius, but notwithstanding this, it is impossible to account for the differences in staining as merely due to differences in the rate of diffusion, because the time factor comes in, as has been shown in chap. xvii (p. 198).

Laurent also adduces, as a further proof that staining is not chemical, the fact that alcohol acts as a solvent for neutral, basic, and acid combinations, and that it is able to extract all dyes from sections. He must, however, show that alcohol does not act chemically, that it does not interfere with the electrical dissociation of the tissue, or its capacity of changing from the pseudo-acid pseudo-basic state into real acids and bases. In the light of the ordinary chemical reactions (p. 87), and also by studying the way it behaves towards dyes (see Gillet, p. 361), it is impossible to regard alcohol as an inert substance (see also Knecht, pp. 451–2).

Krafft[2] is of the opinion that staining in the majority of cases simply means a separating out of colloidal adhesive salts on or in the tissue, and that these colloids adhere to the tissue because of their viscosity, as does, for example, oil colour to a stick. He points out that for mordanting there are used those very metallic hydroxides, which by themselves form colloidal solutions in water, as do, for example, the salts and hydrates of ferric oxide, of alumina, and the oxides of chromium and tin; substances which already Graham[3] had found combining with all stains to form lakes. Tannin, which is also used for mordanting, is likewise a colloidal substance.

Amongst dyes, those with low molecular weights are either not colloidal at all, or only to a small extent. Methylene-blue, fuchsin, and methyl-violet dissolved in absolute alcohol have the molecular weights 319·8, 337, and 407·9, while watery solutions show a much higher molecular weight, indicating a decided colloidal tendency.

It is necessary, therefore, either to use strongly colloidal dyes which will adhere by themselves (benzidene dyes), or if the tissue is not colloidal itself, or if it cannot call forth a colloidal precipitation of the dye, then to impregnate the tissue with colloidal substances such as tannin or metallic oxides, which by their viscosity will make the dyes adhere.

P. D. Zacharias (Athens)[4] has adopted the Hofmeister-Georgievics

[1] Hans Laurent: *Centralbl. f. allg. Path. u. path. Anat.* 9, 86–97.

[2] F. Krafft: 'Über colloidale Salze als Membranbildner beim Färbeprocess,' *Ber. d. chem. Ges.* 32, 1608 (1899).

[3] Graham: *Ann. d. Chem. u. Pharm.* 121, 41. [4] Zacharias: *Färberzeitung*, 165 (1901).

'coefficient of distribution' view to account for the passage of the dye into the tissue, and Krafft's theory of 'colloidal staining,' with a slight modification to explain the fact of dyes remaining in the tissues. This author is of the same opinion as Krafft, that free colour acids and colour bases are colloids with a certain varying tendency to become insoluble on their own account, but while Krafft believes dyes, if colloidal, to adhere mechanically because of their own viscosity, or if they are non-colloidal or crystalline, because of the adhesiveness of the mordant in the tissue, Zacharias believes that wool, to a considerable extent, and cotton, to a much less extent, produce by catalytic action a colloidal precipitation of the dye radical.

'The fixation of the dye-stuff, which means the precipitation of the dye, is a purely chemical process, and the absorption must also be regarded as chemical as all other physico-chemical processes' (l. c., p. 165). On the very next page is found this sentence: 'We have already seen that the differences in the dyeing of wool or cotton do not so much depend on the nature of the fibre itself, as both are chemically indifferent substances, but on the nature of the dyes which are used, and indeed, there are many colour effects which are produced on wool and cotton in exactly the same way.'

This last statement by Zacharias, in which he is alluding to dyes differing in their colloidal tendencies (see Krafft, p. 340), justifies me classing him amongst the upholders of the physical theory of staining, notwithstanding the fact that he himself calls his view 'the new chemical theory.'

Griesbach[1], in 1886, experimenting with azo-blue, found that a watery solution in a test-tube was instantly turned red by ammonia, was decolourized by strong nitric acid, and was precipitated by saturated hydrochloric acid; while sections stained with azo-blue became colourless in both nitric acid and ammonia, and remained unchanged in hydrochloric acid. (See criticism at end of Unna's researches, p. 342.)

Unna[2], in 1887, endeavoured to substantiate Griesbach's postulate, that the tissue and the dye should possess after having combined different chemical properties from those characteristic of their free state. In the test-tube metaphenylene-diamin and nitrous acid unite at once to form Bismarck-brown (p. 390), but when brought separately into contact with the tissue they fail to do so.

Metatoluylene-diamin	+	nitroso-dimethyl-anilin hydrochloride	=	toluylene-blue	+ water
$C_7H_6\begin{cases}NH_2\\NH_2\end{cases}$	+	$C_6H_4\begin{cases}N(CH_3)_2\\NO—HCl\end{cases}$	=	$N\begin{cases}C_6H_4—N(CH_3)_2\\C_7H_5—NH_2\\NH—HCl\end{cases}$	+ H_2O

When sections stained blue with this toluylene-blue are treated with acids, certain tissue elements will turn red while others remain blue; sections placed in 1 per cent. metatoluylene-diamin stain

[1] Griesbach: *Zeitsch. f. wiss. Mikr.* 3 (1886).
[2] Unna: *Monatshefte f. prakt. Derm.* 62 (1887).
[3] Unna: *Arch. f. mikr. Anat.* 30, 39 (1887).

greyish-brown, while in 1 per cent. nitroso-dimethyl-anilin they become greenish-yellow; and sections placed successively in these two dyes never produce blue, but are always stained of the colour of the second bath; because the affinity of the two dye-constituents is greater towards one another than towards the tissue. Traces of blue dye formed temporarily are soon extracted by mass-action of the other radical. Indophenol-violet converted into the leuco-compound[1] stained sections a greyish-blue or greyish-brown, which was not converted into violet by treatment with such oxidizing agents as peroxide of hydrogen or potassium bichromate, while the free leuco-indophenol-violet in a test-tube becomes at once violet with oxidizing media. Reversely, staining sections of the skin first with indophenol-violet[2], and then transferring them into the reducing mixture of zinc and hydrochloric acid, did not reduce the colour. Therefore, indophenol-violet by combining with the tissue elements has lost the power of becoming reduced, just as its leuco-compound has lost the power of being oxidized.

The leuco-compounds of a number of dyes, after uniting with the tissue, gave rise to colours differing from those obtained by the use of their oxidation products, and sections stained in these leuco-compounds, on being treated with oxidizing media, either retained the colour they already possessed or became colourless. Methylene-blue behaved curiously in this respect, because sections put into the perfectly colourless acid solution of leuco-methylene-blue showed that the nuclei became instantly blue, as if the section had been placed in a dilute methylene-blue solution, the leuco-methylene-blue bath remaining colourless all the time.

Unna offers the explanation that nuclei act as alkalies, because it is improbable that oxygen can be given off by the nuclei.

A. Fischer[3] criticizes the results of Griesbach and Unna, without having, it is true, repeated their experiments as far as the tissues or the dyes they used are concerned, but having made an extensive series of experiments, he shows the necessity of taking the greatest possible precautions when investigating the question whether dyes are or are not in chemical combination with the tissues.

Experimenting with albumose granules ($HgCl_2$, '$PtCl_4$,' $K_2Cr_2O_7$, OCH_2); with serum-albumin; with casein coagula and thymus-nucleic acid ('$PtCl_4$'), and such dyes as light-green, acid fuchsin, indulin, picric acid, eosin, safranin, fuchsin, methyl-green, gentian-violet, methylene-blue, haemalum (Mayer), Fischer obtained results in every respect similar to those obtainable in test-tubes, by taking the precaution not to bring preparations directly in contact with such acids as hydrochloric and nitric acid or with ammonia, but to hold the slide with the section in a semi-dry condition over the mouths of the bottles containing the reagents. Personally, I have no prac-

[1] By reduction with zinc filings and hydrochloric or acetic acid and boiling.

[2] This dye stains the superficial layers of the stratum corneum bluish violet, the middle layers blue, and the deep layers colourless.

[3] Fischer: *Fixirung, Färbung und Bau d. Protoplasmas*, 185 (1899).

tical experience as yet in this question, but deem it a very important one. That colours are actually changed in their behaviour after having entered the fibre seems to be shown by Weber's results (p. 345), and the precipitation experiments of Martin Heidenhain (p. 452).

The chemical aspect of staining.

The first step towards the solution of the problem whether staining is a chemical process, was taken by **Miescher** in 1874[1]. He isolated nucleic acid from the nuclear chromatin segments, and found that it united with the colour base methyl-green to form green insoluble salts. But he says: 'In looking for nuclein in tissues we shall not be able to appeal in the last instance to ordinary histo-chemical methods, to the behaviour of solvents and similar reactions. A comparison of the very resisting spermatozoa of the ox with those of the carp, which swell up in water, shows that notwithstanding a very close relationship as regards chemical structure there may coexist the greatest differences in other respects. We must indeed, wherever possible, protect our backs, by depending on a chemical isolation of the substance (Elementaranalyse).'

P. Ehrlich[2] soon afterwards showed that it was possible to fix blood films by purely physical means, namely dry heat, thereby excluding coagulation and chemical changes, and in such blood films to obtain specific colour effects with certain dyes. Having divided stains into acid, basic, and neutral groups, he found, for example, that only acid stains will colour the α-granules of certain leucocytes, which for this reason he termed eosinophilous, that only basic dyes stain the mast-cells containing the γ-granules, and that only neutral dyes or salt colours are taken up by the neutrophil or ϵ-granules of the human blood.

Knecht[3], in 1888, believed in the chemical nature of staining, because he found when dyeing wool and silk with the chlorides of the colour bases fuchsin, chrysoidin, and crystal violet, that only the colour bases were taken up by the fibres, while the hydrochloric acid remained in the bath, if the silk and wool were boiled in the dye-bath till the whole of the colour was taken up[4]. As the free colour base is colourless, and only becomes coloured when in the form of its salts, it follows that between the colour base and the wool a chemical union of the nature of salt formation must have taken place (see also Knecht on p. 451).

Knecht and **Appleyard**[5], in 1889, isolated lanuginic acid from

[1] Miescher: 'Die Spermatozoen einiger Wirbelthiere,' *Verh. d. naturf. Gesellsch. in Basel*, 6, 138–208 (1874).

[2] Ehrlich: *Farbenanalytische Untersuchungen z. Hist. u. Klinik d. Blutes.* Gesammelte Mittheil. Berlin, 1891, Hirschwald.

[3] Knecht: *Ber. d. deutsch. chem. Ges.* 21, 1556 (1888).

[4] Thus a solution of 0·2 grm. of fuchsin contained originally 0·01630 grm. of HCl, and after the wool had taken up the colour base, there was found in the bath 0·01622 grm. of HCl.

[5] Knecht and Appleyard: *Ber. d. deutsch. chem. Gesellsch.* 22, 1120 (1889). See also Knecht, Rawson and Loewenthal: *Handbuch d. Färberei der Spinnfasern*, Berlin, 1895.

wool, and found that this acid formed intensely coloured lakes with all directly staining colour bases. They also established the fact that wool absorbs substantive dyes, if they be offered in great excess, always proportionately to their molecular weight or multiples of the same.

A. Fischer, in criticizing the chemical aspects of staining, admits the correctness of Knecht's contention as regards wool which is boiled, as hereby it is decomposed and thus can liberate the lanuginic acid. He says, however, that in histological work we do not boil our sections, and that therefore we have no right to compare our results with those obtained by dyes. To a certain extent I agree with Fischer, but we must remember that even at ordinary temperatures the tendency towards the formation of lanuginic will be developed in the presence of electrolytes, and that as the result of partial dissociation of the tissue in the presence of a compound already dissociated (namely the dye) a chemical union can be established. I admit that such a union may be less stable than that formed at higher temperatures, but then we are only dealing with the well-known chemical phenomenon that unstable chemical compounds are formed before stable ones, e.g. metaphosphoric acid before the ortho-compound, yellow oxide of mercury before the red, and so on.

Malfatti, in 1891[1], next showed that nucleic acid prepared from yeast, when placed in a mixture of methyl-green and acid fuchsin, combines with the methyl-green and not with the colour acid; that albumin on the other hand unites with the acid fuchsin and not with the colour base. By bringing metaphosphoric acid and albumin together in different proportions he manufactured nucleins, which, according to the amount of phosphorus they contained, stained bluish-red or a neutral colour, or red if only a little phosphorus was present. As nucleic acid is a phosphoric acid compound (p. 283), the affinity for the colour base was taken by Malfatti to be the direct expression of a chemical demand made by the phosphorus for the methyl-green. Test-tube experiments led to the result that yeast-nucleic acid fixed with 'platinum chloride,' and stained in Loewit's safranin-iodine-picric acid, were red like chromatin, while nucleins containing only little nucleic acid became yellow like nucleoli.

E. Zacharias[2], in 1893, dissolved yeast-nucleic acid in dilute ammonia, rendered this solution slightly acid with acetic acid, and filtered it. He next covered a slide with a thin layer of egg-white, and placed a drop of nucleic acid solution on the egg-white, when there was formed an insoluble coagulum owing to the nucleic acid precipitating the albumin. The slide was now placed in absolute alcohol to coagulate the albumin which had not come in contact with the nucleic acid. On staining such slides in a mixture of $\frac{1}{2}$ gram of methylene-blue and $\frac{1}{2}$ gram of acid fuchsin in 100 cc. of water[3], the alcohol coagulated albumin stained red while the nucleic acid-coagulated por-

[1] Malfatti: *Berichte d. naturw. med. Vereins in Innsbruck* (1891-2).
[2] Zacharias: 'Chromatophilie,' *Ber. d. deutsch. bot. Ges.* 11 (1893).
[3] The methylene-blue does not dissolve completely.

tion stained blue. Treating material, for example Galanthus and the roots of Phajus after fixation in alcohol, with the same stain, the nuclei stained blue and the cytoplasm red. Zacharias wanted to determine by these experiments whether a definite mixture of dyes used in a definite manner can be used for determining the distribution in the cell of nucleins, which are compounds formed by the union of nucleic acid and albumins.

Zacharias [1], in 1893, further showed that a considerable part of the cell-plasm is not digested by pepsin-hydrochloric acid, and thus confirmed Halliburton's statement [2] about the presence of nucleins in the cell-plasm. To this non-digestible, nuclein-containing constituent of the cell-plasm, having an affinity for basic dyes as have nuclein compounds, Zacharias has given the name of plastin.

Lilienfeld [3], in 1893–4, made several contributions to the question of micro-chemical staining, and stated that with a mixture of methyl green and acid fuchsin, free nucleic acid stains green, albumin red, while nucleic acid and nucleo proteids become blue. As, further, resting nuclei stained bluish-green, while dividing ones appeared green, he arrived at the conclusion that free nucleic acid was present in dividing nuclei (see Heine, p. 347). His best observation however, which at that time was unexplainable, is that albumin fixed in absolute alcohol has no affinity for either acid or basic dyes (see later, Mathews and myself).

Weber [4], in support of the chemical view of dyeing, stained wool, which contains an amido group, NH_2, and a carboxyl group, COOH, graphically represented thus, $\text{Wool}\langle^{NH_2}_{COOH}$ with naphthol orange, which being an acid dye fixes on to the NH_2 group thus:

$$\text{Wool}\langle^{NH_3\cdot O\cdot SO_2\cdot C_6H_4N=N\cdot C_{10}N_6\cdot OH}_{COOH}$$

which means that 'the amido group of the wool combines with the sulphonic group of the dye, while the carboxyl group of the wool remains unaffected.' Dyeing wool with a basic dye such as magenta

$$\text{Wool}\langle^{NH_2}_{CO\cdot O}\!\!-\!H_2N\!\!-\!\!\!-\!\!\!-\!\!C\langle^{C_6H_4NH_2}_{C_6H_4NH_2}$$

results in the union of the acid carboxylic group of the wool with the basic NH_2 group of the dye (see, however, also pp. 357–8).

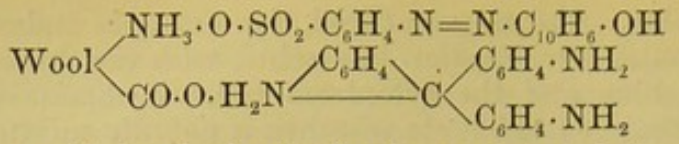
Seeing that in the above formulae in one case the COOH, and in the other case the NH_2, remain unsatisfied, Weber succeeded by staining wool first in the acid scarlet R and subsequently in the basic magenta in satisfying both chemical affinities of the wool :

[1] Zacharias : 'Über chemische Beschaffenheit v. Cytoplasma u. Zellkern,' *Ber. d. deutsch. bot. Ges.* 11 (1893).

[2] Halliburton : *Textbook of Chemical Physiol. and Pathology*, 193 (1891).

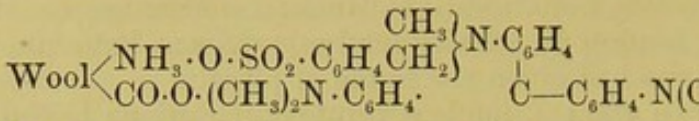
[3] Lilienfeld : 'Über d. Wahlverwandtschaft d. Zellelemente z. gewiss. Farbstoffen,' *Arch. f. Anat. u. Physiol.* (1893) ; 'Über d. Farbenreaction d. Mucins,' *ibid.* ; 'Zur Chemie d. Leucocyten,' *Zeitsch. f. physiol. Chemie*, 18 (1894).

[4] Weber : *Journ. of Soc. of Chemical Industr.* 13, 120 (1894).



That the acid dye had not simply precipitated the basic one was proved by alcohol extracting only traces of the magenta from the wool, while the scarlet R + magenta lake is readily soluble in alcohol.

Even a dual lake-forming function between wool and a dye may take place if the latter possess an acid (sulphonic) and a basic (amidogen) radical, as in the case of acid violet:



Water-blue or methyl-blue, sulphonated methylene-blue (Cassella's thiocarmin), acid violet and acid green, although acid dyes, stain cotton mordanted with tannin in direct proportion to the strength of the unsulphonated colour base, and in inverse proportion to the strength of the sulphonic acid.

Cotton differs from wool in possessing neither amido nor carboxyl groups, but it has a definite although slight acid character (Cross, Bevan and Beadle), forming, with alkalies and several mineral oxides, for example plumbic oxide, salt-like compounds. Weber holds therefore that the acid character of cotton is not sufficiently strong to abstract the colour base from its salt, liberating the acid radical at the same time as does wool,—but that it is strong enough to combine with the undissociated basic colour salt, analogous to normal tannin lakes, which are diacid salts of the colour bases. Cotton, according to this view, unites with one of the free amido groups of the undissociated basic colour salt [1].

Weber also points out that the sulphonic acids of strong colour bases are capable of forming lakes in the amido group as well as in the sulphonic group. Thus, the sulphonic acid dye, acid green, can unite through its sulphonic group with barium chloride, and through its amidogen group with tannin. The acid green-barium lake formed in this manner is deficient in brightness, depth of shade, and fastness to light, but by treatment with tannin the colour is brightened and intensified, and the fastness to light is increased at least threefold.

What holds good for acid green, holds also good for every amido-sulphonic dye in which the amido group of the unsulphonated dye is capable of lake formation, and wool dyed in any of these amido-sulphonic dyes behaves neither towards barium chloride nor towards tannin as if the dyes were simply absorbed. Therefore it follows that the dyes must have combined with the fibre to form lakes comparable to either the barium or the tannin lake.

Benzidene dyes, on the other hand, are changed nowise on passing into the fibre, and therefore the process of direct dyeing amounts to

[1] The electrical dissociation of salts must, however, be taken into account.

'an aqueous solution of a dye with very small coefficient of diffusion, being formed inside the cellular cavities of the cotton fibre by means of a dye-bath possessing a high osmotic pressure.'

It is evident that Weber is a strong upholder of the chemical view, and that he discards Witt's theory of solid solution, which latter postulates that the dye forms a solution with the cellulose. That Witt's theory does not hold good was proved, for benzidene dyes, by staining dinitro-cellulose in its normal spongy and fibrous condition, and also in a perfectly homogenous non-spongy condition, which latter was attained by dissolving dinitro-cellulose in acetone, pouring the solution on glass plates and allowing the solvent to evaporate. The spongy cellulose stains well, while the homogenous cellulose does not.

Heine[1], relying on Lilienfeld's statement as to the differences noticeable between free nucleic acid and nucleins, had hoped to be able to trace the fate of the nucleic acid formed in the chromatin segments by the use of methyl-green and rubin S mixtures, but was disappointed. He arrived at the conclusion that although nucleic acid has an affinity for basic dyes, as yet we have no methods of differentiating micro-chemically between the various types of nucleic bodies, such as nucleo-proteids, nucleins, nucleic and paranucleic acids and their salts. He also expected that a micro-chemical differentiation might result, by substituting for the mixture of a basic and an acid dye a mixture of two basic dyes; but although his experiments in this respect failed, they established the great unreliability of mixtures of basic and perhaps also of acid dyes for microscopic investigations, as the differences seen in sections were produced by physical, not chemical causes[2].

Heine also used Millon's reagent, and found that the chromatin segments gave the most intense colour reactions.

Walker and **Appleyard**[3] showed, generally speaking, that there exists a parallelism between the strength of an acid and the amount which is absorbed by silk, aromatic acids being taken up readily, while non-aromatic acids were absorbed but little. That Witt's theory (p. 330) does not hold good in staining silk with picric acid was proved thus: On dyeing silk with picric acid, a real equilibrium was attained independently of the original distribution of the materials, the coefficient of distribution of picric acid between silk and the water being $v = \frac{1}{2 \cdot 7}$.

'If the equilibrium concentration of the picric acid in the silk be denoted by s, and in the water by w, the relation $\frac{s}{\sqrt{w}} = \text{constant}$

[1] Heine: 'Die Mikrochemie d. Mitose, zugleich eine Kritik mikrochemischer Methoden,' *Zeitsch. f. physiol. Chemie*, 21 (1895).

[2] Even pepsin-hydrochloric acid is not reliable, because after one to one and a half hour's digestion at 40° C., the heads of spermatozoa of the salamander and the mitoses had all their nucleic acid removed.

[3] Walker and Appleyard: 'Absorption of dilute acids by silk,' *Trans. Chem. Soc.* 1334 (1896).

exists between these magnitudes. This formula would indicate, according to the solid solution theory of dyeing, that the weight of the molecule of picric acid, dissolved in water, would be n times that of the molecule of picric acid "dissolved" in silk; but this we know to be incorrect, as n is greater than unity, and the molecular weight of picric acid in water is the smallest consistent with its formula.'

Albert Mathews[1], in 1898, published a most important paper on the chemistry of cytological staining, a paper so short and clear as to be the type of what scientific papers ought to be. He points out that albumins and albumoses, being weak bases (p. 288), readily unite with free acids to form salts which may be either soluble or insoluble. Thus, on adding to albumin or albumose a solution of the free picric, metaphosphoric, molybdic, tungstic, phosphotungstic, tannic, stearic[2], or chromic acids, a coagulum is formed at once. Should these acids, however, be employed in the form of their salts, no precipitate is formed till by the addition of a few drops of acetic or hydrochloric acid the solution containing the mixture of albumose and sodium picrate, for example, has been rendered slightly acid. Whenever the acid is added the albumose picrate is thrown down at once, 'probably because the acetic acid sets free the picric acid.'

All the colour acids used by histologists react in exactly the same manner. These dyes, being employed generally in the form of their neutral salts, require the addition of some acidifier, when they at once combine with the proteid, forming a dense coagulum. Mathews obtained in this way albumin and albumose precipitates with acid fuchsin, acid green, nigrosin, anilin blue-black, erythrosin, congo-red, methyl-blue, carminate of sodium, and indigo-carmin[3], by using an acid or an alkaline (see later) solution of albumoses. 'This reaction of the acid stains indicates beyond doubt that these stains, when in acidulated solutions, will enter into chemical combination with the albumose or albumin molecule like any other acid. Inasmuch as it is possible that the free acids enter one or more of the basic NH_2 groups of the albumin molecule, the acid stains also probably enter this group.'

But not only colour acids but colour bases can be made to unite with albumin and albumoses under conditions to be detailed immediately.

If lead acetate is brought into a neutral solution of albumin or albumose nothing happens, but as soon as the reaction of the fluid is rendered slightly alkaline with sodium carbonate, then an insoluble lead albuminate is thrown down. As gelatin and protamin solutions under identical conditions are not precipitated, and as they

[1] A. Mathews: *American Jour. of Physiol.* 445–454. July, 1898.

[2] 'If a basic dye is added to neutral soap solutions a flocculent, coloured precipitate, consisting probably of the coloured salt of palmitic or stearic acid, is thrown down. Neutral solutions of thyminic acid, a derivative of nucleinic acid, or of the pseudo-nucleinic acid derived from the yolk of hens' eggs show similar reactions.'—Mathews, l. c. 451.

[3] It is recommended by Mathews to determine the acid or basic character of a dye the nature of which we may not know.

are deficient in the phenol group, it follows that in albumin and similarly constituted proteids the basic lead radical must join on to the 'acid hydroxyl' radical (p. 184) of the phenol.

Colour bases used by histologists are neutral salts, being generally the chlorides, hydrochlorates, &c., and react as does lead acetate. Thus, on bringing together a solution of basic fuchsin, methyl-green, thionin, toluidin-blue, safranin, or other basic stains ('with the possible exception of vesuvin[1]'), with a solution of an albumose, nothing happens; but if a similar albumose solution, rendered slightly alkaline with sodium carbonate, be used, then a 'flocculent, coloured precipitate, consisting of the albumose in combination with the dye, is thrown down.'

'These experiments prove that many of the basic dyes enter into chemical combination with the albumose molecule when in alkaline solutions, forming insoluble coloured compounds,' and 'reacting in this respect like basic lead acetate, protamin, histone, or other organic bases.'

'Basic dyes in alkaline solution may thus be used for the detection of albumins in the cell, and indeed of albumins possessing a phenol or tyrosin group.'

It is thus beyond all doubt that the ordinary colour acids and colour bases will combine under suitable conditions with albumins and albumoses, to form definite salt-like compounds (see also M. Heidenhain's work on p. 452).

The next question is: Does the same hold good for albumin which has been coagulated by alcohol or by acid media, not containing such metallic salts as corrosive sublimate or chloro-platinous acid?

Egg-white, coagulated by heat or by alcohol, does not stain in neutral solutions of either colour acids or colour bases. 'It is true it will imbibe a certain amount of colour and will appear stained, but this colour is easily and quickly removed by washing in water.'

'A most striking contrast is shown by two pieces of coagulated albumin, one of which has been immersed in a neutral, the other in an acid solution of acid fuchsin. After washing, the former will be found to be colourless, the latter a brilliant red.' 'Towards basic stains coagulated albumin reacts on the whole like the albumoses,' but as egg-albumin coagulated by heat is normally alkaline, it is necessary either to neutralize it or to render the solution of a colour base slightly acid, if we desire to prevent staining. Placing normal heat-coagulated egg-albumin into neutral or slightly alkaline solutions of basic stain, without neutralization, causes it to become coloured immediately. 'If two pieces of (alcohol) coagulated egg-albumin be brought, the one into slightly acid and the other into alkaline solutions of thionin, the stain poured off after a few seconds, and the albumin washed in water, the piece that has been in the alkaline solution will be an intense purple, the other barely tinged with colour.'

'These reactions clearly indicate that the staining of coagulated

[1] This dye is a mixture of colour acids and colour bases (see p. 390).

albumin depends on chemical combinations similar in all respects to those which the albumoses enter into with the same stains. In neutral solution, neutral coagulated albumin combines neither with acid nor with basic stains; in alkaline solutions, it combines only with the basic; in acid solutions, only with the acid stains.'

Freshly prepared haematoxylin does not precipitate albumose in either acid, neutral or alkaline solutions, but its oxidation product, haematein, as well as sodium carminate give at once a coloured precipitate in an acid solution, showing that both the haematein and carminic acid react like ordinary anilin colour acids. 'In alkaline solutions of the albumoses the addition of solutions of carmalum (p. 245) or haemalum (p. 238) caused heavy flocculent coloured precipitates, which may have been simply the stain which is insoluble in alkaline solutions, but it is also possible that it was the stain in combination with the albumose. In any case the aluminium salts of carminic acid and haematein no longer react towards the albumoses or tissues like acid stains, but like basic stains. This is possibly due to the strong basicity of the aluminium and its tendency to form double acid salts. It will probably be found that the aluminium salts of the acid anilin colours stain like the basic dyes.'

In applying the above results to the staining of sections fixed in alcohol, Mathews reasons thus: Basic stains in neutral and slightly acid solutions have no affinity for albumins and albumoses, and yet they stain the nuclear chromatin, mucin and hyaline cartilage, all of which elements contain 'organic acids in salt combinations with strong bases[1].'

Therefore it must be borne in mind that basic dyes are indicators of all organic acids, but they do not tell us the nature of the organic acid. Mathews remarks: 'It is still not uncommon to find, in cytological works, methyl-green and other basic stains regarded as microscopical reagents for the detection of chromatin, and some cytoplasmic bodies, because of their affinity for basic dyes, have been looked upon as chromatin or derivatives of chromatin.' The readiness with which nucleic acid in tissues reacts towards colour bases depends on the firmness of its union with organic bases: thus nucleic acid in the spermatozoa of the fish and the sea-urchin (Miescher, Kossel, Mathews) is joined to histone or protamin; in the thymus, in leucocytes, and in the red blood-corpuscles of birds, Kossel has shown nucleic acid to be a histone salt. All of these tissues stain readily with basic dyes, but the vertebrate pancreas exhibits only slight affinity for basic dyes, and in the pancreas, as macro-chemical methods prove, nucleic acid is united much more firmly after the manner of Lilienfeld's artificial nucleins, which stained with basic dyes only as long as they were not saturated with albumin; after saturation they developed a preponderating attraction for acid stains.

'This is strong evidence that the acid stain enters the albumin

[1] In nuclear chromatin it is the nucleic acid, as pointed out by Miescher and others (see p. 343), while the acid radical in both mucin and hyaline cartilage is chondro-sulphuric acid (p. 285).

molecule, while the basic enters the nucleinic acid molecule in these nucleins.'

Mathews fixed some pieces of liver, kidney, and voluntary muscle of the frog in neutral 95 per cent. alcohol, and other pieces in similar alcohol containing 1 per cent. acetic acid; imbedded the tissues in paraffin, and cut as usual.

The basic dyes used were: 'vesuvin, methyl-green, methylene-blue, safranin, toluidin-blue, thionin, and dahlia.' The acid stains were: 'indigo-carmin, sodium carminate, nigrosin, methyl-blue, erythrosin, acid green, congo-red, orange G, and acid fuchsin.'

The dyes were used in weak aqueous solutions, as strong solutions only lead to a physical absorption of large quantities of the stain. The physically absorbed dye is readily removed by water. The sections were well washed, then left in the staining fluid for a few seconds up to three minutes, were well washed again and examined.

The tissues mentioned above, fixed in either neutral or acid alcohol, and stained with neutral solutions of basic dyes, exhibited only the chromatin stained, but with slightly alkaline solutions gave an intense colouring of the cytoplasm, behaving therefore in every respect as does albumose and albumin (p. 348).

Similar sections, placed in neutral solutions of acid dyes, gave results differing entirely from one another according as to whether the tissues had been fixed in neutral alcohol or in acid alcohol. Neutral alcohol material did not stain at all in neutral solutions, but did so readily in slightly acid solutions of the acid dyes. Acid alcohol material, on the other hand, combined at once with the acid dyes in neutral solutions, probably because the acetic acid radical, which had united with the albumin of the molecule at the time of fixation, was now replaced by the colour acid of the staining fluid.

Mathews finishes up by remarking: 'It would be interesting to know what influence the introduction of mercury or other metals and of acids into the albumin molecule may exert on its staining properties.' This point has been investigated to some extent by Burchardt and by A. Fischer, who did not know of Mathews' researches.

Burchardt[1] found if intestines were left for 24 hours in 2 to 5 per cent. acetic acid, and then were washed in water and hardened in alcohol, that neither methyl-green, Bismarck-brown, or other basic dyes, nor haematoxylin, would stain the nuclear chromatin, although if similar pieces of intestine were treated simultaneously in the acetic acid + methyl-green mixtures of Carnoy or in Mayzel's acetic acid + Bismarck-brown, the nuclei did stain well. Similarly, while pure sublimate fixation gave good staining with methyl-green, sublimate + acetic acid fixation did not. Sublimate in any form excludes the typical staining by van Giesson's method, and calcium bichromate for unknown reasons also gives much worse results with this staining than do all other bichromate solutions[2].

[1] Burchardt: *La Cellule*, 12, 335 (1897).

[2] Burchardt prefers Kultschitzky's patent acid rubin to the ordinary acid fuchsin. He makes up van Giesson's stain by mixing picric acid (1 : 300 of water) 9 parts, and saturated watery rubin S 1 part.

After bichromate fixation, cells can be stained readily by overstaining sections in methyl-violet or basic fuchsin; washing; transferring for a few minutes to picric acid + chromic acid mixtures, consisting either of one per cent. picric acid 1 part + one per cent. chromic acid 2 parts, or one per cent. picric acid 1 part + two per cent. chromic acid 2 parts.

Alfred Fischer[1] divides, as already stated (p. 256), fixing reagents into three classes:

(*a*) Indifferent fixatives requiring no subsequent washing (alcohol, formaldehyde, acetic acid, and the almost indifferent picric acid).

(*b*) Partially indifferent fixatives (chromic acid, potassium bichromate, Flemming's solution, and corrosive sublimate).

(*c*) Total colour enemies (platinum chloride, Hermann's solution, tannic osmium tetroxide, Altmann's fluid, and iodine alcohol).

Fischer terms the colour affinity exhibited by tissues after fixation in what he supposes to be indifferent media[2], the primary chromatophily, while 'secondary chromatophily' is the term given to the staining reactions obtained after the use of the two classes of fixatives enumerated above under (*b*) and (*c*).

Fischer obtained as regards primary chromatophily (see above) the following results:

	Fixative.	*Acid dyes.*	*Basic dyes.*
Serum-albumin	alcohol	stain intensely	stain intensely except with methyl green.
Serum-globulin	sublimate	,, ,,	,, ,, but methyl green stains slightly.
Deutero albumose	sublimate; formol	stains equally well in acid and basic dyes.	
Casein	alcohol; sublimate		stains more deeply than albumin and globulin. stains deeply after alcohol, less deeply in indulin and picric acid after sublimate.
Nuclein	acetic acid	stains much more slowly in acid dyes than in basic ones, but after ten minutes stains deeply in both.	
Yeast-nucleic acid	sublimate	does not stain	intense colouration.

[1] A. Fischer: *Fixirung, Färbung u. Bau d. Protoplasmas*, Gustav Fischer, Jena, 1899.

[2] The following objections to the so-called indifferent fixatives naturally suggest themselves. With the exception of alcohol, if it act only for a short time, all other fixatives, such as formaldehyde and acetic acid, cause enormous changes not only by forming additive compounds (formol), but also by causing electrolytic decomposition satisfying basic tendencies of the proteid, and extracting cell constituents (acetic acid).

	Fixative.	*Acid dyes.*	*Basic dyes.*
Serum-albumin	nucleic acid	stains equally well in acid and basic dyes, but from mixture of methylene-blue and acid fuchsin stains blue, therefore takes up blue more quickly.	
Haemoglobin Red blood-corpuscles	alcohol 10 per cent. formaldehyde	stain deeply	deeply in methylene-blue, fuchsin, gentian-violet, not deeply in safranin, not at all in methyl-green.
Eosinophilous leucocytes	formaldehyde ; sublimate	deeply	not at all.
Bacteria	dried sublimate	very little affinity deeply in acid fuchsin and eosin	great affinity. deeply.
Pollen mother-cells of *Funkia ovata* Kidney of mouse + anthrax	alcohol sublimate	acid dyes on diffusing into the preparation stain the cytoplasm first and only after a few seconds the nuclei. The basic dyes, reversely, stain the nuclei and cell-plasm 'about simultaneously' and the nuclei at once much deeper.	

The chief results are, therefore, that after precipitation with 'indifferent' media (alcohol, formaldehyde, acetic acid), nucleic acid is strictly acidophobe, but there is no corresponding basophobia. Nucleins are slightly acidophobe, and only in heterogeneous mixtures, such as Ehrlich-Biondi's or acid fuchsin methylene-blue mixtures, appears 'basophily,' because 'basic dyes are taken up more readily than are acid ones.'

No reference is made by Fischer as to the reaction of the serum-albumin or globulin he used ; on p. 45, l. c., it is stated that commercial serum-albumin is faintly acid, and that an alkaline reaction prevents alcohol precipitation, so Fischer probably used acid serum-globulin. Nothing is stated as to the reaction or strength of the basic dyes, nor for how long the preparations were washed after having been stained. In a previous chapter it is mentioned that borax greatly increases the staining power of methyl-green. In the light of the researches of Mathews, these points must be carefully noted in the future.

The secondary chromatophily (see p. 352) imparted by partial and total colour enemies is as follows :

Chromium (sp. gravity 6·8)[1] prevents all staining with methyl-green in those cases where the proteid, after precipitation with alcohol, stains only feebly or of medium strength, as do casein and albumose ; but if the primary affinity for methyl-green be great, as in nucleic acid and the nucleins, then chromium does not interfere. Safranin stained albumose, albumin, and casein distinctly less than when acting on alcohol coagula. The chromates of albumose, albu-

[1] Fischer believes, apparently, the specific weight to have some influence.

min, casein, and haemoglobin stain apart from methyl-green with acid dyes and such basic stains as fuchsin, gentian-violet, methylene-blue, like formaldehyde coagula.

Fischer further states, and this is certainly wrong, that chrome does not establish an affinity for acid dyes. If, as he states, nucleic acid and nucleins do not stain more readily in acid dyes after chrome-fixation, it is for the simple reason that they are acid compounds themselves; albumins do stain much more deeply after chrome-fixation, because of the mordant action of the basic hydrate.

Iron (sp. gravity 7·84), chemically nearly related to chromium, produces effects similar to the latter. It is suggested that the 0·3 to 0·5 per cent. of iron occurring in haemoglobin is the cause of the latter not staining with methyl-green.

Mercury (sp. gravity 13·6) increases the affinity for methyl-green to such an extent that even haemoglobin becomes stained, and simultaneously lowers the affinity for acid dyes.

Platinum (sp. gravity 21·5) and *Osmium* (sp. gravity 21·4) diminish the affinity for all acid dyes and increase that for basic stains, especially for methyl-green, which colours deeply all platinum and osmium precipitates of albumose, albumin, and globulin, and the platinum coagulum of haemoglobin. Nucleic acid, precipitated by platinum, neither stains in 2 per cent. light-green or acid fuchsin if boiled even for one hour on a water-bath, nor if it be left in the stain for 20 hours at the ordinary temperature. This 'acidophobia' Fischer overcomes by albumose impregnation, or adding sulphuric acid or alum to the stains (see pp. 202, 362–365).

Fixing solutions: 'Just as every component has its own specific coagulating power, and exerts its influence independent of others, so does each component impart a specific adsorptive power[1]. In Flemming's solution (p. 95), for example, the osmium has exactly the opposite effect to the chromic acid, while in Hermann's solution (p. 96) it has the same effect as "platinum chloride." For this reason, albumose precipitated with Hermann's solution is strongly acidophobe, while, if Flemming's solution be employed, the albumose precipitate stains readily with such acid dyes as eosin, acid fuchsin, and light-green. The percentage amount of such antagonistic reagents as chromic acid and osmic acid (osmium tetroxide) determines in each individual case the affinity for stains.'

How the fixing reagent, if allowed to remain in the tissue, influences the primary and secondary chromatophily, Fischer has worked out with great care for methyl-green, as the following table, taken from his book, shows. (Three crosses + + + means very great affinity, + + medium affinity, + slight affinity, and O no affinity.) See also chap. xx.

[1] Fischer's view of staining, as mentioned on pp. 200 and 334, is a purely physical one.

PRIMARY AND SECONDARY ADSORPTIVE POWER FOR METHYL-GREEN.

Fixing reagent in parentheses.	*Primary adsorption.*	*Secondary adsorption after fixation in*			
		Chromic acid.	*Sublimate.*	*'Platinum chloride.'*	*Osmium tetroxide.*
(1) albumin (alcohol)	0	0	+	+ +	+ +
(2) globulin (alcohol)	0	0	+	+ +	+ +
(3) casein (alcohol)	+	0	+	+ +	
(4) haemoglobin (alcohol)	0	0	+	+	
(5) albumose (formaldehyde)	+ +	0	+ +	+ + (+)	++ (+)
(6) nucleic acid (acetic acid)	+ + +	+ + +		+ + +	
(7) nuclein (acetic acid)	+ + +	+ + +		+ + +	

L. Vignon[1] endeavoured to determine whether staining cotton by the direct method (p. 195) depends on a chemical process. He therefore chose various substances which are chemically related to the dyes, boiled cotton with these reagents in neutral solutions for 15 minutes, weighed the cotton, and also estimated the residue of the substances left in the bath. He always employed 1 gram of cotton and 1 gram of the dye substance to be tested, dissolved in 250 cc. of water rendered alkaline by the addition of 3·5 grams of sodium carbonate. The bases which Vignon incorporated with the cotton were neutral hydrochloride salts. His results are shortly these: Diphenyl ($C_6H_5—C_6H_5$), ammonia (NH_3), hydroxylamin ($NH_2OH + H_2O$), and azobenzene ($C_6H_5 \cdot N:N \cdot C_6H_5$), are not absorbed, and therefore the fixation of direct colours (p. 195) is not due to free nitrogen atoms or to the azo group (p. 182).

On the other hand, diamins, $C_6H_4(NH_2)_2$, except *o*-phenylene-diamin, and hydrazins[2] are absorbed to a considerable extent, and the absorption is independent of the degree of substitution of the azotized molecular group. Direct staining, therefore, is due to the grouping

$$\rangle N-R-N\langle \quad \text{or} \quad \rangle N-N\langle$$

which schemes express that two hydrazinic nitrogen atoms are united either by means of aromatic residues (R) or directly. The two N-groups may be further combined with hydrogen, H, methyl, CH_3, or with N-atoms forming azo groups, when in the last case the N by uniting with the cellulose may become pentavalent,

$$\rangle N—N\langle$$

[1] L. Vignon: *Compt. Rend.* 357–360 (1897); abstract in *J. Soc. Chem. Ind.* 1014 (1897).

[2] Hydrazins are obtained by reducing diazo-compounds, e. g. diazo-benzene chloride, $C_6H_5 \cdot N:NCl + (4H) =$ phenyl-hydrazin, $C_6H_5-NH-NH_2 + (HCl)$.

as is proved by benzidene, $NH_2-C_6H_4\cdot C_6H_4-NH_2$, and tetramethyl-benzidene,

$$\begin{matrix}NH_2\\(CH_3)_2\end{matrix}\!\!>\!C_6H_2\cdot C_6H_2\!<\!\!\begin{matrix}NH_2\\(CH_3)_2\end{matrix}$$

being absorbed by cotton while the methyl-iodide compound of tetra-methyl-benzidene, in which the nitrogen atoms are already penta-valent, is not absorbed.

Gnehm and Roetheli [1] are of the opinion that staining is not a homogeneous process at all. Gnehm, in his earlier experiments, had shown that glass beads immersed in hot aqueous solutions of fuchsin (magenta), fuchsin + ammonia, and the colourless fuchsin-carbinol base (p. 421), and left for eleven weeks, looked all alike, being deeply stained. Alcohol, however, extracted the colour from the glass beads stained by the two solutions mentioned last, while the beads stained in the ordinary fuchsin, or fuchsin chloride salt, did not lose their colour even after prolonged extraction with alcohol.

The authors found that silk could never be decolourized completely by alcohol, and that therefore the dye which remained in the fibre was bound chemically, while the remainder which was extractable was bound only physically. Weak staining solutions allowed to act for a long time gave much more resisting stains than strong solutions which had acted for only a short time. This observation is fully borne out by ordinary histological staining, and is explainable, I believe, on the theory that weak solutions of dyes are more completely dissociated electrolytically and therefore more active.

The carbinol base of fuchsin (magenta) is a tolerably stable compound, and requires somewhat strong acids to become converted into the ammonium derivative, and apparently silk is a sufficiently strong acid to bring about this change. That dyeing is really a question of salt-formation they proved in this way: Skeins of silk dyed with magenta were allowed to stand at the ordinary temperature in a mixture of methyl iodide and methyl alcohol, side by side with the rosanilin base, the rosanilin hydrochloride, the rosanilin stearate and amido-stearate. All the samples remained unaltered with the exception of the free base, which turned deep blue, but on warming from 35 to 40° C. for 20 hours, the salts as well as the skein were alkylated. Animal fibres, therefore, unite in all probability with the dyes to form chemical compounds.

Weber's observation that benzidene dyes (p. 346) attach themselves as salts to the fibre, was confirmed by Gnehm and Roetheli, as 100 grms. of cotton immersed in boiling solutions of 1 per cent. of the pure barium salt of benzopurpurin 4B, and benzazurin 3G, took up the whole of the salts. Barium salts, having only a small coefficient of diffusion, dye only slowly, and are also not extracted from the fibres; further, as barium salts are impervious to Röntgen's rays, it was easy to determine that the cotton had taken up these salts.

[1] This work was done in the Technisch-chem. Laborat. d. Polytechnik, Zürich.

To answer the question whether other dyes, not belonging to the benzidene group, are decomposed on dyeing, the authors made the following experiments: Dye radicals were combined with acids and bases having high molecular weights; thus colour bases were united with palmitic and stearic acids, while colour acids were used in combination with organic bases [1].

On dyeing silk in these solutions; drying it; washing off with benzene any acid which was adhering mechanically and dissolving the silk in hydrochloric acid, it was found that no acid had been taken up by the fibre. Therefore the colour salt must have been decomposed, and only the colour base have been taken up by the silk.

A further proof of the chemical theory of dyeing is the existence of salts which are formed by colour bases uniting with relatively weak acids, if these have a high molecular weight, such as amido acids. Further compounds of colour acids with faintly basic bodies are possible; for example, naphthol yellow S combines in equimolecular proportions with anilin, benzidene, and also with amido-palmitic acids.

Gnehm and Roetheli have arrived at the following conclusions:

(1) Substantive dyes (p. 195) + animal fibres = mixture of chemical compounds + physically absorbed dye.

(2) Adjective dyes + animal fibres = partly chemical compound with the mordant + partly physically absorbed dye.

(3) Substantive dyeing of cotton with benzidene colours results in a solution of the dye salt in the cell-sap. The dye remains in the fibre owing to its small coefficient of diffusion.

(4) Indigo and basic substantive dyes are taken up by adsorption according to the view of v. Georgievics (see p. 331).

(5) Pigments and azo colours formed on the fibre are precipitated purely mechanically.

(6) Adjective dyes, on cotton, form lakes which are precipitated mechanically.

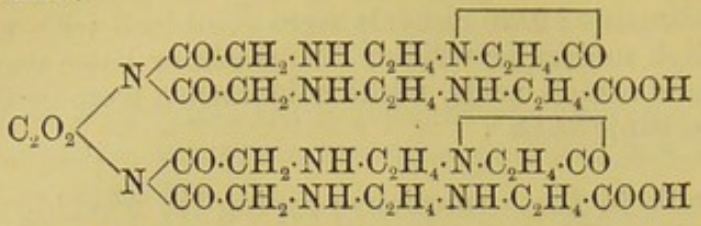
Schützenberger has investigated the products of hydrolysis of wool by baryta water, and has found them to be analogous to those

[1] To prepare palmitates proceed thus: Precipitate the dye in the cold with NH_3, add the finely powdered base to the molten acid, and purify the salt after cooling with alcohol, when metallic powders with no crystalline appearance are obtained. Thus palmitate of pararosanilin has a melting point of 85° C., and the stearate of 98°. Both are easily soluble in alcohol, chloroform, acetone; insoluble in benzene, carbon bisulphide, and petroleum ether. The following salts of new-rosanilin were prepared and were found to stain wool and silk from dilute alcoholic solutions, the respective acids remaining in the bath:

(1) The amido-acetate, (2) the amido-caproniate, (3) the tyrosin salt, (4) the amido-stearate, and (5) the amido-palmitate.

Similarly acid dyes united with feeble bases: (1) naphthol yellow S + anilin salt; + benzidene salt; + amido-palmitate; (2) Helvetia-blue and benzopurpurin with anilin salt; (3) β-naphthol orange and picric acid with benzidene; and (4) picric acid with amido-palmitate.

of ordinary gelatin and albumin. The formula he attributes to gelatin is this:



and similarly albumin contains the typical groups $C_2O_2\langle{}^{N=}_{N=}$ and the carbamide group $CO\langle{}^{N=}_{N=}$

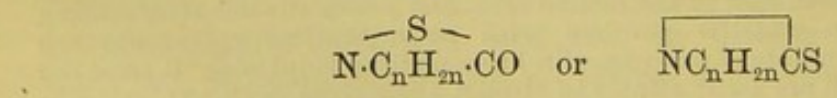
In these formulae the complete absence of the amido group NH_2 will be noticed. This view is also held by v. Flick and Prud'homme [1], and is further supported by the experimental work of the two following observers.

Bentz and Farrel [2] made experiments to determine whether the affinity between acid dyes and silk and wool depended on the basic amido radical NH_2 which the latter are supposed to possess, being amido carboxylic acids, $H_2N—R—COOH$. They de-amidated the fabrics by treatment with nitrous and hydrochloric acids at the ordinary temperature for 10 to 12 hours, followed by washing with (1) alcohol, (2) cuprous chloride and hydrochloric acid, and (3) water. As both silk and wool behaved towards acid colours as ordinary wool and silk, the authors arrived at the conclusion that the amido groups present in silk and wool either play no part at all or at most an unimportant one.

Prud'homme [1] is a strong believer in the chemical theory of staining, and the very ingenious method of liquid artificial fibres, described below, allows of accurate determinations being made.

In his first paper Prud'homme, adopting the view of Schützenberger (p. 357), Bentz, and Farrel, assumed that wool contains the group $\overline{N \cdot CH_{2n} \cdot CO}$, which with sulphurous acid would form the compound $N \cdot C_n \cdot H_{2n} \cdot C\langle{}^{OH}_{SO_3H}$ Thus the acid function of the molecule becoming accentuated, its affinity for basic colouring matters is increased.

The sulphur radical in wool Prud'homme believes to be combined as



Wool absorbs nitrous acid, which, combining with the imide group NH, may give rise to nitrosamins, and by assuming that the NH radical combines with phenols in alkaline solutions, staining reactions may be readily explained on chemical lines.

[1] Prud'homme: *Rev. Gén. des Mat. Col.* 2, 213 and 4, 72.
[2] Bentz and Farrel: *J. Soc. Chem. Industr.* p. 405 (1897).

Two years later, in 1900[1], Prud'homme constructed the liquid artificial fibres above referred to. He took neutral solvents, which are not or only slightly soluble in water, such as benzene, chloroform, or amyl-alcohol, and dissolved in them acids, bases, mixtures of acids and bases, or substances containing both basic and acid groups in strengths of 10 per cent. An acid character was given to the amyl-alcohol by means of benzoic $C_6H_5 \cdot CO-OH$, and salicylic $C_6H_4 \cdot OH \cdot CO-OH$ acids; a basic character by weak bases such as imides, for example acetanilide (antifebrin), $C_6H_5 \cdot NH \cdot CO \cdot CH_3$, and diphenylamin, $C_6H_5-NH-C_6H_5$; while bodies containing both an acid and a basic group were represented by phenyl-glycocoll, $C_6H_5 \cdot CH\langle^{NH_2}_{COOH}$, and anthranilic acid, $C_6H_4\langle^{NH_2}_{COOH}$

Next, small quantities of the bases of pararosanilin, malachite-green, hexamethyl-violet, methylene-blue, rhodamin, safranin, Bismarck-brown, and the oxyazins sky-blue and Nile-blue (see p. 407) were dissolved in 1 per cent. caustic soda.

On shaking rapidly together equal quantities of the solutions of the dyes just mentioned, and 10 per cent. salicylic acid amyl-alcohol, the latter became coloured like a salt of the base; thus the violet methylene-blue became blue, the rhodamin changed from yellow to orange, safranin from carmin to orange red, Nile-blue from orange to blue, and Bismarck-brown from yellow to orange.

The less basic a colour base, the less stable are its compounds; for example, rhodamin + amyl-alcohol = yellow orange colour + fluorescence, while rhodamin + amyl-alcohol + naphthol showed the same red colour as is exhibited by wool.

As dye radicals of weak basicity, such as carmin blue G, acid violet 7B, and magenta, are more freely absorbed from water by amyl-alcohol containing β-naphthol than by alcohol alone, we may use β-naphthol as an indicator as to how colour bases will react with stronger acids, such as wool which has been treated with sulphuric acid, because with the stronger acids insoluble compounds will be formed.

The acidity of wool is distinctly greater than its basicity, while the opposite holds good for those dyes which stain wool directly (substantive dyes), and therefore a chemical union between the wool and the dye is very probable.

Naphthylamin-β, $C_{10}H_7NH_2\beta$, extracts in an amyl-alcohol solution carmin blue less rapidly than does a solution of naphthol-β, $C_{10}H_7OH\beta$, in amyl-alcohol. The former is a stronger base than wool, for dissolved in amyl-alcohol it extracts free colour acids from aqueous solutions, while wool does not, but cannot form compounds of great stability with acid dyes.

If neutral instead of acid amyl-alcohol was taken, then the alcohol turned the same colour as the free base if the latter was coloured, or remained colourless if the carbinol base (p. 421) was colourless.

As silk dyes the same colour as is exhibited by the salicylic acid

[1] Prud'homme: *Rev. Gén. des Mat. Col.* 189–192 (1900).

amyl-alcohol mixture, the process of staining must be due to a combination of the colour base with an acid group in the fibre (see above, Prud'homme's early paper).

The salts of basic dyes are more or less soluble in neutral amyl-alcohol, but fuchsin, methylene blue, and safranin salts dissolve more readily in acid alcohol, because the salicylic acid acts on the free amido groups of the dye. Analogously silk is supposed to take up a certain quantity of the undissociated magenta salt.

Corresponding experiments were made with the sulphonic acid dyes; acid fuchsin (acid magenta), acid green, acid violet, patent blue, cyanol, ponceau, crocein, orange III; chromotropes and indigo-carmin.

Amyl-alcohol, whether neutral or acidified with sulphuric acid, became only slightly coloured; but amyl-alcohol containing 10 per cent. of acetanilide gave an intense colouration differing from that of neutral or acid alcohol. Thus indigo-carmin is greenish blue in neutral alcohol, and pure blue in acetanilide alcohol.

From an acid cherry-red solution of the salt of orange III, neutral amyl-alcohol takes up the yellow colour acid, while acetanilide amyl-alcohol shows an orange colouration. Silk and wool in an acid dye-bath act analogously to amyl-alcohol, as under these conditions they are capable of decomposing acid salts[1].

Prud'homme thinks that the HSO_3 of the dye combines with the NH radical of the fibre, while Gillet believes that the NH radical of the chromophore of the dye (—NH—N=) unites with the CO·OH group of the fibre, because salicylic acid cannot extract the azo-dyes from their acid solutions, although it is a stronger acid than any which can be supposed to exist in the fibre.

The free colour acid tetrabrom-fluorescein of the salt eosin dissolves in neutral or acid amyl-alcohol with the yellow colour of the free base; and the acetanilide + amyl-alcohol reagent does not act on a solution of eosin till some salicylic acid, or phenyl-glycocoll, is added to the alcohol, when the red colour of the eosin salt is obtained on bringing the alcohol mixture in contact with the free, yellow colour acid tetrabrom-fluorescein.

As regards the mordant stains, the oxyketones, and the carboxylic compounds, it is necessary for the mordant to be at least diatomic or divalent, to allow it firstly to become fixed to the tissue, and secondly to fix the dye, which latter event is brought about by the interaction between one or more of the free hydroxyl radicals of the metal with the hydroxyl or carboxyl groups of the dye, leading to the formation of condensation products, accompanied by the elimination of water.

As all animal and vegetable fibres are semi-permeable membranes (Hofmeister, p. 330), they possess the power of decomposing certain compounds such as acid salts, and therefore, because of their structure alone, tissues will repel certain stains (Prud'homme). It is better, however, to put it this way: Tissues being semi-permeable mem-

[1] To be accurate we should say abstracting the colour radical from the dye which has undergone electrolytic dissociation.

branes will allow only of the migration of certain ions through them, after the ions have previously been formed by the electrical dissociation of the electrolytes.

These observations of Prud'homme were attacked by Sisley [1], who found that the colourless rosanilin base of crystal-violet reverts to the coloured form on being boiled with water, or on being shaken up with neutral amyl-alcohol. Sisley attributed the negative results which Prud'homme got to Prud'homme having used the colour bases dissolved in 1 per cent. alkaline solution, by which process the colouration of the amyl-alcohol is prevented.

Knecht [2], however, has shown Prud'homme's work to be correct, and Sisley to be in the wrong, for these reasons: Ordinary amyl-alcohol always contains traces of both organic acids, and also of carbon dioxide, a fact overlooked by Sisley; and if amyl-alcohol be purified by contact with powdered caustic potash and subsequent distillation, it is not coloured by rosanilin solutions, unless the amyl-alcohol and the watery solution be shaken together in the presence of air, when, owing to the absorption of CO_2, the alcohol becomes rose-coloured. Pure amyl-alcohol (but not ethyl or heptyl alcohol) containing heptylic, $CH_3{\cdot}(CH_2)_5{\cdot}CO{\cdot}OH$, oleic $CH_3CH{:}CH{\cdot}(CH_2)_{14}COOH{\cdot}$ or carbonic, CO_2, acids is quickly and strongly coloured by the colourless rosanilin base.

Gillet [3] likewise points out the existence of valeric and other easily hydrolysable acids in ordinary amyl-alcohol, and to remove them and the carbonic acid, amyl-alcohol is first shaken up with moist soda, and then distilled in a current of nitrogen. To obtain the rosanilin base quite colourless he distils it freshly over soda into ether. Having taken these precautions the rosanilin base does not colour the amyl-alcohol in the cold, and only slowly on heating.

Gillet further noticed that a number of triacids in watery acid solution when shaken up with amyl-alcohol stained the latter of the colour of the diacid; thus the triacid hydrochloric acid salt of magenta is of a yellow colour, and stains amyl-alcohol a blue colour. The explanation offered is that under the influence of water and mineral acids the neutral alcoholic solvent takes up the colour acid, and then brings about an intra-molecular union between the basic (NH) and the acid (SO_2OH) groups of the dye. Should the compound formed in this manner be soluble in water, as is the case with azo-blue, then the intra-molecular union is broken down in watery solvents. These results of Gillet have been mentioned to point out the importance of remembering that dyes in alcoholic solutions may have entirely different properties than when dissolved in water, and also to emphasize, if we desire to study the effect of stains offered in a watery solution to tissues, that we should examine sections in water before acting on them with alcohol.

[1] P. Sisley: *Rev. Gén. des Mat. Col.* 4, 251 (1900).
[2] E. Knecht: *ibid.* 182 (1900).
[3] C. Gillet: *ibid.* 183–189 (1900).

Gillet[1] has modified Prud'homme's plan of using salicylic and acetanilide reagents in constructing 'artificial fibres,' because these reagents are readily soluble in water, and has substituted for them naphthalene derivatives, in particular β-naphthol $C_{10}H_7OH\beta$ (see p. 388) in 5 per cent. strengths in amyl-alcohol. This reagent has no action upon aqueous solutions of strongly basic dye radicals such as fuchsin (magenta), methyl-violet, or safranin, because the phenol is too weak an acid to displace those which are joined to the colour bases of these dyes.

Naphthol-β resembles in its behaviour cotton rather than wool, as it extracts the free colour bases from their aqueous solutions. It is coloured by the colour bases because these unite with the phenol radical to form salts: $C_{10}H_7O$ + colour base, which latter being a stronger kat-ion replaces the hydrogen kat-ion of the naphthol.

Gillet believes Prud'homme to be wrong in supposing that the dyeing of wool by means of strongly acid dye radicals takes place through the agency of the sulphonic acid radical, because the colours on wool are neither produced quickly nor are they 'fast.' He considered the free colour acids to be in reality feeble basic compounds, owing to the azo group $-N=N-$ or $=N-NH-$, but it has been pointed out that dihydroxy-naphthalene disulphonic acid is also absorbed by wool in exactly the same way as are the acid azo-dyes, notwithstanding the fact that the azo group is absent in this compound.

If we suppose the sulphonic acid radical capable of being split off the remainder of the dye molecule by means of free sulphuric acid, we may still consider the dyes as basic radicals. Such a view is supported by the observation that the presence of acid in the wool is necessary to bring about staining, for Knecht showed in 1888[2] that wool treated with sulphuric acid and then well washed in water still retains a certain quantity of acid in combination with it. When in this state the wool dyed even more rapidly than if the sulphuric acid was merely present in the dye-bath, an observation confirmed by the behaviour of nucleic acid (see p. 335). (See also p. 365, under acid fuchsin.)

Gillet's view that the so-called acid dyes belonging to the sulphonic group are in reality feeble bases is supported by the fact that the greater the number of sulphonic radicals there are present in the dye molecule, the less quickly does it dye, while the very opposite should hold good if they were really acid dyes joining on to the basic radical of the wool.

Nietzki inclines strongly to the chemical view. In the last edition of his *Chemistry of Organic Dyes*, in 1901, he says: 'Certain facts speak for the view that the unions of dyes with fibres are nothing but salt-like unions, in which the fibre, analogously to an amido-

[1] Gillet: *Rev. Gén. des Mat. Col.* 4, 305–7 (1900); abstract in *J. Soc. Chem. Indust.* 1105 (Dec. 1900).

[2] E. Knecht: 'Communication from the chemistry and dyeing department of the Bradford Technical College,' *Journ. Soc. Dyers and Colourists* (1888), pp. 104–107; and *Journ. of Soc. Chem. Indust.* 621–623 (Sept. 1888). See also p. 451.

acid, plays in the one case the part of an acid, in the other case that of a base. Thus rosanilin in the form of its free base (carbinol base, p. 421) is colourless, while its salts are coloured. If one place in the colourless solution of the colour base a skein of silk or wool and then heat the solution, the skein will be stained as intense a red as if the corresponding amount of rosanilin hydrochloride, or other rosanilin salt, had been used.' 'The fibre in this union plays the part of an acid.'

'That towards a colour acid the fibre may play the part of a base, may be shown in a very instructive manner with the quinoid ethyl-ether of tetrabrom-phenol-phthalein (p. 428). This ether in the free state is pale yellow, and dilute solutions appear almost colourless, while its alkali salts are of an intensely blue colour. If one acidify the blue solution of this salt with acetic acid till it has become colourless, a skein of silk may be stained in this colourless solution an intense blue.'

As further many free colour acids, for example the sulphonic acids of the amido-azo compounds, possess a different colour from their alkali salts, and as the free colour acids do not stain fibres with their own colour but that of their alkali salts, it follows that the fibre must play the part of a base. 'As a rule the fibre is unable to decompose the alkali salts of strong colour acids. The latter, therefore, only stain, if by the addition of a stronger acid the colour acid has been liberated.'

In connexion with this last sentence of Nietzki's, I have to point out that dyes which are not pseudo-compounds (see p. 189) are dissociated electrolytically in watery solutions, but that the tissues being pseudo-compounds are not dissociated. Therefore the addition of acids leads to the partial electrolytic dissociation of the tissue, and thus enables its ions to come into relationship with the coloured ions of the dye.

Lehmann states that crystals stain more deeply than the mother liquor (which, of course, is due to a difference in the coefficient of distribution), but also that the dyes in some way participate in the formation of the crystals because metachromatic effects are produced. As further different surfaces of a crystal may show varying affinities for the same dye; as certain dyes increase the power of crystallization; as two dyes together will stain when either of them taken individually will fail to do so; as only certain dyes are taken up by certain crystals, and as the staining can be used for chemical analysis, it is difficult to arrive at any other conclusion but that staining is a chemical process.

Professional dyers are in the habit of adding to dye-baths, in addition to acid, sodium sulphate in different proportions so as to procure 'level' or even staining.

Hallit[1] has investigated the action of sodium sulphate as well as

[1] Hallit: *J. Soc. Dyers and Colourists*, 15, 30 (1899); abstract in *J. Soc. Chem. Industries*, 368–370 (1899).

that of sulphuric acid, and has also made experiments with free colour acids instead of the colour acid salts usually employed.

He finds that sodium sulphate, as an assistant to sulphuric acid in dyeing wool with acid dyes, does not act by raising the boiling-point of the bath nor by improving the state of suspension of the dye. Fifty per cent. sodium sulphate extracts acid colours more readily than pure water or 5 per cent. sulphuric acid. He therefore holds that the 'levelling' action of the sodium salt is due to its power of extracting the dye more readily from those areas where it has been deposited in excess, and that it does so by breaking down the chemical compound which dyes form with water. This dye + water compound can be completely decomposed by adding sufficient sodium sulphate, as then the dye, having been rendered insoluble, is precipitated.

The definite state of equilibrium, which at the end of the dyeing process is arrived at, is due, on the one hand, to the attraction between the dye radical and the fibre, and on the other hand to the solvent action of the exhausted dye-bath on the dye already fixed on the fibre. This result is brought about by the laws of chemical equilibrium in dilute solutions, according to which 'two acids present in equivalent quantities in solution containing any salt, the base of which is insufficient for both, will each secure a proportion of the base corresponding to their respective avidities.'

These relative acid avidities or intensities are for

Hydrochloric acid.		*Sulphuric acid.*		*Oxalic acid.*		*Acetic acid.*
1	:	0·49	:	0·24	:	0·03

Thus in the acid dye-bath containing sulphuric acid and a colour salt, both the sulphuric acid and the colour acid will compete for the sodium base. 'On introducing yarn into the dye-bath, both the colour acid and the sulphuric acid are partially absorbed and neutralized by the fibre, the colour acid in larger amount than the sulphuric acid. In consequence of this, the interaction between the remaining colour salt and the sulphuric acid proceeds a step further, more colour acid being set free and being absorbed by the yarn.' This process would go on till practically the whole of the colour acid had been taken up by the yarn, if it were not for the fact that the dye in the yarn is still exposed to the solvent action of the exhausted dye-bath.

Dyes with strongly acid elements, such as the nitro and sulphonic acid radicals (p. 184), stain most evenly and exhaust the dye-bath least because they resist the rapid displacement of their salts by sulphuric acid.

Not only is the more intense colour acid liberated more slowly from its base and therefore dyeing made more gradual, but the staining does not proceed, so far as regards intensity, because of the solvent action of the dye.

Sulphuric acid is said, on boiling, to be neutralized by wool to a greater extent than other acids, and therefore to act less intensely as a solvent for the dye already fixed in the fibre, because, having been taken up by the fibre, its total mass is of course diminished.

Hallit, on experimenting with the free colour acids scarlet 2R, orange G, cardinal-red, found that they dye wool very feebly indeed, but after the addition of 3 per cent. sulphuric acid or equivalent amounts of hydrochloric acid, the dyeing proceeds in the same way as if colour salts were present. It follows that it is not sufficient for dyeing purposes to have a free colour acid present, but there must also be present some assisting factor, such as mineral acids, which by acting on the wool-fibre prepare the latter to receive the acid dye-radical.

Acid fuchsin, in a watery solution, is undoubtedly an acid dye owing to the presence of the SO_2OH radical, the hydrogen kat-ion of which is replaceable by stronger kat-ions. But does acid fuchsin remain a colour acid on the addition of sulphuric acid? There is strong evidence that it does not. Gillet (p. 361) was the first to point out that colour acids behave as feeble bases, and this I believe is due to the fact that by the addition of sulphuric acid to the staining solution the acid fuchsin (composed of basic fuchsin + a sulphonic acid radical + sodium) dissociates primarily into the kat-ion radical Na and the an-ion radical:—basic fuchsin + sulphonic acid,—and that secondarily this an-ion still further dissociates into the basic fuchsin kat-ion and the sulphonic acid an-ion.

Owing to this dissociation having taken place the basic fuchsin may link on to acid radicals in the tissue, and thus Fischer is using, in reality, a basic dye when he stains nucleic acid by means of a solution containing both acid fuchsin and sulphuric acid.

My own views as to staining have already been given when criticizing the views of others, but a short résumé may be useful. Before discussing whether chemical or physical factors are at work in staining the question must be asked, Is there any difference between chemistry and physics? There is not. Frankland used to teach that chemistry is but a branch of physics, and the whole recent development of chemistry points to the importance of studying chemical interaction apart from those conditions where substances join in definite molecular proportions. Even adsorption (p. 331), which is perhaps the best example of physical action, has certain resemblances to chemical action, for, according to Ostwald, substances under certain conditions are taken up in definite proportions; further, the more complex a substance, be it liquid or gas, the more readily it is absorbed, and not only are certain substances condensed, for example on carbon, but others are actually expelled or kept out.

What must take place when a body is adsorbed and stored in quantities larger than those in which it is present in the solution, is a lessening of its osmotic power by the adsorptive agent. This effect may be due to a squeezing together of the individual molecules owing to surface tension, as in the case of formation of mechanical coagula (p. 50); or it may be that the adsorbent matter exerts the same influence in condensing a substance as does an electrolyte in producing coagulation. According to the latter view there is established,

during adsorption, an electric field in the adsorbing medium owing to the strain the adsorbed matter is subjected to when it adapts itself to the surface of the adsorptive agent.

Again, on theoretical grounds, a physico-chemical staining along the lines of Krafft's colloidal theory is easy to understand, for nucleic acid and nucleins, because of their acid reaction, may have the same effect on the precipitation of colloidal dyes, in an insoluble form, as has the addition of acids to soap [1]. On the other hand a direct chemical union between the compounds mentioned cannot be excluded.

This matter has been gone into to make the reader realize that hard and fast lines cannot be drawn between chemical and physical action.

Gierke, Fischer, and Laurent explain all their results as due to adsorption, or due to differences in the rate of diffusion ; Hofmeister, v. Georgievics, Rawitz, and Spiro, as due to differences in the coefficient of distribution ; Krafft, and after him Zacharias (Athens), to the behaviour of colloid solutions ; while Miescher, Knecht, Ehrlich, Unna, E. Zacharias, Lilienfeld, Heine, Mathews, Gnehm, Roetheli, Nietzki, Prud'homme, Gillet, and I believe that in addition to the physical, also chemical factors come into play.

That under certain conditions differential staining by purely physical means may be brought about, especially if mixtures of basic or acid dyes are chosen, has already been shown.

That, either accompanying the physical staining or quite apart from it, true chemical staining is not only possible but actually does occur, I have pointed out in criticizing the views of Fischer.

When on the basis of macro-chemical knowledge we discuss the micro-chemical action of stains, we have to bear in mind what previous treatment the tissue has undergone, whether it was allowed to coagulate or whether coagulation was prevented. Thus, Ehrlich's method of drying films spontaneously, and then rendering them insoluble by heat (p. 143), or Altmann's method of freezing tissues, dehydrating them at a low temperature, and impregnating them with paraffin when quite dry (p. 143), are ideally the best methods for micro-chemical research, because coagulation is prevented.

The next best method is fixation by non-electrolytes, which act physically, as does, for example, alcohol, because with most tissues such treatment amounts essentially to dehydration, if the alcohol be not allowed to act too long. All these fixing methods, by preserving the tissues in a 'normal' state, allow us to study and to control the effect of staining reagents by knowledge gained macro-chemically.

It is different when we have employed non-electrolytes, forming additive compounds such as formaldehyde and osmium tetroxide. The former of these is by no means a reagent which can be regarded as indifferent to stains, as held by Fischer and Laurent, for Ehrlich has shown that the dimethyl-amido-benzaldehyde reaction (p. 298) does not take place with cartilage, if the latter has been fixed in

[1] This view seems to be confirmed by the coagulation experiments of Heidenhain (pp. 452–459).

formaldehyde; and quite apart from this the formic acid usually present acts on the pseudo-bases, converting them into true bases.

Still more difficult is it to apply physiological knowledge gained by acting on tissues macro-chemically to tissues fixed by electrolytes. Thus sublimate sections by treatment with iodine can have the sublimate removed, but iodine radicals are substituted, and these, when removed by alkalies, as I have seen some histologists do, leave the tissue in an unknown state, perchance to be coagulated again by alcohol or dissociated by water.

Notwithstanding the fact that I feel intensely the difficulties we have to encounter in all histological work, I yet believe macro-chemistry to have certain fundamental facts in common with micro-chemistry, and this belief will justify chapter xxii, on the chemistry of some tissue constituents. If certain organs macro-chemically are rich in nucleic or chondro-sulphuric acid, and these very same organs micro-chemically show an affinity for basic dyes, as do for example nuclei, cartilage, and mucus, we cannot arrive at any other conclusion than that chemical union has taken place in our sections between the acid tissue component and the colour base. Again, if albumin reacts towards nucleic acid as a base, forming nucleins, why should we scorn the idea that an analogous chemical union takes place between it and a colour acid?

To be able to explain all staining by physical means is possible, but to say that therefore it must take place in this way is impossible.

The conception I have formed of precipitated proteids is that each component molecule, in addition to adhering probably only physically to its neighbour, is still endowed with a number of side-chains, which are unsatisfied after the removal of the fixing reagent, and which, under suitable conditions, may attract towards themselves colour radicals of the opposite electrical sign. Thus, for example, a tissue side-chain may be a sufficiently strong kat-ion to combine with the an-ion radical $C_6H_2O.(NO_2)_3$ of picric acid, and the unsatisfied nucleic acid radical in nucleins may withdraw the kat-ion methylene-blue from its an-ion chlorine. Thus dyes and tissue-molecules can adhere chemically to one another by their side-chains.

I do not think it far-fetched to assume that these side-chains are comparable to pseudopodia of an amoeba or the tentacles of a hydra; for just as the latter are directed by chemotaxis in certain directions, so do I hold that side-chains are able to exert their own chemical influences when released from their intra-molecular combinations under conditions of special chemical stress.

The chief factor in liberating the side-chains is moisture, for anhydrous tissues do not stain (p. 198), and it is for this reason also that I believe alcohol is a much better extractive of dyes than water, as by acting on the side-chains of both the tissue and the dye it diminishes their mutual chemical affinity (see however p. 451).

That tissues require to be coagulated or rendered firm before permanent staining is possible has been pointed out repeatedly, the probable reason being that otherwise the proteid molecules become dissociated in the watery media, owing to the action of the water

and the action of the ions derived from the dyes. Instead of having the dye drawn towards the tissue, the tissues will dissolve in the dye-bath (see also p. 198).

Each side-chain must be imagined to have its own sphere of influence, and if many are placed together the total effect is quite different from what it would be if they were separated; and therefore it follows that a rigid system, namely the coagulated proteid, will attract suitable dyes by its unsatisfied side-chains, which are held together by the rest of their molecules having united. It is want of chemical tension which prevents methyl-violet, for example, from being taken up completely from very dilute watery solutions by gelatin, quite apart from the fact that no chemical interaction goes on to completion.

It was pointed out (p. 329) that the coefficient of distribution for succinic acid between ether and water is as 6:1, and it follows according to the laws given above that this acid cannot be in the same physical state in the ether as in the water. It is generally assumed that the substance aggregates into bigger molecules, or that it polymerizes in that solution in which it develops the least osmotic pressure, or, what amounts to the same, in which it is more soluble.

As iodine dissolved in different menstrua shows a variety of colours owing to its molecules being in different states of aggregation, so do certain dyes stain wool differently from cotton. There is thus produced a metachromatic effect, a phenomenon which also holds good for many histological stains such as kresyl-violet, methyl-violet, safranin, toluidin-blue, and azo-blue.

From the metachromatic effects we may reason that tissues staining differently have different chemical natures, but no more, because iodine with alcohol gives a brown colour, but it dissolves with a violet tint not only in chloroform, but also in carbon bisulphide.

Fischer has endeavoured to explain all metachromatic effects as due either to impurities in the stain (methyl-violet in methyl-green, P. Mayer), or due to the 'ripening' of solutions (methylene-red in old alkaline methylene-blue solutions, Unna, p. 405); or, as in the case of haematoxylin, on the theory of acid indicators[1]. 'Just as methyl-orange shows a colour change on passing from the state of the combined molecule (red) into the free state of an ion (yellow), so will haemateic acid give an analogous colour change from blue to red (free ion). Or it may be a pluri-basic acid which is coloured differently in its acid salts from what it is in its neutral state.' To get the chloro-platinous precipitate of nucleic acid stained in haematoxylin to change from red to blue, required one hour's washing. 'That the red "acid" colour disappears also here completely, is the best proof of the purely physical part played by nucleic acid.'

It must be remembered that there is always sufficient alkali present in running water to turn haematein (haematoxylin) blue.

The two first 'explanations' of Fischer are not explanations at all, as it is self-understood that mixtures of dyes will stain different

[1] Ostwald: *Die wissenschaftlichen Grundlagen d. analytischen Chemie*, 104 (1894); and *Lehrbuch d. allgem. Chemie*, 1, 799 (1891).

elements differently, either physically or chemically. As regards the comparison with acid indicators I cannot follow Fischer, for, in the first instance, we do not know that blue litmus is not dissociated, while red litmus is; and, secondly, when the 'red' passes into 'orange' methyl-orange, or, according to Fischer, when an electrolyte dissociates, he assumes that no chemical action takes place, a view which is quite untenable. If water causes dissociation, and thereby produces a colour change, we know it to act chemically, and why should we deny chemical activities to tissues, knowing the great number of acid and basic principles present in the cell? (See ch. xxii.)

The chief factors which influence staining are summarized in the following table:—

A. Fixative.
  I. Physical considerations:
    (*a*) Spontaneous drying,
    (*b*) Alcohol,
    (*c*) Heat (?).
  II. Chemical considerations:
    (*a*) Non-electrolytes:
      (1) Aldehydes,
      (2) Osmium or ruthenium tetroxides.
    (*b*) Electrolytes:
      (1) Acids,
      (2) Bases,
      (3) Salts:
        (α) Neutral,
        (β) Acid,
        (γ) Basic.
    (*c*) Mordants.
    (*d*) Non-mordants (?).

B. Tissue.
  I. Physical considerations:
    (*a*) Solubility
    (*b*) Porosity
    (*c*) Moisture
    } According to the nature of the solvent of the dye.
  II. Chemical considerations:
    (*a*) Has the state existing before fixation been preserved as regards
      (1) Organic constituents:
        (α) Pseudo-acids or pseudo-bases,
        (β) Real acids or bases;
      (2) Presence of inorganic salts?
    (*b*) After fixation with chemically active reagents
      (1) Has the fixative been removed?
      (2) Does it act as mordant for the dye?
      (3) What are the effects of secondary and tertiary mordanting?
    (*c*) What radicals show an affinity for stains? Can the tissue cause a coagulation of the dye?

C. Stain.

I. Physical considerations :

(*a*) Solubility in different solvents; coagulability by tissue-constituents.

(*b*) Diffusibility depending on size of molecules.

(*c*) Saturation or concentration (osmotic pressure).

(*d*) Fastness of physical union dependent on the size of the dye molecule and the pores of the tissue.

(*e*) The 'covering power' by which one dye hides another one to our eyes.

II. Chemical considerations:

(*a*) Non-electrolytes :

(1) Oxy-compounds,

(2) Pseudo-compounds.

(*b*) Electrolytes (the amount of dissociation) :

(1) Colour acids,

(2) Colour bases,

(3) Colour salts.

(*c*) What radicals show an affinity for tissues?

(*d*) Does the dye react with the tissue directly?

(*e*) Does the dye combine with the mordant?

(*f*) Is differentiation required or not?

(*g*) Is the chemical union 'fast'?

D. Solvent of the dye. Its effects on the mordant, the tissue and the dye.

I. It is neutral to litmus paper :

(*a*) Water,

(*b*) Alcohol,

(*c*) Acetone,

(*d*) Glycerin.

II. It is acid, due to acid accentuators (p. 213).

III. It is basic, due to basic accentuators (p. 212).

IV. The dyes by the addition of other salts, such as sodium sulphate, are on the point of precipitation.

E. Temperature.

F. Time.

In conclusion the object of staining may be stated to be—

1. To help us in determining morphological facts. In this case all methods, no matter whether physical, chemical, or physico-chemical, are justified.

2. To recognize micro-chemically the existence and distribution of substances which we have been made aware of by macro-chemical means. It is not sufficient to content ourselves with using acid and basic dyes and speculating on the basic or acid nature of the tissues, or to apply colour radicals with oxidizing and reducing properties; but we must endeavour to find staining reactions which will indicate not only the presence of certain elements such as iron or phosphorus, but the presence of organic complexes such as the carbohydrate group, the nucleins, protamins, and others.

PART VIII.

CHAPTER XXV.

How to render Preparations permanent.

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On fixing sections to slides . . . . . p. 372
On mounting sections . . . . . p. 376

On cleaning Slides and Cover-glasses.

Glass is a mixture composed of the silicates of alkalies (potash and soda) and alkaline earths (calcium), and contains in addition usually aluminium and iron, and, if flint-glass, also lead. The silicates of the alkalies are non-crystalline and readily soluble in water, while those of the alkaline earths are crystalline and are readily dissolved by acids; but when these two different kinds of silicates occur together, as they do in glass, they seem to mutually protect one another to a considerable degree.

Notwithstanding this protection, glass constantly undergoes decomposition, the alkaline silicates separating out and forming a coat on the surface of the glass, as is most readily shown by Mylius' test (p. 214).

Because of the alkaline silicates separating out it is necessary to thoroughly clean both slides and cover-glasses. Jenner gives the following directions: (1) Clean cover-glasses by throwing them singly into 500 cc. of boiling water, and let them boil for half an hour; then move them about in ordinary sulphuric acid, one at a time, by holding each cover-glass with a pair of forceps; rinse in several changes of distilled water; transfer to absolute alcohol till the whole number of cover-glasses have passed through the sulphuric acid bath; dry the cover-glasses with a perfectly clean linen handkerchief, and keep them dry under a bell-jar (see also p. 374).

I have adopted the following plan:

In a solution containing 1 per cent. of chromic and sulphuric acids slides are left for at least one hour, and cover-glasses for ten minutes; then they are rinsed in distilled water to remove the acids, and dried with a clean cloth.

A cover-glass after treatment with acids is much more brittle, and is best cleaned by being placed on a piece of clean paper, as I first saw Carlier do, or on American cloth[1], being subsequently rubbed with a clean handkerchief.

To clean slides containing balsam preparations, heat the slide over a Bunsen burner till the cover-glass can be pushed off with a blunt instrument; allow the cover-glass to fall into a vessel containing a strong and hot solution of Hudson's soap, and place the

[1] American cloth, which was recommended to me some years ago (I forget by whom), has this advantage, that the cover-glass lies firmly on it without having to be held.

slide in a similar solution. Boil the slides for ten minutes, and then rinse them in clean water; if necessary, add some more soap and boil up once more. Finally, treat the slides as indicated above with the chromic and sulphuric acid mixture.

On fixing Paraffin Sections to Slides.

To enter fully into all the methods which have been described for fixing sections to slides is impossible.

Gaule, in 1881, was the first to cover a slide with spirit; to float sections on the spirit; to flatten them by gently heating the slide; to allow the alcohol to evaporate; to cover the sections with a cover-glass to prevent them shifting, and, finally, to remove the paraffin with xylol.

Paul Mayer, in 1883, used egg-white as a fixative for paraffin sections. His solution is made by thoroughly mixing together 50 cc. of egg-white, 50 cc. of glycerin, and 1 grm. of sodium salicylate, and then filtering into a clean flask. When required it is spread out on the slide in a very thin layer by rubbing the slide with a clean finger[1]. It is an excellent fixative, but does not allow of crumpled sections being spread out as evenly as can be done by Gulland's method, and has the further disadvantage that stained sections cannot be mounted directly in balsam after the removal of the paraffin, because the glycerin would cause opacity. Therefore it is essential first to remove the glycerin with absolute alcohol, then to clear, and finally to mount in balsam.

Born and **Wieger**[2] introduced quince-jelly for fixing paraffin sections to slides. They used quince-jelly of the German Pharmacopoeia 2 parts, and glycerin 1 part. It owes its adhesive properties to a gum-like substance called bassorin.

Frenzel[3] used to fix sections with gum arabic prepared in this way: To a dilute watery solution of gum a watery solution of chrome alum and a little glycerin and alcohol were added. Slides were covered with this mixture, and the sections pressed against the gum mixture with a brush. The slide was then heated up to the melting-point of the paraffin, and kept for fifteen minutes, not longer, at a temperature of from 30 to 45° C., to render the gum insoluble.

Gulland, in 1891, floated paraffin sections on warm water (or alcohol); immersed a slide in the water, arranged the sections on the slide, and let the water evaporate spontaneously. If the slides are clean, sections fixed in sublimate will adhere firmly, while bichromate and osmium sections have a tendency to float off when placed in water.

Author's Method of albuminizing Slides.

This fact led me, in 1892, to disbelieve in the theory which attributes the adhesion of the sections to capillary attraction. The

[1] Lee and Mayer: *Grundzüge d. mikr. Technik* (1898), p. 118.
[2] Born and Wieger: *Zeitsch. f. wiss. Mikr.* 2, 346 (1885).
[3] Frenzel: *Arch. f. mikr. Anat.* 25, 51 (1885).

true explanation seems to be, that both bichromate and osmium trioxide render tissues insoluble, while sublimate tissues are readily soluble in water, especially if free salts be present. Applying this theory to practice, the idea suggested itself to me to supply a very thin layer of albumin, which would act as a cement. I therefore diluted the white of one egg (30 cm.) up to 300 cm. with distilled water, shook the mixture vigorously for five minutes, and then filtered the solution twice through the same filter-paper. This weak solution answers well for sublimate sections.

A number of slides are coated on one side with the albumin solution by immersing one slide in the solution, and drawing it over one side of a clean slide. The albuminized slides are then arranged vertically against a ledge, the albuminized surface looking towards the support, to prevent dust settling on the albumin, while the superfluous egg-white is sucked up by the filter-paper on which the slides stand. When dry, the slides are packed into parcels of ten or twenty, and are stored in an incubator to keep them dry.

Method of using Albuminized Slides.

Float the paraffin sections on water heated from 40–45° C., if the melting-point of the paraffin is 52 to 56° C., till they are quite flat; breathe on the albuminized slide, when that side containing the albumin will remain clear; immerse the slide in the water on which the sections float, arrange them on the albuminized side, and gently withdraw the slide. To allow the water to evaporate rapidly place the slide in an incubator heated up to ten degrees below the melting-point of the paraffin, or use a copper table or support heated up by a gas flame as first practised by G. Born (1888).

Instead of floating the sections on water, the following plan may be adopted: Place the glass slide on the warm support, then drop a sufficient quantity of water on the slide to cover it, and lay the sections on the water. As soon as the sections have become quite flat, allow the superfluous water to drop off, and lay the slide again on the warm table. In five to ten minutes, after the paraffin sections have become quite dry, dissolve off the paraffin with xylene while the slide is still warm.

If the sections are stained, a few drops of Canada balsam are placed on them, and the cover-glass is adjusted; if, however, staining is contemplated remove the xylene with alcohol, and the latter with water. Sublimate sections should now for ordinary work be treated with a 1 per cent. of sodium chloride, or with iodine potassium-iodide solution to remove the free sublimate; but it should be borne in mind that these processes also liberate proteids, and particularly albumoses and peptones (see pp. 77, 78, 113).

Method for Class Purposes.

When it is desirable to save time and to make sure that every student has a good section, the following plan will be found useful: Procure from Hollborn and Grübler large sheets of mica cover-

glasses, measuring about 10 × 20 cm., albuminize these on one side, arrange the sections in series, and treat each mica plate as if it were an ordinary slide. After staining, the sections may be dehydrated and cleared collectively by placing the mica plate in a photographic dish and treating it as an ordinary slide, or sections are cut off with a pair of scissors, and are then given to the student as required. Each student dehydrates and clears his own section, and if of an economic mind may use the mica as a cover-glass by mounting the section face lowermost. As, however, the mica is apt to become scratched proceed rather thus: Place a drop of balsam on the slide, and on it the mica with the section uppermost; now put a second drop of balsam on the section, and then finish mounting by applying an ordinary cover-glass.

Method of fixing Bichromate Sections to Slides.

For bichromate sections of the central nervous system which are to be stained by Weigert's or Heller's method, slides should be coated either with a 1 in 5 watery egg-white solution (30 cc. egg-white diluted to 150 cc. with water and filtered), or with a half to 1 per cent. gelatin solution. The paraffin sections are floated on warm 5 per cent. bichromate solution; they are quickly arranged on the slide; the slide is withdrawn and exposed to bright sunshine or daylight, when the albumin or gelatin will be rendered insoluble. Experiments are being made now with gelatin and formaldehyde, which seem promising.

Van Walsem[1] uses a quince-jelly method for bichromate sections, which in his hands is safe. Proceed thus: Slides are thoroughly cleaned by boiling in aqua regia (hydrochloric acid 4 parts, and nitric acid 1 part); they are then rinsed in water; washed in absolute alcohol; dried with a clean linen cloth; immersed in alcohol, and placed at once in water.—Quince kernels 1 part and distilled water 30 parts are shaken vigorously for fifteen minutes, and filtered through a fine linen cloth; the filtrate may be preserved after the addition of a small piece of thymol.—Slides cleaned in the above manner are covered with the quince-jelly; the superfluous jelly is allowed to flow off by placing the slides in an upright position for a short time; the slides are next dried in a dust-free place in a horizontal position, and, after drying, are stored.

Paraffin sections are floated out on warm water; the slides are immersed in the water; the sections are arranged; the water allowed to evaporate; the slides are fixed in absolute alcohol for some hours; the alcohol allowed to evaporate; the paraffin is dissolved, and then the slides are placed once more in absolute alcohol for some hours. They may now be transferred directly to water.

On fixing Celloidin Sections to Slides.

If the tissue was stained before being imbedded in celloidin, the sections need only be arranged on the slides in rows; be covered with

[1] Van Walsem: *Verhandl. d. Kon. Akad. van Wetensch. te Amsterdam*, 2. Sect., Dl. 7, No. 1 (1899); and *Zeitsch. f. wiss. Mikr.* 17, 227 (1900).

a drop of origanum oil or any similar substance which does not dissolve celloidin, and, when quite clear, be mounted in balsam.

If the sections have to be stained in basic anilin dyes, which have a great affinity for the acid celloidin, the celloidin has to be removed before the stain is applied, and to fix the sections to the slide the following plans may be tried:

Bolles Lee[1] recommends P. Mayer's glycerin-egg-white (p. 372). Having pressed celloidin sections very firmly to the slide covered with glycerin-egg-white, the celloidin may 'subsequently be safely removed with alcohol and ether.' I have not been successful with this method.

Jordan's procedure is more reliable[2]. Slides are covered with egg-white, or P. Mayer's glycerin-egg-white; celloidin sections are transferred to the slide from 80 to 96 per cent. alcohol, but on no condition from water, and are pressed down by means of the tissue-paper which served to transfer them; the tissue-paper is left covering the sections to prevent them from becoming dry, and a second slide is pressed down on the paper; pressing both slides firmly together the albumin is coagulated by holding the slide containing the egg-white lowermost, over a flame; having coagulated the albumin the two slides are transferred together to 96 per cent. spirit, and are separated from one another under spirit. Now the celloidin may be removed with a mixture of alcohol and ether, or acetic ether or clove oil.

In some instances it is desirable to keep a number of celloidin sections in series without having them attached to glass slides, when Obregia's modification of Weigert's original method answers best.

Weigert's Celloidin Section Method.

Weigert[3] covers a plate with a thin layer of celloidin, and allows it to dry. He further cuts toilet-paper into strips a little wider than the breadth of the celloidin sections, and soaks these strips in 70 per cent. spirit. They are kept moist by being placed on blotting-paper in a flat dish also containing 70 per cent. spirit.

As soon as a section is cut it is drawn off the knife by means of one of the papers, the first section being placed close to the left hand of the operator. Each successive section occupies a position nearer to the right hand holding the strip of paper. When the first strip is covered with sections, a second one is taken, and this process is continued till a sufficient number of sections have been cut to cover the collodionized glass plate. During the whole of this time care is taken to prevent the sections from drying by putting them on filter-paper moistened with spirit.

To transfer the sections to the glass plate, the strips are laid down in order, with the sections in contact with the collodium; the paper is firmly squeezed down to make the sections adhere to the collo-

[1] Bolles Lee and Paul Mayer: *Grundzüge d. mikr. Technik*, 132 (1901).
[2] H. Jordan: *Zeitsch. f. wiss. Mikr.* 15, 50 (1898).
[3] Weigert: *ibid.* 2, 490 (1885).

dium, and then the strips of paper are gradually and carefully lifted up so as not to disturb the arrangement of the sections.

Now all free spirit is removed with blotting-paper, and before the sections have become quite dry a thin solution of collodium is poured over the sections; and as soon as this collodium has set and feels dry to the touch the glass plate is transferred, directly, into the staining bath, when the double celloidin film enclosing the sections separates from the glass. After the staining has been completed the celloidin film may be cut with scissors, and either all or only some of the sections be kept. Dehydrate the sections in 95 per cent. spirit, clear in origanum oil, and mount in balsam; or if complete dehydration is essential, follow Nikiforow's plan of using equal parts of absolute alcohol and pure chloroform[1].

Obregia's Modification of Weigert's Method[2].

The disadvantage connected with Weigert's original method is that staining takes a very long time, because the dyes have to act through the collodium which encloses the sections. To obviate this difficulty Obregia covers a glass plate not with a layer of collodium, but with a mixture of cane-sugar syrup 3 parts, dextrin syrup 1 part, and 95 per cent. alcohol 2 parts. After two days, when this solution has become dry, the glass plates are used in exactly the same way as are Weigert's collodionized plates. On placing the glass plates in warm water the sugar dissolves, and the celloidin film floats off. The sections stain readily, as they are covered only on one side by a film of celloidin.

On Mounting.

Mounting aims at surrounding tissues with any medium which will show the structural elements to their greatest advantage, and also, if possible, preserve them for future reinvestigation. Further to safeguard the specimens and microscopic lenses against injury, it is the custom to cover tissues with some transparent medium, either a photographic varnish as first recommended by Weigert for large brain-sections, or with mica or glass.

Mounting media should be chosen according to their refractive indices. If the tissues are of low refractive index, choose media with higher indices, and vice versa. Some elements of high refractive index are converted into, apparently, lowly refracting ones, by being mounted in substances possessing exceedingly high indices—such a medium being, for example, methylene biniodide.

The following table[3] gives some of the more common media and their indices, calculated for yellow light or D at 15 to 20° C.:

[1] Nikiforow: *Zeitsch. f. wiss. Mikr.* 8, 189 (1891).

[2] Obregia: *Neurol. Centralbl.* 9, 295 (1890).

[3] With the exception of the three last substances, the refractive indices have been compiled from Behrens, *Tabellen z. Gebrauch f. mikr. Arbeiten*, Braunschweig, 1898.

Refractive Indices of Mounting Media.

Air	1·000	Xylene	1·497
Methyl-alcohol	1·330	Benzene	1·501
Distilled water	1·333	Cedar-wood oil (ordinary)	1·510
Sugar solution, 10 per cent.	1·347	Cedar-wood oil (inspissated)	1·520
Sodium chloride, 10 per cent.	1·350	Dammar	1·520
Albumin solution [1].	1·350	Clove oil	1·533
Ether	1·357	Mastic	1·535
Absolute ethyl-alcohol	1·361	Canada balsam	1·535
Potassium acetate, saturated watery solution	1·370	Creosote	1·538
Glycerin and water, equal parts	1·397	Colophonium	1·545
Amyl-alcohol	1·407	Phenol	1·549
Chloroform	1·449	Aniseed oil	1·557
Glycerin	1·456	Anilin	1·580
Bergamot oil	1·464	Cinnamon oil	1·619
Liquid paraffin	1·471	[2] Metacinnamene + equal weight of phenyl-thiocarbimide	1·639
Turpentine	1·472	[2] Phenyl-thiocarbimide (C_6H_5CNS)	1·654
Toluene	1·496	[2] Methylene diiodide (CH_2I_2)	1·743

Personally I prefer to mount in media having a low refractive index, namely in potassium acetate, 10 per cent. glycerin made with thymol water, liquid paraffin, bergamot oil + Canada balsam, and turpentine dammar; but when doing so I am aware that the lenses in ordinary use have been corrected approximately for a refractive index of 1·520, which means that the index of ordinary balsam mounts is too high, while that of glycerin and water is too low.

To ensure permanency of stains, especially if coal-tar products are used, it is absolutely necessary to employ neutral mounting media, which remain neutral. For this reason all substances prone to undergo oxidation must be avoided, firstly because acting as reducers they convert colour salts into leuco-products, as Unna [3] has shown, and, secondly, because having become oxidized they turn into acid bodies, and as such extract the basic dyes.

Thus toluene (toluol) or methyl-benzene ($C_6H_5CH_3$) by oxidation is converted into benzoic acid ($C_6H_5CH_3 + O_3 = C_6H_5COOH + H_2O$); xylene (xylol) or ortho-dimethyl-benzene ($C_6H_4(CH_3)_2$) by oxidation gives rise to orthotoluyl acid ($C_6H_4 + CH_3 + COOH$, 1:2) and ortho-phthalic acid ($C_6H_4 + (COOH)_2$, 1:2).

Turpentine is a mixture of resins (colophonium) and liquids. The latter, called oil of turpentine, consist essentially of the hydrocarbon pinene ($C_{10}H_{16}$), which by oxidation gives rise to benzene-*p*-dicarboxylic acid ($C_6H_4(CO{\cdot}OH)_2$), and also to terpenylic and terebic acids.

Turpentine is even less to be recommended for anilin colours than toluene or xylene, while for sections stained in carmin it may be used (p. 380).

How enormous the oxidation processes going on in ordinary

[1] Albumin solution consists of egg-white 10 cc., distilled water 100 cc., sodium chloride 0·5 grm.

[2] These three substances were kindly suggested to me, and given me by H. G. Madden.

[3] P. G. Unna: 'Die Entwickelung d. Bakterienfärbung,' *Centralbl. f. Bakter. u. Parasitenkunde*, p. 3 (1888).

balsam are, becomes apparent from the experiments of Zoth[1], who determined that under normal atmospheric pressure 20 cc. of new fluid turpentine will absorb in ten days 40 cc. of gas, the rate of absorption during the first few days being about 0·4 cc. in one hour. Similarly 30 cc. of xylene absorbed in ten days 11 cc. of gas.

The thinner a balsam solution, the more rapid is the absorption of air-bubbles in a preparation, or, what amounts to the same, the greater is the process of oxidation. By stirring air-bubbles into balsam and controlling their disappearance under the microscope, Zoth obtained the following figures, which express in minutes the time bubbles took to disappear:

Dammar dissolved in xylene (xylol).	*Diameter of air-bubbles, expressed in μ, and time they took to disappear, expressed in minutes.*			
	90	83	54	36
very thin	1¾	1½	½	¼
50 per cent.	6	4½	1½	¾
70 per cent.	—	—	2¼	1
80 per cent.	—	—	5½	2
Canada balsam dissolved in turpentine.				
very thin	5	3½	1	¼
thin	7½	5¾	1¾	¾
thick	—	17½	5½	2½
semi-solid (83 per cent.)	98	92	34	9

Commercial balsam is always acid because of oxidation processes which have taken place in it, and therefore 'neutral' balsam, as specially prepared by such firms as Hollborn and Grübler, Leipzig, or by P. Ferman, Amsterdam, should be employed[2]. My own attempts at neutralization with fixed alkalies and with pyridin or picolin have not given such uniform results as to warrant publication.

To use chloroform ($CHCl_3$) as a solvent is perhaps also bad, because of a possible oxidation by the action of light into carbonyl chloride, or phosgene gas and hydrochloric acid, thus: $CHCl_3 + O = COCl_2 + HCl$.

The best solvent for neutral balsam is pure benzene (C_6H_6), because it is exceedingly stable towards concentrated alkalies, and such strong oxidizers as potassium permanganate and chromic acid. When exposed, however, to ozone in the presence of sunlight it is converted first into phenol (C_6H_5OH), and later into oxalic, acetic, and formic acids, while peroxide of hydrogen gives rise to phenol and oxalic acid. Concentrated nitric acid converts the hydrocarbon into nitrobenzene ($C_6H_5 \cdot NO_2$), and concentrated sulphuric acid slowly

[1] O. Zoth: 'Notiz ü. d. Aufsaugung v. Luftbläschen in Harzeinschlüssen,' *Zeitsch. f. wiss. Mikr.* 15, 192 (1898).

[2] P. Ferman's firm is recommended by A. C. Hoff: 'Topik d. Alkalivertheilung,' *Bot. Centralbl.* 83 (1900).

changes it into benzene-sulphonic acid ($C_6H_5 \cdot SO_3H$). With chlorine and bromine in the presence of direct sunlight or at high temperatures, it forms additive compounds such as benzene hexachloride ($C_6H_6Cl_6$), while at the ordinary temperature the benzene nucleus is attacked, yielding substitution products such as chlorobenzene (C_6H_5Cl), dichlorobenzene ($C_6H_4Cl_2$), &c.[1]

The '90 per cent. benzol' of commerce contains about 70 per cent., while the '50 per cent. benzol' contains about 46 per cent. of pure benzene. The remainder (about 25 per cent.) in the '90 per cent. benzol' is toluol, while in '50 per cent. benzol' there is contained 50 per cent. of xylol. For reasons given above (see p. 377) it is essential to use pure benzene and not a mixture of benzene and toluene or xylene.

Pure benzene is usually sold as 'benzene free from thiophene.' To prepare pure benzene from so-called 90 per cent. benzene proceed as follows: Cool water by dissolving ice in it to almost the freezing-point; place a vessel containing the impure benzene in this mixture, when the benzene crystallizes out while the toluene remains fluid; remove the toluene or xylene by rapid filtration and repeat the freezing process several times; finally, shake up the benzene purified in this manner repeatedly with small quantities of sulphuric acid to remove the thiophene, which is a sulphur compound, C_4H_4S. To determine whether the benzene is really pure, use the indophenin reaction by shaking it up with a little sulphuric acid and adding a trace of isatin, a derivative of indigo, when, in the presence of thiophene, the acid will turn a beautiful blue colour.

The chief disadvantage connected with the use of benzene is its high refractive index, but this may be overcome by using dammar resin instead of Canada balsam, and also by choosing stains which have a considerable covering power.

Experiments are now in progress with various petroleum ethers or benzines as solvents for balsam, and the results obtained so far seem to be satisfactory.

For ordinary use a resinous mounting medium should be of the consistency of syrup, and more should not be used than is just sufficient to cover the space under the cover-glass. Excess of balsam is best removed by placing a piece of thick blotting-paper on the slide, and by gently pressing it down with the tip of a finger, or by placing on the filter-paper metal weights. These weights are best made by cutting off pieces of a thick lead pipe. To evaporate the solvent of the balsam as quickly as possible keep the preparations for twenty-four hours at a temperature of 30 to 35° C. If the balsam is acid the basic dyes will be discharged.

The greatest enemy to the permanency of all stains is moisture. Having taken every care to exclude water vapour, we still run the risk of seeing some preparations out of a series fade much quicker than others. The probable cause of this fading is that, in holding the slide or cover-glass, the moisture of our hand or breath

[1] Perkin and Kipping: *Organic Chemistry* (1900), p. 303.

was condensed on the preparation or balsam. To avoid such occurrences the cover-glass should be held in a pair of forceps, and care be taken not to breathe on the preparation while dehydrating, clearing, and mounting it.

The special methods required for mounting Golgi preparations are given on p. 273, and there the use of solid balsam has been described; but the latter is also used with a different object in view, namely, not so much to prevent deterioration of the specimen as to mount sections in some medium which will not enter the preparation, and which cannot dispel the air present in the tissue.

Thus preparations of hard bone and teeth show the lacunae, canaliculi, and dentinal tubules plainly as long as these are filled with air. If the tissues were mounted in fluid balsam, all the air-spaces would be filled in the course of twenty-four hours with the balsam, for we have seen how balsam absorbs air (p. 378), and the preparations would thereby be rendered worthless, as now the light is not reflected from those elements which previously enclosed air. Should preparations accidentally become filled with balsam, then boil the sections in xylene and ether to remove all traces of the resin, and remount.

Bone and teeth sections, as well as blood-films, scales, and hairs, make very instructive preparations if they are mounted in air. The tissues are placed on a slide and covered with a clean cover-glass, or the blood-film on a cover-glass is mounted face downwards. To secure the cover-glass, either a ring of Krönig's cement may be applied, or a microscopic label is folded twice, and the corner corresponding to the centre of the label is cut off with a pair of scissors; the label is then unfolded, wetted, and placed over the cover-glass. Care must be taken not to wet the label too much, as otherwise moisture runs in between the cover and the slide, and may ruin the blood-film.

Watery mounting media containing alcohol cannot be used for permanent preparations, as no cement can withstand the action of alcohol; but the alcoholic balsam medium of Seiler and Vosseler can be recommended for carmin sections, especially for class purposes, as such sections need not be completely dehydrated. Carmin preparations, so Paul Mayer has told me, are quite permanent. Haematein and coal-tar colours cannot, however, be preserved in these solutions.

Seiler[1], in 1881, recommended dissolving dry balsam in pure absolute alcohol, and filtering the solution through absorbent cotton. Vosseler[2] mixes equal volumes of 96 per cent. alcohol and Venetian turpentine (which is obtained from *Pinus larix*), and after some weeks decants the upper clear portion. The presence of the alcohol ensures a low refractive index, which has certain advantages.

Watery mounting media may be divided into two classes, namely, those which ultimately retain the water, and those which do not.

[1] Seiler: *Proc. Amer. Soc. Micr.* 2, 50 (1881).
[2] Vosseler: *Zeitsch. f. wiss. Mikr.* 6, 292 (1889).

1. **Water** itself is a good medium for preserving tissues which have been fixed in osmium tetroxide and formaldehyde, or picric acid (provided this acid is not washed out completely) and mineral acids, but it should not be used for sublimate preparations as they become granular. To prevent the growth of bacteria in the preparations distilled water is boiled, and while hot some camphor is added to it.

2. **Potassium acetate** in the form of a saturated watery solution was introduced by Max Schultze[1]. I use it principally for osmium preparations.

3. **Ten per cent. glycerin** in thymol water for delicate, fresh tissues stained in picro-carmin.

4. **Glycerin jelly,** according to Lee and Mayer, was introduced in 1862 by Schacht[2].

Squire's formula is especially to be recommended, as by the coagulation of the egg-white the impurities are all removed. Proceed thus: Soak 100 grms. of gelatin in chloroform water for three hours, and when quite soft drain off the water which has not been absorbed; melt the gelatin on a water-bath; add 750 cc. of glycerin; mix the gelatin well with the glycerin, keeping the mixture at a temperature of about 70° to 80°; then allow it to cool to about 50° C. Now dissolve the white of two eggs (50–60 cc.) in 400 cc. of chloroform water, add this solution to the gelatin-glycerin, and mix well. Finally, boil the jelly for five minutes to coagulate the albumin; add sufficient chloroform water to raise the bulk of the fluid to 1,550 cc., and filter while hot through new flannel.

Before use melt the glycerin jelly, put one or two drops on the section and cover with a cover-glass. After the gelatin jelly has set, remove the superfluous mounting medium if there should be any; clean off all traces of glycerin from the cover-glass and the slide, and either apply a ring of balsam or Krönig's cement.

Lee points out[3] the importance of thoroughly soaking the tissues for some time in that strength of glycerin in which they are to be mounted ultimately, as otherwise the tissues may swell up and exert such force as to overcome any cement. Paul Mayer finds that a small air-bubble under the cover-glass is the best means of counteracting the injurious influences of changes in temperature, and, as the primary aim of histologists is not to turn out show specimens, the plan is to be recommended on theoretical grounds.

5. **Farrants' solution:** Suspend in 150 cc. of distilled water 40 grms. of white gum arabic in a muslin bag. When the gum has dissolved, mix the gum solution thoroughly with the white of one egg; boil; filter through a double layer of muslin; inspissate to 80 cc.; add 1 grm. of crystalline carbolic acid and 20 cc. of glycerin.

6. **Brun's mixture** was especially recommended by Henneguy to Lee, as it is said not to extract anilin dyes from sections. This

[1] M. Schultze: *Arch. f. mikr. Anat.* 7, 184 (1872).
[2] Schacht: *Das Mikroskop*, 281, Berlin, 1862.
[3] See also Rousselet: *Journal Quecket Micr. Club* (2), 7, 93 and 129 (1898).

mixture is composed[1] of distilled water 14 parts, grape-sugar 4 parts, camphorated spirit 1 part, and glycerin 1 part. Filter off the camphor which precipitates.

7. **Mounting media for iodine preparations:** Ehrlich uses a thick gum solution containing 1 per cent. of Lugol's solution (p. 223); v. Kahlden[2] employs equal parts of glycerin and Lugol's solution; and Langhaus, after staining in Lugol's solution, dehydrates in tincture of iodine 1 part + absolute alcohol 4 parts, and preserves in origanum oil.—I have used Welch's plan (p. 445) in combination with origanum oil.

On Cements.

Every worker fancies a special cement, and if what different histologists say of their favourites be true, then bad cements do not exist.

Krönig's[3] **cement** for general purposes I like best. Mix two parts of wax with 7 to 9 parts of colophonium, filter while hot into a vessel made of metal. Remelt before use.

Amberlack is made by E. Pfannenschmidt in Danzig, and may be obtained from Grübler and Hollborn. It is recommended by Behrens because of its great toughness, which prevents it from becoming brittle.

Tolu balsam cement of Carnoy[4] is composed of balsam of tolu, 2 parts; Canada balsam, 1 part; saturated solution of shellac in chloroform, 2 parts. Dilute to the consistency of syrup with chloroform.

Bell's cement, to be obtained from J. Bell and Co., 338 Oxford Street, London, is considered by Lee to be the best for watery media. It is soluble in ether and chloroform, and is not attacked by cedar-wood oil.

Mask cement (Maskenlack) No. 3, to be obtained from Beseler, 66 Schützenstrasse, Berlin, first recommended by Schacht in 1862, is still recommended by Paul Mayer.

Asphalt cement, obtained from Grübler and Hollborn, is reliable, and may be used either by itself or mixed with a little gold-size (Kitton[5]).

Gold-size, according to Lee and Mayer, is essentially inspissated linseed oil, and is soluble in turpentine. It is sold in good quality by Grübler and Hollborn. The object of these various cements is to prevent watery mounting media from drying up, or glycerin mounts from absorbing water.

[1] Lee and Mayer: *Grundzüge d. mikr. Technik*, 249 (1901).
[2] v. Kahlden: *Technik d. histol. Untersuchung* (1895), p. 52.
[3] Krönig: *Arch. f. mikr. Anat.* 27, 657 (1886).
[4] Carnoy: *Biol. cell.* 129.
[5] Kitton: *Month. Micr. Journ. London*, 11, 34 (1874).

APPENDIX.

THE CHEMISTRY OF DYES.

The general nature of dyes is discussed in chapter xiv, pp. 180–190.

The following account is based essentially on R. Nietzki's *Chemie der organischen Farbstoffe* (Julius Springer, Berlin); on Victor Meyer's and Paul Jacobson's *Lehrbuch der organischen Chemie* (Veit & Co., Leipzig), and Schultz and Julius (*Tabell. Übersicht d. künstl. organ. Farbstoffe*).

Dyes may be classified as follows:

A. Simple dyes.

I. Artificial dyes:

(A) The nitro group (picric acid) . . . . . p. 383
(B) The azo group (methyl-orange, Bismarck-brown) . . . p. 385
(C) The hydrazon and pyrazon groups . . . . . p. 395
(D) The azo-methin group (rubifuscin) . . . . . p. 396
(E) The stilbene group (sun-yellow) . . . . . p. 397
(F) 1. The oxyquinones (alizarin) and . . . . . } p. 397
2. Quinone-oximes (fast green) . . . . . }
(G) The quinone-imide group (toluene-blue, methylene-blue, Nile-blue, safranin, neutral red, indulin) . . . . . p. 401
(H) Phenyl-methane group (auramin, malachite-green, fuchsin, methyl-violet, methyl-green, methyl-blue, eosin, erythrosin) p. 417
(I) Quinolin (cyanin-blue) and acridin group (benzo-flavin) . . p. 431
(K) Sulphide group (cachou de Laval, Vidal-black) . . . p. 434

II. Natural dyes:

(*a*) Flavones . . . . . p. 435
(*b*) Pyrocatechin group (haematoxylin, brazilin) . . . . p. 436
(*c*) Carmin . . . . . p. 438
(*d*) Orcein and litmus . . . . . p. 441
(*e*) Alcannin . . . . . p. 441

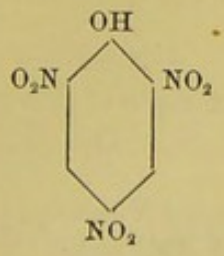
B. Compound dyes. . . . . p. 441

I. Artificial Dyes.

(A) The Nitro group.

The chromophore radical is NO_2, with an acid character.

Trinitro-phenol or **Picric acid**, $C_6H_2(NO_2)3OH$.



Benzene hydroxyl (1), nitrogen peroxide (2, 4, 6). It is formed by the action of nitric acid on phenol and many other organic substances.

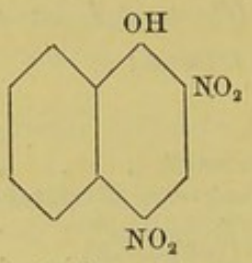
Its ammonium salt, or ammonium picrate, has the formula

$$C_6H_2(NO_2)3ONH_3,$$

and is best made as follows: Add ammonia to a 1 per cent. solution of picric acid at 25° C., till free ammonia can be detected by the smell; cool the fluid to 10° C.; filter off the crystals of ammonium picrate, and place them in a porcelain dish just covered with distilled water, to allow the free ammonia to evaporate. Then transfer the moist crystals to a bottle, cover them with distilled water, and saturate the latter by repeated shaking. It is best to preserve the crystals in a moist state, because I once had an explosion, drying crystals on blotting-paper in the sun.

The potash salt of picric acid is very insoluble.

Dinitro-α-naphthol or **Martius' yellow**, $C_{10}H_5(NO_2)_2OH$,



is obtained by the action of nitric acid on α-naphthol disulphonic acid. (The constitution of naphthalene is given on p. 388.) It is almost insoluble in water, and only slightly soluble in alcohol, ether and benzene. Its sodium salt is readily soluble in water, and stains wool and silk in an acid bath a golden-orange colour. It does not possess the bitter taste of picric acid.

Tetranitro-diphenol,

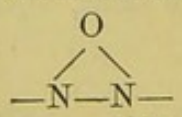
$$\begin{matrix}(NO_2)_2\\OH\end{matrix}\!\!\rangle C_6H_2-C_6H_2\langle\!\!\begin{matrix}(NO_2)_2\\OH\end{matrix}$$

is sold as an ammonia salt under the name of Palatine-orange.

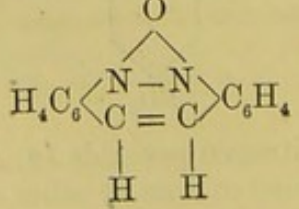
Aurantia or **Hexanitro-diphenylamin** or **amin-dipicric acid** is sold in the form of its ammonia salt. Its deep orange colour, as contrasted with the pale yellow colour of picric acid, is due to the further introduction of the chromophore group NO_2:

$$NH_4-N\begin{matrix}C_6H_2(NO_2)_3\\C_6H_2(NO_2)_3\end{matrix}$$

In this class of nitro bodies we may also place the nitroxy bodies, which, according to Lefêvre, possess the chromophore group:



Sun-yellow is the best example of this group:



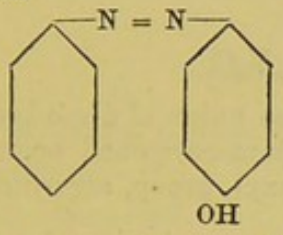
(B) The Azo-dyes.

The chromophore radical is the azo group $-N=N-$ with basic characters. It forms in every case the link between benzene rings or benzene and naphthalene groups, or naphthalene groups, thus

$C_6H_5-N=N-C_6H_4OH$ = (oxy-azo-benzene).
$C_6H_5-N=N-C_{10}H_6OH$ = (benzene-azo-α-naphthol).
$C_{10}H_7-N=N-C_{10}H_6NH_2$ = (amido-azo-naphthalene).

Whenever possible, the azo group takes up a paraposition to the hydroxyl (OH) or amidogen group (NH_2) of the benzene or naphthalene-rings. Should, however, the paraposition be occupied, then the ortho-position is taken up, but never the metaposition.

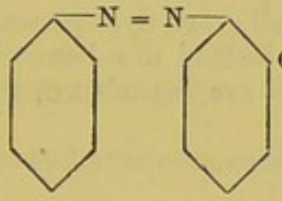
Now, whenever the azo group $-N=N-$ occupies the paraposition to the hydroxyl group of a benzene radical, the resulting dye behaves as a phenol,



(The OH occupies the paraposition)

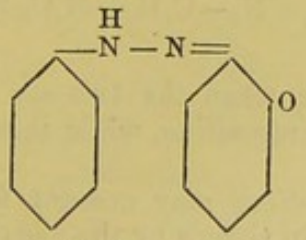
Phenol, in a watery solution, dissociates thus, $C_6H_5O' + H^*$, the hydrogen ion giving an acid character to the dissociated phenol solution. On displacing the H^* by stronger kat-ions such as sodium, salts of phenol are formed. Azo-dyes with the hydroxyl and the azo groups in a paraposition also liberate the hydrogen ion, and can therefore form salts.

If, however, the azo group and the hydroxyl group occupy the ortho-position



(The OH occupies the orthoposition)

then the hydrogen leaves the oxygen and attaches itself to the azo group, thus:



Such a compound has no acid characters, and does not form salts, because the hydrogen is linked on to N.

To sum up, para-azo-dyes can form salts, while ortho-azo-dyes cannot; the latter must therefore be considered as oxides. The importance of this is shown on p. 309, where the staining of fat by ortho-azo-compounds is discussed.

Nietzki has drawn special attention to the tautomery of azo-dyes with the hydrazones of the quinones and quinone-imides, thus :

$C_6H_5-N=N-C_6H_4OH$	$C_6H_5-\overset{H}{\underset{\vert}{N}}-N=C_6H_4=O$
oxy-azo-benzene	benzoquinone = phenyl-hydrazone.
$C_6H_5-N=N-C_6H_4NH_2$	$C_6H_5-\overset{H}{\underset{\vert}{N}}-N=C_6H_4=NH$
amido-azo-benzene	phenyl-hydrazone of quinone-imide.

Whenever phenyl-hydrazones are formed out of phenyl-hydrazin and quinones, the resulting dyes are identical with those obtained from the corresponding phenol and diazo-benzene; thus α-naphthoquinone and phenyl-hydrazin form the hydrazone

$$C_6H_5-\overset{H}{\underset{\vert}{N}}-H=C_{10}H_6O$$

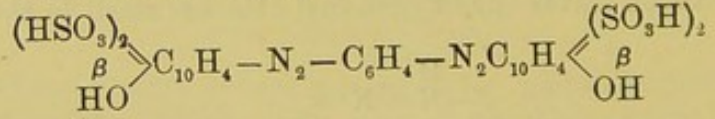
but the same substance is also formed by the union of diazo-benzene and α-naphthol, although the latter being a para-compound ought to give rise to a substance containing the hydroxyl group, according to the formula $C_6H_5-N=N-C_{10}H_6OH$.

By vigorous reduction the azo-group is convertible into amido groups because the double nitrogen bond is broken up, hydrolysis taking place[1]. Amido-azo-benzene is thus split up into one molecule of anilin and one molecule of paraphenylene-diamin :

$$H_5C_6-N=N-C_6H_4NH_2+4\,H=H_5C_6NH_2+H_2N-C_6H_4-NH_2.$$

As regards colour, the simplest dyes, namely those which contain only the azo group $-N=N-$ once, are yellow ; all green dyes possess a nitro group ; the dyes containing one naphthalene instead of a benzene radical are red, while if several naphthalene groups are introduced, the colour becomes blue.

How the relative position of the chromophore groups influences colour is well shown by the following dye :



which is of a blue colour when the two azo groups in the central benzene ring occupy the paraposition, while it is red if they occupy the metaposition.

Analogous changes I believe may account for some of the metachromatic effects produced in tissues by the use of simple dyes, although in most cases we are dealing with different sized aggregates of the dye.

Mono-amido-azo colours do not stain readily, being feeble bases, and therefore it is customary to either introduce into the nucleus a second amido group in the orthoposition, as is done for example in chrysoidin,

[1] This reaction is employed to determine the composition of azo-dyes.

$H_5C_6-N=N-C_6H_3(NH_2)_2$, or to convert the azo-dyes into sulphonic acid compounds.

Most azo-dyes are such amido-sulpho acids, and behave as acid dyes, the acid sulphonic group serving to satisfy the basic demands of the tissue, and thereby forming the link between the tissue and the free azo-dye. For this reason azo-dyes stain a tissue always with the colour of the alkali salt, or that of the free amido-base. If for example the violet-coloured solution of the sulphonic acid of phenyl-amido-azo-benzene or tropaeolin OO be employed, an orange stain is produced with the tissue, as the sodium salt of tropaeolin OO has an orange colour.

Azo-dyes are used for dyeing purposes in one of the three following ways:

(1) by staining directly (substantively).
(2) after mordanting tissues with metallic oxide (adjectively).
(3) by being produced in the tissues (synthetically).

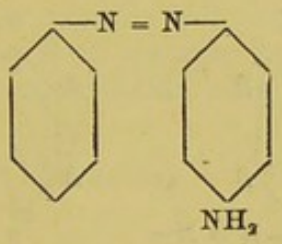
Subdivision of the azo-dyes.

I. The azo group $-N=N-$ is only contained in the molecule once.

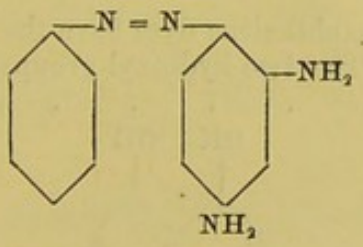
Monazo-dyes.

A. Amido compounds.

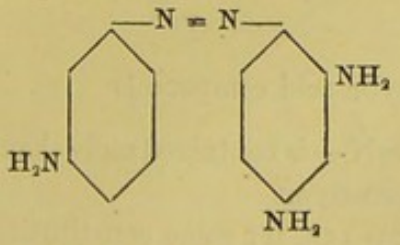
(1) Mono-amido compounds . . . . . p. 389



(2) Diamido compounds . . . . . p. 390



(3) Triamido compounds . . . . . p. 390

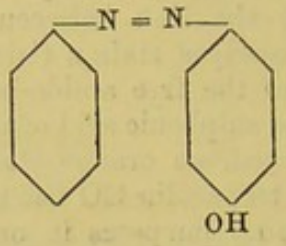


(4) Azo-dyes obtained from diazo-ammonium bases . p. 390

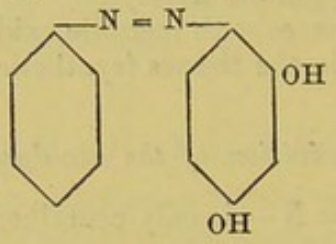
B. Hydroxyl compounds.

(*a*) Benzene series.

(1) Monoxy-azo compounds . . . . . p. 391

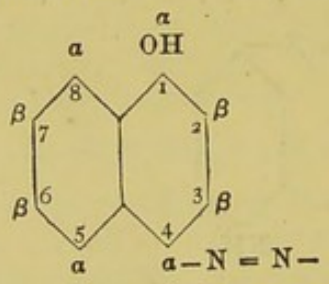


(2) Dioxy-azo compounds . . . . . p. 391

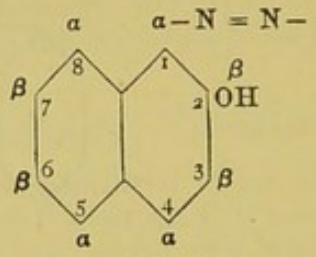


(*b*) Naphthalene series . . . . . p. 391

(1) Monoxy-azo-dyes . . . . . p. 392

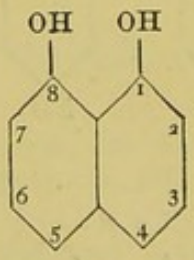


α-naphthol



β-naphthol

(2) Dioxy-azo-naphthalene dyes have the azo group replaced by a hydroxyl group, OH. The two hydroxyl groups occupy the periposition (1 : 8). . . . . p. 393



(*c*) Hydroxy-carboxylic acid compounds . . . p. 394

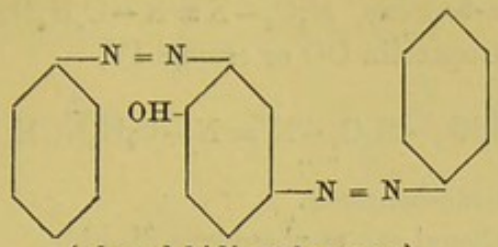
II. The azo group —N=N— is contained more than once in the molecule.

The tetrazo- or disazo-dyes.

See Addendum, pp. 453–455, for some constitutional formulae.

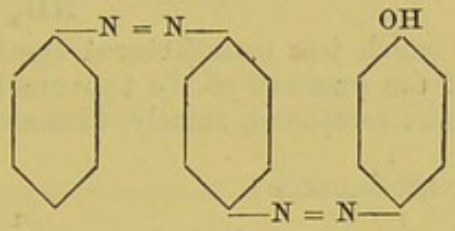
A. The amido and hydroxyl compounds . . . . p. 394

(1) The two azo groups as well as the auxochrome OH or NH_2 groups are joined to the same benzene nucleus.



(phenol-bidiazo-benzene).

(2) The two azo groups are joined to one benzene nucleus, while the auxochrome group is attached to another benzene nucleus.



(benzene-azobenzene-azophenol
or tetrazo-benzene-phenol).

B. The benzidin and analogous derivatives. Benzidin or para-diamido-diphenyl has the composition $NH_2 \cdot C_6H_4 \cdot C_6H_4 \cdot NH_2$, and possesses a strongly basic character. It is obtained by treating hydrazo-benzene $C_6H_5 \cdot NH \cdot NH \cdot C_6H_5$ with strong acids, and thus producing an intra-molecular change . . . . . p. 395

C. The diazo-α-naphthol derivatives . . . . . p. 395

Azo-dyes of histological importance[1].

I, A, 1 (see p. 387). Mono-amido-azo-benzene

$$H_5C_6-N=\overset{4}{\overline{N}}-C_6H_4\overset{1}{\overline{NH_2}}.$$

Methyl-orange.

Dimethyl-amido-azo-benzene, $H_5C_6-N=\overset{1}{\overline{N}}-C_6H_4\overset{4}{\overline{N}}(CH_3)_2$, on sulphonation forms helianthin: $\overset{1}{\overline{HSO_3}}-H_4C_6-\overset{4}{\overline{N}}=\overset{1}{\overline{N}}-C_6H_4-\overset{4}{\overline{N}}(CH_3)_2$, and the sodium salt of the latter is known as methyl-orange.

Methyl-orange (or tropaeolin D, or orange III) is used chiefly as an indicator for mineral acids, which colour it red, while acetic or amido-sulphonic acids produce no effect. It also serves for titrating free sulphurous acid as the bisulphite. Chloride of lime ($CaCl_2$) forms insoluble lime salts with the watery solutions of the alkali salts of the monosulphonic acids.

[1] See also Addendum, pp. 453–455.

Tropaeolin OO.

Phenyl-amido-azo-benzene, $H_5C_6-N=\overset{1}{\overline{N}}-C_6H_4\overset{4}{\overline{N}}HC_6H_5$, on being sulphonated forms tropaeolin OO or orange IV,

$$-\overset{1}{\overline{H}}SO_3-H_4C_6-N=N-C_6H_4\overset{4}{\overline{N}}CH_6H_5.$$

I, A, 2. Diamido-azo-benzenes.

Chrysoidin or phenyl-azo-*m*-phenylene-diamide,

$$H_5C_6-N=\overset{4}{\overline{N}}-C_6H_3\begin{cases}\overset{1}{\overline{N}}H_2\\ \overset{3}{\overline{N}}H_2\end{cases}$$

is the first azo-dye which was manufactured synthetically by Witt. It is a base, because of the presence of the two amido groups. This also holds good for the next compound, namely, Bismarck-brown.

I, A, 3. Triamido-azo-benzene

$$[H_2\overset{1}{\overline{N}}-H_4C_6-\overset{3}{\overline{N}}=\overset{4}{\overline{N}}-C_6H_3\begin{cases}\overset{1}{\overline{N}}H_2]\\ \overset{3}{\overline{N}}H_2\end{cases}$$

usually known as phenylene-brown, Vesuvin, or Bismarck-brown.

Bismarck-brown. The commercial salt is generally the hydrochloride, and consists, according to Nietzki, essentially of the disazo-body:

$$H_4C_6\begin{cases}N=N-C_6H_3(NH_2)_2\\ N=N-C_6H_3(NH_2)_2\end{cases}.$$

According to Täuber and Walder[1] it consists, on an average, of 70·5 per cent. phenylene-disazo-*m*-phenylene-diamin and 8 per cent. triamido-benzene, both of which are soluble in benzene, while another base, amounting to 15·4 per cent., is insoluble. The remainder consists of substances like tar, and is insoluble in acids. The phenylene-disazo-*m*-phenylene-diamin and the base insoluble in benzene stain cotton readily, while triamido-benzene shows hardly any affinity for the unmordanted fibre.

It follows that by rights Bismarck-brown ought to be classified amongst the diamido compounds.

I, A, 4. Azo-dyes obtained from diazo-ammonium bases. On diazotizing amido-derivatives of trimethyl-phenyl-ammonium chloride or its analogues, the diazo radical unites with phenols and amines to form strongly basic quaternary ammonium compounds. Thus indoin, an azo-dye obtained from diazotized safranin and β-naphthol, is a blue dye with strong basic character.

The 'Janus dyes' of the dye manufactory at Höchst also come under this heading, some being disazo-dyes of *m*-amido-trimethyl-phenyl-

[1] Täuber and Walder: *Ber. d. deutsch. chem. Gesellsch.* 2897-9 (Oct. 1900).

ammonium chloride, a compound which is joined up on the one side to metatoluidin, and subsequently on the other side with a phenol.

Janus red, formed by the union of the ammonium base with *m*-toluidin and β-naphthol, has this constitution :

$$(CH_3)_3\equiv N-C_6H_5-N=N-C_6H_3(CH_3)_{(1)}-\overset{3}{N}=NC_{10}H_6\overset{\beta}{OH}.$$
$$\qquad\qquad\quad Cl$$

Janus green is described on p. 444, under the heading of compound dyes.

I, B, a, 1. Monoxy-azo-benzene dyes.

Tropaeolin Y.

Oxy-azo-benzene or phenol-diazo-benzene, $H_5C_6-\overset{1}{N}=N-C_6H_4\overset{4}{OH}$,

when sulphonated becomes tropaeolin Y: $\overset{1}{H}SO_3-H_4C_6-\overset{4}{N}=\overset{1}{N}-C_6H_4\overset{4}{OH}$.

I, B, a, 2. Dioxy-azo-benzene, $H_5C_6-N=N-C_6H_3(\overset{1}{OH})(\overset{3}{OH})$.

Tropaeolin O is the para-sulphonic acid of dioxy-azo-benzene :

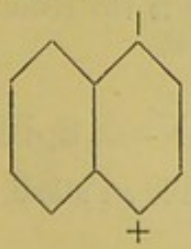
$$\overset{1}{H}SO_3-H_4C_6-\overset{4}{N}=\overset{1}{N}-C_6H_3(\overset{2}{OH})(\overset{4}{OH}).$$

I, B, b, 1. Monoxy-azo-naphthalene dyes, as pointed out above, belong either to the α or β naphthol series, of which commercially the β or ortho-compounds are considered best, because they are not so liable to change their tint under the influence of acids or bases. The α-naphthol dyes are especially subject to colour changes, if the hydroxyl group occupies the paraposition.

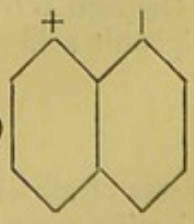
Nearly all naphthalene dyes in the trade are sulphonic acid compounds, being manufactured from the following naphthol sulphonic acids, in which the hydroxyl group is represented by | and the sulphonic acid (SO_3H) by + (Nietzki).

α-naphthol sulphonic acids.

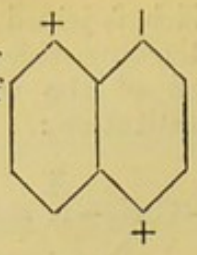
monosulphonic acid of Nevile and Winther (1 : 4)



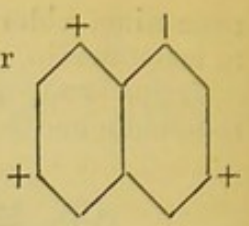
monosulphonic acid of Schöllkopf (1 : 8)



disulphonic acid S or δ-acid of Schöllkopf (1 : 4 : 8)

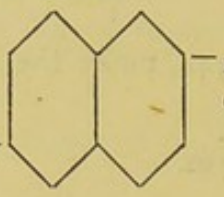


trisulphonic acid for chromotrope (1 : 3 : 6 : 8)

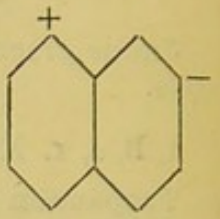


β-naphthol sulphonic acids.

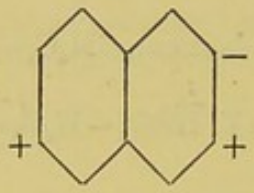
monosulphonic acid β or Schäffer's acid (2 : 6)



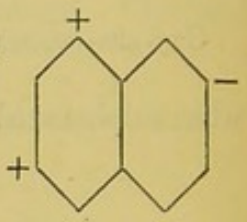
monosulphonic acid α or crocein acid (2 : 8)



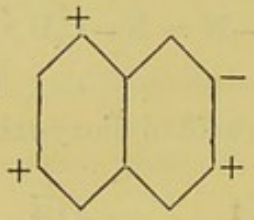
disulphonic acid R (2 : 3 : 6)



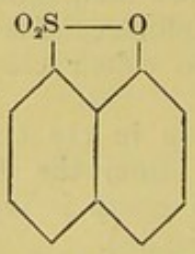
disulphonic acid G (2 : 6 : 8)



trisulphonic acid (2 : 3 : 6 : 8)



Sultones. Those sulphonic acids of α-naphthol containing the hydroxyl and sulphonic groups in the 1 : 8 or periposition, are apt to undergo an intramolecular anhydration, giving rise to substances called sultones. The simplest sultone is that obtained by heating Schöllkopf's acid, and possesses this formula :



I, B, b, 1 (see p. 388). Monoxy-azo-dyes.

Orange I (tropaeolin OOO No. II) is the sodium salt of the monosulphonic acid of benzene-azo-α-naphthol,

$$HS\overset{1}{\overline{O_3}}\cdot C_6H_4-\overset{4}{\overline{N}}=\overset{4}{\overline{N}}-C_{10}H_6\cdot\overset{1}{\overline{OH}}.$$

Excess of alkali turns this salt red, while an alkali has no effect on the following compound :

Orange II (tropaeolin OOO No. I) is the sodium salt of the monosulphonic acid of benzene-azo-β-naphthol:

$$HSO_3^{1}\cdot C_6H_4-N^{4}=N^{1}-C_{10}H_6^{2}\cdot OH.$$

Orange G is the sodium salt of the disulphonic acid of benzene-azo-β-naphthol:

$$C_6H_5-N=N-C_{10}H_4\begin{cases}(HSO_3)_2\\ {}^{2}OH\end{cases}$$

Azo-dyes derived from β-naphthol disulpho-acid and the higher homologues of diazo-benzol, play in the dyeing of wool a very important part. These dyes were introduced by Meister, Lucius, and Brüning, who made use of the fact that isomeric disulpho-acids of β-naphthol in combination with diazo compounds, give rise to dyes varying greatly in their colour. The disulphonic acid G (see p. 392) will thus form orange colours with the diazo-compounds of the benzene series, but scarlet tints with naphthalene derivatives; the disulphonic acid R (see p. 392) with diazo-bodies of the benzene series forms red dyes; with diazo-bodies of the xylenes and the higher homologues of benzene, scarlet dyes (called **Ponceau R, RR, RRR, and G**).

The most important azo-dyes derived from α-naphthalene-azo-β-naphthol or αβ oxyazo-naphthalene,

$$C_{10}H_7-N^{1}=N^{1}-C_{10}H_6^{2}OH,$$ are

Roccelene or Echtroth, which is a monosulphonic acid, staining wool and silk with a bluish-red colour. It is this compound which has superseded carmin for dyeing purposes.

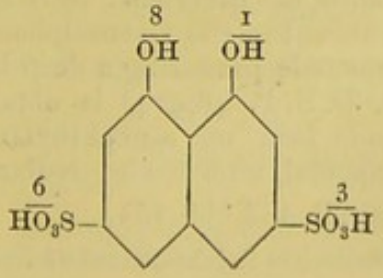
Bordeaux B (Höchst), which is obtained by acting on α-diazo-naphthalene with β-naphthol-disulphonic acid R.

Crystal ponceau 6R (Cassella & Co.), which is obtained by acting on α-diazo-naphthalene with the spirit-soluble G or γ-disulphonic acid, and then joining the resulting acid dye with a sodium salt.

Carmin-naphtha, which is an oxyazo-naphthalene prepared from β-diazo-naphthalene and β-naphthol, and is used for colouring varnishes.

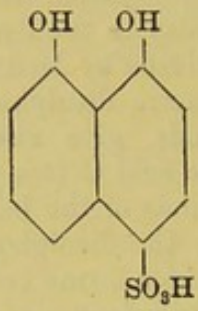
I, B, b, 2 (see p. 388). The dioxy-naphthalene dyes, possessing the two hydroxyl groups in the 1 : 8 or periposition, behave as if the hydroxyl groups were in the orthoposition, for they show a great affinity for metallic mordants. This property is especially well marked in the dyes which have been called chromotropes.

Chromotropes, possessing the radical chromotropic acid, with this formula:



are characterized by having their colour altered by different metallic mordants; thus the dye prepared from diazo-benzene stains wool in an acid bath an eosin-red colour, which by aluminium salts is converted into a violet, and by chrome salts into a deep blue-black. It is usual when using chromotropic dyes to impregnate the material with them in an acid bath, and subsequently by boiling with the mordant, for example potassium bichromate, to produce the desired colour. The ordinary procedure of mordanting is thus reversed.

Azofuchsin is a name given to compounds formed by the union of dioxy-naphthalene-sulphonic acid S:



with diazo-toluols and diazo-benzene-sulphonic acid. Azofuchsin stains wool with a colour very similar to that of acid fuchsin, but is not so good a mordant for metallic oxides as 'chromotrope.'

I, B, c. **The hydroxy-carboxylic acid azo-compounds.**

All dyes belonging to this group show a special affinity for metallic mordants.

Alizarin yellow (Höchst). The paranitro-diazo-benzene in conjunction with salicyclic acid stains tissues mordanted with chrome-compounds an orange colour (alizarin yellow R), while the isomeric meta-combination or alizarin yellow GG produces a bright yellow shade.

Diamant flavin (Bayer & Co. D. R. P.[1] 60,373) belongs also to this group, and has the following constitution (Nietzki):

$$\overset{1}{\overline{HO}}-C_6H_4-\overset{4}{C_6}H_4N=N-C_6H_3-\overset{4}{\overline{OH}}\cdot\overset{3}{\overline{COOH}}.$$

II, A. **The amido and hydroxyl-tetrazo compounds.**

Biebricher Scarlet. Tetrazo-benzene-β-naphthol, or benzene-azo-benzene-azo-β-naphthol $[H_5C_6-N=\overset{4}{\overline{N}}-C_6H_4-\overset{1}{\overline{N}}=\overset{1}{\overline{N}}-C_{10}H_6(\overset{2}{\overline{OH}})]$ forms a disulphonic acid:

$$HSO_3-H_4C_6-N=N-C_6H_3HSO_3-N=\overset{1}{\overline{N}}-C_{10}H_6\overset{2}{\overline{OH}},$$

the sodium salt of which is the Biebricher scarlet. The commercial salt also contains a certain amount of the monosulphonic acid.

Azo-blacks are compounds possessing a deep blue-black colour; thus jet-black (Bayer & Co. D. R. P. 48,924) is obtained by the action of amido-benzene-disulphonic acid on α-naphthylamin, and subsequently combining the new compound, after having rediazotized it, with phenyl-α-naphthylamin (see also pp. 453 and 454).

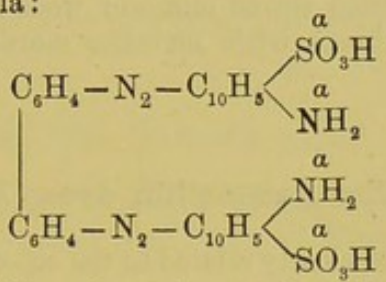
[1] D. R. P. = Deutsches Reichs-Patent or German Patent.

Diamond-black (Bayer & Co. D. R. P. 51,504) is formed similarly by acting on α-naphthylamin with amido-salicylic acid, rediazotizing the resulting compound and then joining it to α-naphthol-sulphonic acid 1 : 5. Because of the presence of the salicyclic acid radical this dye unites readily with metallic mordants.

II, B and C. **Benzidin-tetrazo compounds.**

Benzidin and analogous bases after their conversion into tetrazo-bodies by the action of nitrous acid are combined with phenols and amins to form yellow, red, blue, or violet colours, the alkali salts of which unite directly with cotton, and then become mordants for other basic dyes. The two dyes belonging to this class which are most used in histology are congo-red and benzo-purpurin 4 B.

Congo-red results from the action of tetrazo-diphenyl on naphthionic acid and has this formula:

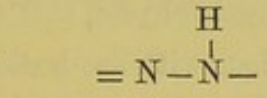


Its sulphonic acids are blue, the salts scarlet-red. Its colour is readily changed by weak acids into blue (see also p. 445).

Benzo-purpurin 4B is a compound of the diazotized base ortho-toluidin with β-naphthylamin-β-sulphonic acid (2 : 6).[1]

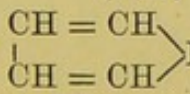
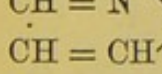
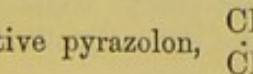
(C, 1) The Hydrazone dyes.

In the introduction to the azo-dyes attention has already been drawn to the tautomery existing between some azo-dyes and hydrazones (see p. 386). The chromophore group has this structure:



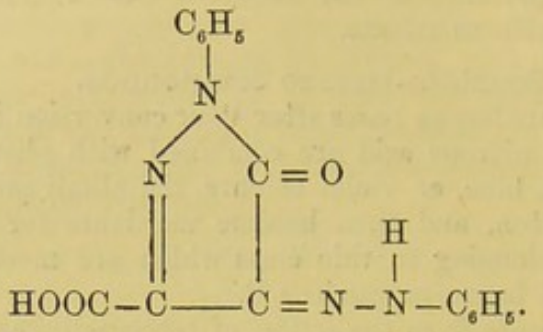
The hydrazones are not used by professional dyers because they do not stain deeply enough; their importance from a micro-chemical point is shown on p. 309, where the staining of fat is discussed.

(C, 2) The Pyrazolon dyes.

Pyrrol,  , turns a chip of pinewood a fiery red colour, and hence its name. One of its substitution products is pyrazol,  , a feeble base which may be converted into the ketonic derivative pyrazolon,  (see also p. 325).

[1] An enumeration of the sulpho-acids of β-naphthylamin will be found in Nietzki's *Chemie d. organ. Farbstoffe*, p. 73 (1901).

Tartrazin is prepared by heating together dioxy-tartaric and phenyl-hydrazin-sulphonic acid, when a closed pyrazolon ring-compound is formed having this constitution:



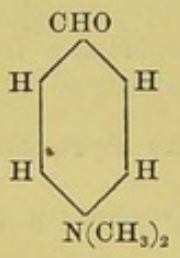
Tartrazin in an acid bath stains silk and wool a golden-yellow colour, and also readily combines with metallic mordants, such as chrome-compounds.

(D) The Azo-methin dyes (*Nietzki*).

These compounds are closely related to the azo-dyes. The chromophore of the latter, $-N=N-$, has one nitrogen displaced by the trivalent methin group $\equiv CH$, there being formed the azo-methin chromophore $-\overset{H}{\overset{|}{C}}=N-$. The chromogens in the azo-methins are benzylidene derivatives, benzylidene being a name applied to the group $C_6H_5 \cdot CH=$, which is analogous to ethylidene, $CH_3 \cdot CH=$.

Benzylidene-anilin is only coloured a feeble yellow, but by the introduction of the auxochrome amido groups a deep red colour is produced.

Para-dimethyl-amido-benzaldehyde, $C_6H_4\langle^{CHO^{I}}_{N(CH_3)^{II}}$ (This dye is included because of its reactions with azo-bodies as described below.)



$$C_6H_5CHO + C_6H_5NH_2 = C_6H_5C=NC_6H_5 + H_2O$$
Benzaldehyde + anilin = benzylidene-anilin + water.

This benzylidene-anilin or phenyl-azo-methin-phenyl contains the chromophore radical $C=N$, which Möhlau has called the azo-methin group to express its relation to the azo group.

By itself $-C(H)=N$ is coloured only feebly yellow, but on introducing the auxochrome radical NO_2 and also methyl groups as side-chains

(p. 187), its colour tendencies are greatly increased, as is shown by para-dimethyl-amido-benzaldehyde (J. R. Geigy and Co., Basel).

This latter compound, in a slightly acid solution, on being brought into contact with aromatic bases, forms at once deeply-coloured insoluble azo-methin dyes, but this reaction fails if aldehydes be also present. (This dye is used by Ehrlich to obtain a typical red reaction with urine; see also Pröscher's account in *Zeitsch. f. physiol. Chemie*, 31.) Ehrlich obtained a definite reaction with cartilage as described on p. 298.

Rubifuscin was obtained by Möhlau by acting on nitroso-dimethyl-anilin with dimethyl-anilin, and it possesses this formula:

$$(CH_3)_2N-C_6H_4-\overset{H}{\underset{|}{C}}=N\cdot C_6H_4\cdot N\cdot(CH_3)_2.$$

It is a yellow base which forms red salts with acids.

(E) The Stilbene dyes.

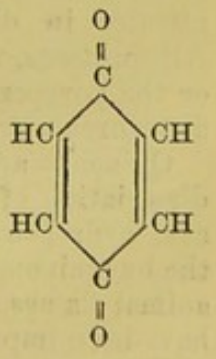
Stilbene is symmetrical diphenyl-ethylene, $C_6H_5\cdot CH{:}CH\cdot C_6H_5$, and dyes obtained from it possess, according to Nietzki, the chromophore group $-\overset{H}{\underset{|}{C}}=\overset{H}{\underset{|}{C}}-$, which latter plays the same part as does the azo group in the azo-dyes.

The Sun-yellow or Mikado yellow representing this series has been already described on p. 384 as a nitroxy compound, adopting Lefèvre's view.

Direct yellow (Kalle) is a dinitro-stilbene-disulphonic acid.

(F) Oxyquinones and Quinone-oximes.

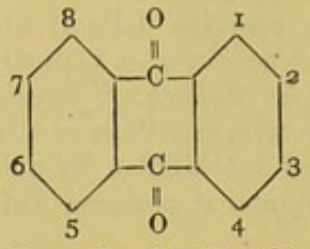
Quinone is a benzene in which two hydrogen atoms in the para-position have been replaced by two atoms of oxygen. Constitutionally it may be regarded as a ketone, or a body containing the carbonyl group CO linked on both sides to an alcohol radical; e. g. the methyl group CH_3 on either side of the CO group forms dimethyl-ketone or acetone (see p. 88).



The radical [C = O] if standing in a closed carbon-ring is one of the most important chromophores. It has its colour-bearing tendencies still more developed by substituting for its oxygen atom other divalent radicals such as sulphur [C = S], or by linking it with two valencies of such a trivalent substance as nitrogen [C = N−].

The oxyquinones are derived from anthracene: $H_4C_6\langle\overset{CH}{\underset{CH}{|}}\rangle C_6H_4$,

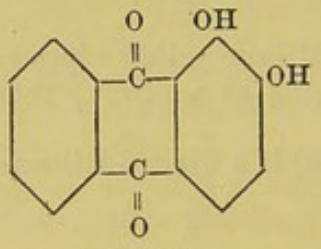
a compound which by chromic or nitric acid is oxidized into the neutral substance anthraquinone $H_4C_6\langle{CO \atop CO}\rangle C_6H_4$.



Formula after Nietzki.

The latter when treated with ordinary sulphuric acid becomes sulphonated to form anthraquinone-β-monosulphonic acid $H_4C_6\langle{CO \atop CO}\rangle C_6H_3 + SO_3H$, while anhydrous sulphuric acid produces the α and β disulphonic acids: $C_{14}H_6O_2(SO_3H)_2$. These in their turn, on being melted with alkalies, have firstly the sulphonic groups replaced by hydroxyl, and secondly undergo oxidation according to the formula: $C_{14}H_7O_2{\cdot}SO_3Na + 3NaOH = C_{14}H_6O_2(ONa)_2 + Na_2SO_3 + H_2 + H_2O$.

The substance obtained by the hydroxylation and oxidation of the sulphonic acids of anthraquinone is called alizarin. It forms orange-red



Alizarin.

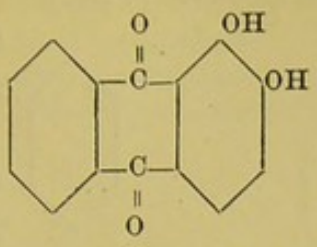
crystals and behaves towards ammonia and caustic alkalies as a weak acid, dissolving with a violet colour; but towards alumina, tin, and other bases it acts as a strong acid, decomposing their chlorides and nitrates in dilute solutions, and liberating the free mineral acids. All quinones, no matter whether the quinone group occupies the ortho- or the paraposition, are strong chromogens, which by the introduction of auxochrome groups are readily converted into real dyes.

Quinones are further chromophores which cause a ready electrolytic dissociation of the auxochromic hydroxyl radicals, and thus set free the acid hydrogen ion. This acid character, manifesting itself especially in the oxyquinones, explains why these dyes can fix themselves directly to animal tissues, although they do so to a much greater extent if the fibres have been impregnated previously with metallic oxides.

The faculty of readily combining with metallic oxides to form lakes is seen, however, only in those oxyquinones which possess at least one hydroxyl group in the orthoposition to the quinone-oxygen atom, and in this respect oxyquinones and chromotropes agree (see above, p. 393).

(F, 1) **Oxyquinones.**

Alizarin, $C_{14}H_8O_4$,



occurs in the natural state in the root of the madder (*Rubia tinctorum*) as the readily decomposable glucoside, ruberythric acid. Besides alizarin there is also found in this plant purpurin (see below).

Alizarin will stain animal tissues directly with a feeble yellowish-red colour, because it contains the hydroxyl groups in the orthoposition, while intense red colours called 'lakes' are produced with alumina, bluish-violet tints with iron, and brownish-violets with chrome-compounds. To obtain red tints on cotton the presence of lime salts in addition to alumina is essential, and the precipitate so formed in the fibres is soluble in carbonic acid; while if, instead of using alizarin, its nitro derivative $[C_{14}H_7(NO_2)O_4]$ be employed, a violet alumina-lime-lake is obtained which is insoluble in carbonic acid (see also p. 456).

Trioxy-anthraquinones.

These are oxy derivatives of alizarin. The best-known representative of this group is purpurin.

Purpurin, $H_4C_6\langle^{CO}_{CO}\rangle C_6H(OH)_3$ (in the 1 : 2 : 4 position), occurs along with alizarin in madder, and differs from its fellow in being soluble in seething alum solution, and forming with alumina scarlet-red lakes.

Anthragallol is a brown dye, having the hydroxyls in the 1 : 2 : 3 position.

Tetraoxy-anthraquinones.

Alizarin-bordeaux contains the hydroxyls in the positions 1 : 2 : 5 : 8, while **Anthrachryson** contains them in the position 1 : 3 : 5 : 7. The latter cannot act as a dye because it does not conform to the rule laid down for alizarin, as it does not contain the hydroxyl groups in the orthoposition. By its sulphonation, nitration, and subsequent reduction there is formed, however, diamido-anthrachryson-sulphonic acid, which behaves like an ordinary acid dye, staining animal tissues, in an acid bath, yellow. By its action on tissues which have been mordanted, and which contain chromic oxide, the blue colour acid, alizarin-blue BB[1], is formed.

Pentaoxy-anthraquinones (see also p. 456).

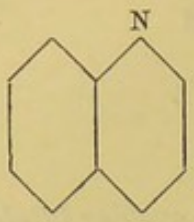
Alizarin-cyanin has its hydroxyls in the position 1 : 2 : 4 : 5 : 8.

Hexaoxy-anthraquinones.

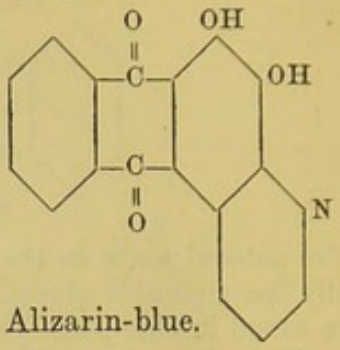
Rufigallic acid, with the hydroxyls in the position 1 : 2 : 3 : 5 : 6 : 7, dissolves in alkalies with a violet colour.

[1] D. R. P. 75,490.

Alizarin-blue [$C_{17}H_9NO_4$] stands to alizarin in the same relationship as does quinolin to benzene (see also p. 456).



Quinolin.



Alizarin-blue.

It acts as a weak base, stains directly, and also forms lakes, those with chromic oxide being of an indigo-blue colour. The sodium bisulphite salt (alizarin-blue S) is colourless, but on heating decomposes into alizarin-blue.

(F, 2) **Quinone-oximes** (nitroso-phenols).

These bodies are quinones, in which one or more of the oxygen atoms have been replaced by the divalent oxime group [=N−OH].

Quinone.

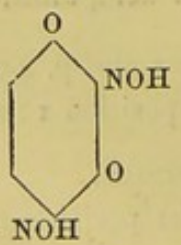
Quinone-oxime.

Analogously to the oxyquinones, those dyes which possess the oxime group in orthoposition to the oxygen atom of the quinone, readily combine with metallic oxides to form lakes.

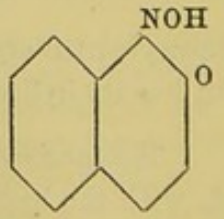
Dinitro-resorcin [$C_6H_2O_2(NOH)_2$] is a strongly dibasic acid, which forms salts with alkalies. With oxide of iron it forms an intensely coloured, green lake.

Fast green or **Echtgrün** is an alkali salt of dinitro-resorcin.

Naphtho-quinone-oxime [$C_{10}H_6O{\cdot}NOH$] forms a sulpho-acid, which in combination with an iron salt is known as naphthol-green (D. R. P. 28,065, January 19, 1884). It stains tissues as an acid dye.



Dinitro-resorcin.



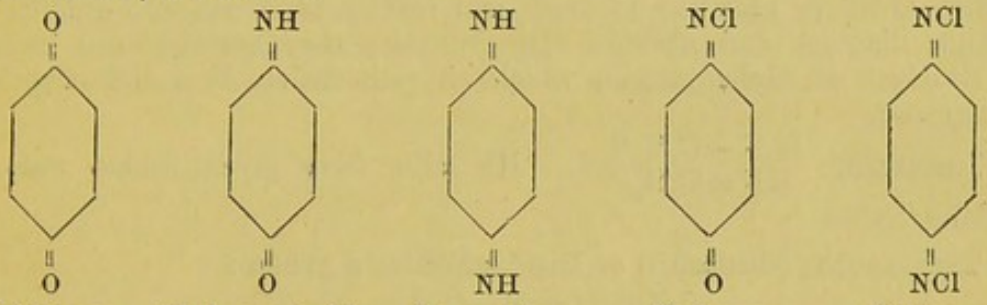
Naphtho-quinone-oxime
(α-nitroso-β-naphthol).

G. The Quinone-imide Dyes.

These compounds are represented by the following groups:

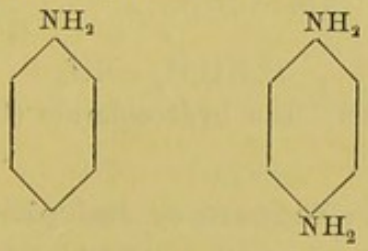
I. Indamins (p. 401), and indones . . . p. 402
II. Thiazins (p. 403), and thiazones . . . p. 405
III. Oxazins (pp. 406–7), and oxazones . . pp. 406–7
IV. Azins . . . . . p. 408
V. Indulins . . . . . p. 416
VI. Quinoxalins . . . . . p. 416
VII. Fluorindins . . . . . p. 417

All substances in this group are derivatives of paraquinone-diimide (compare with the azins, p. 408). If in quinone one of the oxygen atoms could be replaced by the imide group (NH) there would be formed quinone-imide, or if both oxygen atoms were so affected, the compound paraquinone-diimide would result. Neither of these two substances has been prepared so far, but substitution products, namely mono- and di-chlorimides, are known.



Quinone. Quinone-imide. Paraquinone-diimide. Quinone-chlorimide. Quinone-dichlorimide.

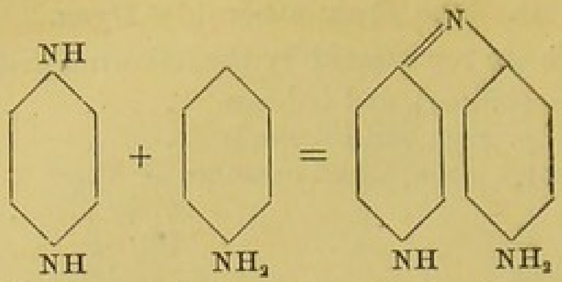
Further, on substituting in benzene one of the hydrogen atoms by the amidogen group NH_2, the substance amido-benzene is formed, which has also received the name of phenyl-amin or anilin. If similarly two hydrogen atoms in the paraposition are replaced by two amidogen groups, the substance paraphenylene-diamin results.



Anilin. Paraphenylene-diamin.

I A. The Indamins.

On oxidizing a mixture of anilin and paraphenylene-diamin, the latter is converted into paraquinone-diimide, $NH{=}C_6H_4{=}NH$, and this compound on further oxidation attaches itself by means of one of its nitrogen atoms directly to that carbon atom in the anilin ring which occupies the paraposition to the amidogen group, NH_2, of the anilin (Nietzki), thus:



Paraquinone-diimide + Anilin = Indamin.

The indamins contain the chromophore group :

in which R stands for hydrogen or the alcoholic group CH_3, C_2H_5, &c., while R′ indicates an aromatic (benzene, naphthalin or other) group.

They are weak bases, and form unstable green, blue, or violet salts, which are very sensitive to acids, and readily break up into quinones, amins, diamins, and phenols. By reduction they become leuco bases, derivatives of diphenylamin, which on reoxidation form the original compound.

Indamin, $\left.\begin{matrix} H_2N-C_6H_4 \\ HN=C_6H_4 \end{matrix}\right\rangle N$. Its salts form greenish-blue water-soluble bodies.

Tetramethyl-indamin or **Bindschedler's green** :

$$\left.\begin{matrix} (CH_3)_2=N-C_6H_4 \\ (CH_3)_2=\underset{\displaystyle Cl}{\underset{|}{N}}=C_6H_4 \end{matrix}\right\rangle N$$

The iodide ($C_{16}H_{20}N_3I$) and the double zinc chloride ($C_{16}H_{20}N_3Cl_2$) are both water-soluble.

Toluylene-blue

$$\left.\begin{matrix} (CH_3)_2=\overset{\displaystyle Cl}{\overset{|}{N}}C_6H_4 \\ H_2NC_7H_5-NH_2 \end{matrix}\right\rangle N$$

is an amido-indamin, its mono-acid salts are blue. The hydrochloride ($C_{15}H_{19}N_4HCl$) is water-soluble. A weak base.

I B. Indones or Indophenols.

The **indones** or **indophenols** are obtained by oxidizing paradiamins or para-amido-phenols in conjunction with phenols, when there are formed feeble basic substances, which in the free state are of a deep blue colour. The leuco-compounds are, however, acid, and behave like phenols in an alkaline solution, and are readily oxidized in the air into the coloured indophenol. This dye is very susceptible to acids, being turned into a grey colour by ten per cent. hydrochloric acid. Dyers impregnate material with the leuco-compound, and allow the blue colour to be generated by the oxidizing action of the air or potassium bichromate.

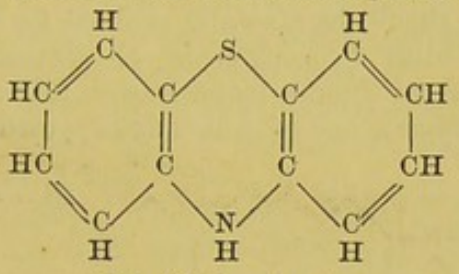
The absence of an acid reaction makes it highly probable that no hydroxyl group is present, therefore the formula $O=C_6H_4=N-C_6H_4-NH_2$ is more probable than the formula $HN=C_6H_4=N-C_6H_4-OH$.

Liebermann's phenol dyes.

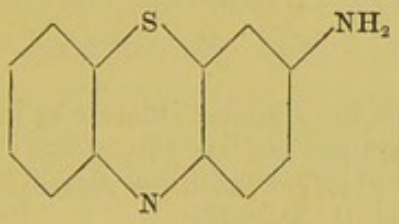
The simplest indophenols, according to the formula $HO-C_6H_4-N=C_6H_4=O$, are probably the so-called Liebermann's phenol dyes, which are obtained by acting with nitrous acid on phenols dissolved in concentrated sulphuric acid, at a temperature of 45 to 50° C. They are acid bodies of a blue or violet colour, and are soluble in alkalies.

II. Thiazins and Thiazones.

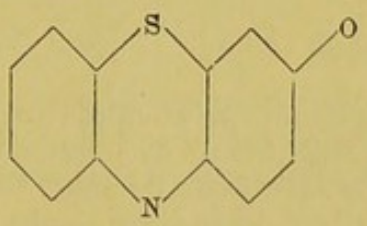
These thio or sulphur-containing indamins consist of two benzene nuclei which are linked together by means of a closed ring, built up of one sulphur, one nitrogen, and four carbon atoms. The nitrogen in the sulphur ring corresponds to one of the amidogen groups of the para-phenylene-diamin nucleus; it is in the orthoposition to the sulphur atom, and in the paraposition to the nitrogen atoms in the two benzene rings. Thiazins are thus derived from thio-diphenylamin:



Thio-diphenylamin.



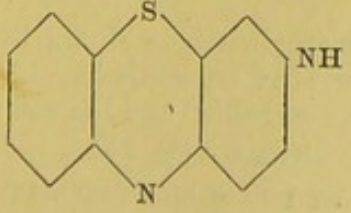
Thiazin.



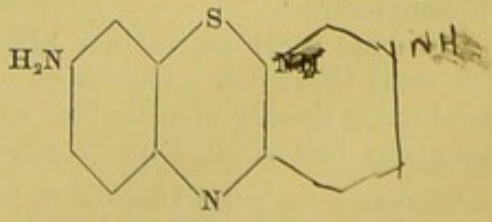
Thiazone.

II A. Thiazins.

(A) **Imido-thio-diphenylimide** is the simplest thiazin, containing only one imido (NH) and no amido (NH_2) radical. Its hydrochloride ($C_{12}H_8N_2SHCl + 1\frac{1}{2}H_2O$) is soluble in water with a bluish-violet tint. It also forms a double salt with zinc chloride, $(C_{12}H_8N_2SHCl)_2ZnCl_2$:



Imido-thio-diphenylimide.

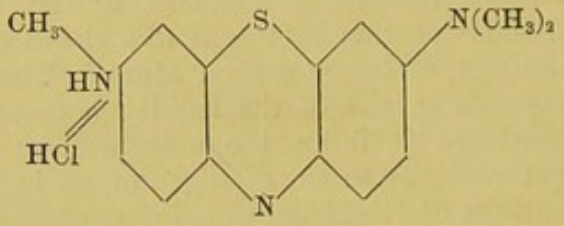


Thionin.

(B) **Thionin, Lauth's violet,** or **Amido-diphenthiazin.** In constitution it differs from the most simple indamin in possessing a sulphur atom. The base ($C_{12}H_9N_3S$) is soluble in alcohol with a reddish-violet colour, while its hydrochloride ($C_{12}H_9N_3SHCl$) is water-soluble. By reduction it becomes para-diamido-thio-diphenylamin. Its formula is given above.

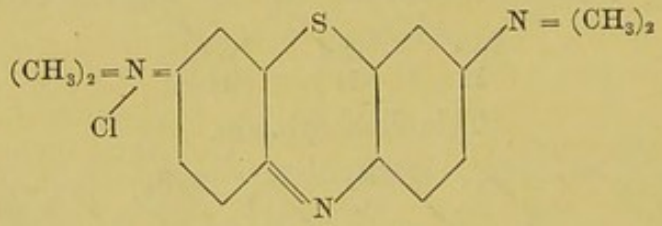
Oxonin is thionin, in which the sulphur atom has been replaced by an atom of oxygen, and belongs to the class of oxazins (p. 407). This dye was first introduced by Ehrlich [1].

(C) **Toluidin-blue** has three methyl groups (CH_3), of which two replace the two atoms of hydrogen in the amidogen group of thionin, while the third methyl radical is attached to the imide group (NH) of thionin:



The double zinc salt should be carefully avoided for intra vitam staining.

(D) **Methylene-blue** (the chloride of tetramethyl-amido-diphenthiazin). It differs from thionin by possessing four methyl groups joined on to two nitrogen atoms, one of which is supposed to be pentavalent:



and to link on to an atom of chlorine to form the chloride of methylene-blue ($C_{16}H_{18}N_3SCl$). The latter is therefore an ammonium base. To obtain the base from its salts, the chloride must be decomposed with oxide of silver, when there results ($C_{16}H_{18}N_3SOH$).

It is this base which Laveran calls Bleu Borrel [2]. To obtain the base he treats a saturated solution of methylene-blue for a whole week with freshly precipitated silver oxide, prepared by adding sodium carbonate to silver nitrate, and carefully washing the precipitate.

The commercial methylene-blue is a double zinc chloride, and should be avoided in histology for intra vitam staining. Leuco-methylene-blue, the formula for which is given on p. 189, is tetramethyl-diamido-thio-diphenylamin.

As most other ammonium bases, methylene-blue shows little affinity for wool (Nietzki). The action of alkalies on methylene-blue and the production thereby of methylene-violet (see below) and the red methylene-azur was first studied by Bernthsen (*Liebig's Ann.* 1885, 1889). Unna

[1] Through the great kindness of Ehrlich I have been enabled to investigate the properties of this dye. It is not to be obtained commercially.

[2] *C. R. Soc. d. Biol., Paris,* (11) t. 1, 250 (1899).

discovered that the same change occurs spontaneously in methylene-blue solutions which have been kept for a long time, and it is this mixture of dyes which is found in the 'polychromic methylene-blue' advocated by Unna[1].

Rosin and Schimmelpfeng[2] have also investigated the action of alkalies on other basic dyes, namely toluidin-blue, safranin, dahlia, magenta-red, neutral red, methyl-green, methyl-violet, and congo-red.

Nocht and Rosin found, independently of one another, that on bringing methylene-blue and eosin together there is formed methylene-azur, along with other compounds, which will be dealt with later (p. 442).

G. Cohn[3] has prepared benzoyl-, anisyl-, propionyl-, butyryl-, and valeryl-leuco compounds from the methylene-blue double zinc chloride, from ethylene-blue and new-methylene-blue. All these leuco bodies are permanent outside the body, but when injected or brought in contact with living tissues, become decomposed into the coloured methylene-blue.

New-methylene-blue N (Cassella & Co.) is a compound analogous to the ordinary methylene-blue, but is prepared from para-nitroso-ethyl-ortho-toluidin, instead of nitroso-dimethyl-anilin.

(E) **Methylene-green** is mono-nitro-methylene-blue, and acts as a base. It is obtained from methylene-blue by the action of nitrous acid. It gives good results in combination with eosin, and also as a substitute for methyl-green in Ehrlich's mixtures (see p. 221).

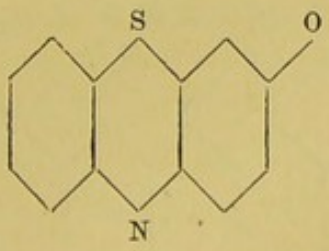
(F) **Thio-carmin** is obtained from nitroso-ethyl-benzyl-anilin-sulpho acid, and is an acid dye, because it contains in each of the two benzy-remainders a sulphonic radical.

(G) **Methylene-violet.** On boiling methylene-blue with an alkali, there are formed (1) a violet basic substance ($C_{14}H_{12}SON_2$), methylene-violet, containing only two methyl groups, and (2) sulphonic acid methylene-blue or methylene-azur ($C_{16}H_{17}N_3SO_2$) (Bernthsen).

(H) **Methylene-red**, $Cl(CH_3)_2N = C_6H_3\langle\!\!\begin{matrix}N\\S\end{matrix}\!\!\rangle S$, is formed along with methylene-blue by the oxidation of dimethyl-para-phenylene-diamin in the presence of a large excess of H_2S_2. Its hydrochloride is extracted from watery solutions by phenol. Alkalies destroy, while acids regenerate the colour.

II B. Thiazones.

The thiazones bear to the thiazins the same relation as do the indophenols to the indamins, as the amidogen group NH_2 is replaced by an atom of oxygen:



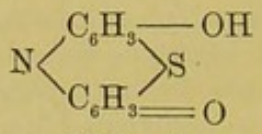
[1] Unna called the red methylene-azure, methylene-red, a term which had better be restricted to the substance mentioned above under F.

[2] Rosin and Schimmelpfeng: *Centralbl. f. Physiol.* 13, 25-6 (1899).

[3] G. Cohn: *Ber.* 33 [10], 1567-68.

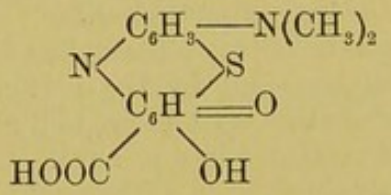
Thionolin is obtained from thionin by boiling with alkalies, the imide group being replaced by an atom of oxygen. This compound bears to thionin the same relation as methylene-violet does to methylene-blue. It is a basic substance.

Thionol (dioxy-thio-diphenylimide) differs from thionin in having the imide group replaced by oxygen, while the amide group is substituted by hydroxyl (OH). It has the basic character of thionolin, but, owing to the hydroxyl, acts also as an acid, and this latter characteristic preponderates:



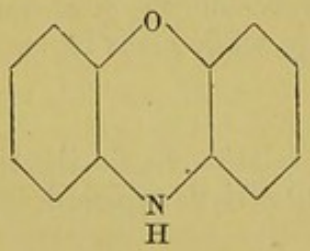
Thionol.

Gallo-thionin. This substance also acts both as an acid and as a base, for it can form salts with acids and with bases:

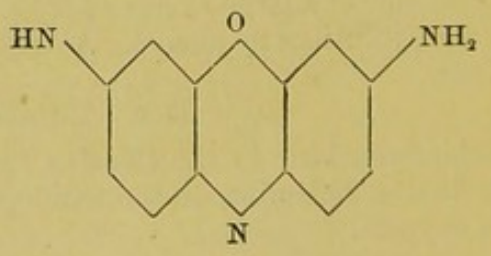


III. **Oxy-indamins** (Oxazins) and **Oxy-indones** (Oxazones).

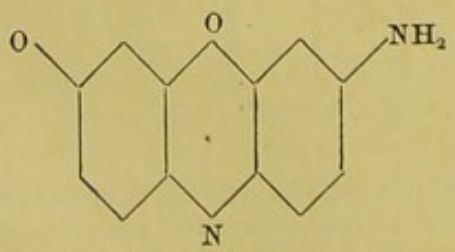
These dyes are derivatives of phenoxazin, and they correspond as regards general molecular structure with the thio-compounds, only that instead of possessing a sulphur atom in the ring which joins two indamin or indophenol nuclei, they contain an oxygen atom. In the oxazones the imide group of the oxazins is replaced by oxygen, thus:



Phenoxazin.



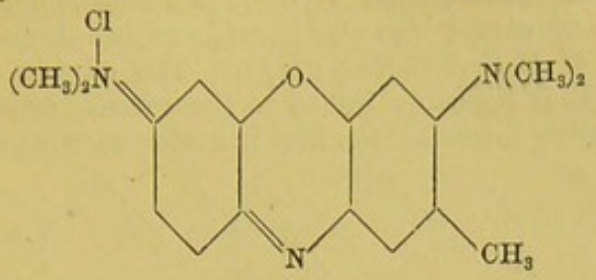
Oxazin.



Oxazone.

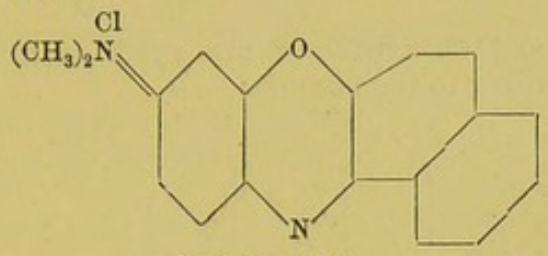
III A. Oxazins.

Capri-blue [chloride of tetramethyl-amido-diphenazin]. A base.



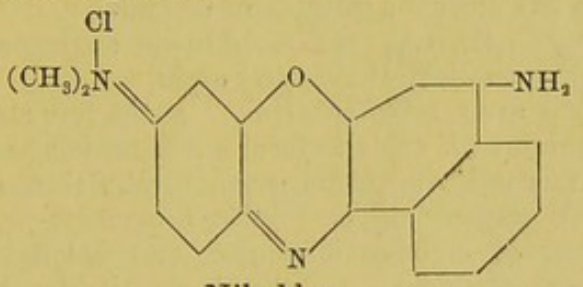
Capri-blue.

Naphthol-blue (Meldola) [chloride of dimethyl-naphtho-phenazin] ($C_{18}H_{15}N_2OCl$). A base.



Meldola's blue.

Nile-blue is naphthol-blue containing an additional amido group, and is for this reason a strong base:

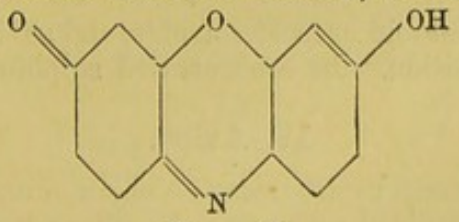


Nile-blue.

New-methylene-blue is dimethylated naphthol-blue, or the chloride of diamido-tetramethyl-naphtho-phenazin. (See above, p. 405, for New-methylene-blue N.)

III B. Oxazones.

Resorufin (Diazo-resorufin of Weselsky) is an acid dye because of the

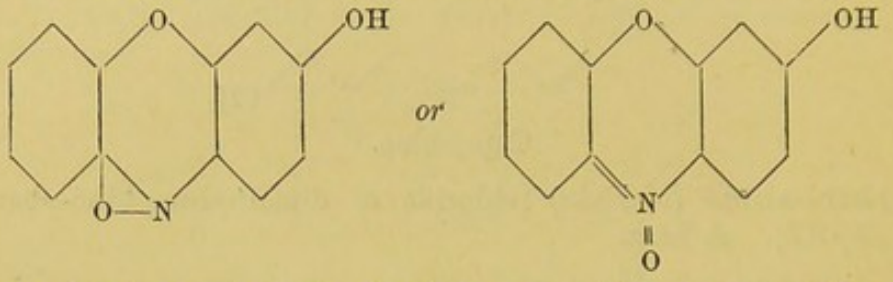


Resorufin.

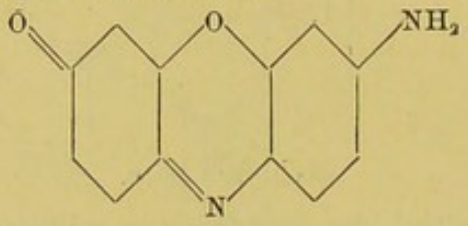
hydroxyl group it contains; the potash salts are readily soluble in water with a vermilion-like fluorescence. Its tetrabrom derivative forms with

alkalies a blue solution, and stains animal tissues a bluish red, and is called fluorescing-blue. The sodium salt is $C_{12}H_2Br_4NO_3Na + 2H_2O$.

Tetrabrom-resazurin, $C_{12}H_3Br_4NO_4$, is a non-fluorescent dye, which by reduction is converted into the fluorescent tetrabrom-resorufin. Its sodium salt is $C_{12}H_2Br_4NO_4Na + 2H_2O$. The oxygen atom, which is readily given off, is probably joined to the nitroso or isonitroso group. Of the two following formulae, the first is analogous with azo-oxybenzene:



Resorufamin and **Orcirufamin** are amido-oxazones of resorufin and orcirufin, according to the formula:

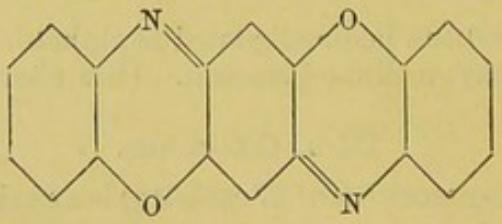


Resorufamin, $C_{12}H_8N_2O_2$, is obtained by the action of quinone dichlorimide on a hot alcoholic solution of resorcin.

Orcirufamin, $C_{13}H_{10}N_2O_2$, is formed in an analogous manner by the interaction of quinone dichlorimide and orcin.

Both these dyes are soluble in alcohol in the free state. Being weak bases, they combine with acids to form red fluorescing salts.

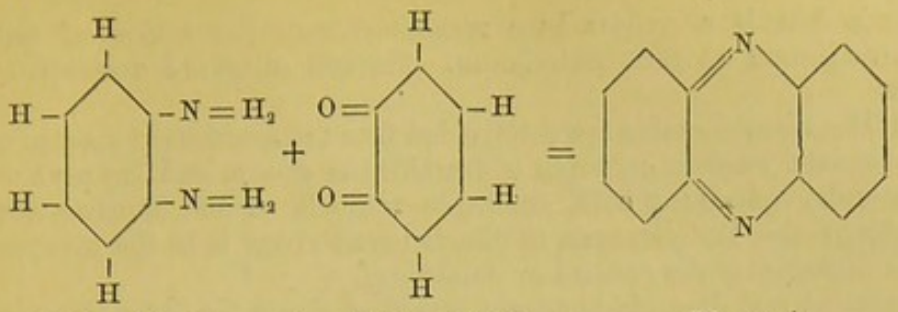
Triphendioxazin is the name given by P. Seidel (*Ber.* 23, 182) to a substance first prepared by G. Fischer (*Journ. f. pr. Chem.* 19, 317) by the oxidation of ortho-amidophenol, and belonging, according to Nietzki, to the oxazone dyes. It has this constitutional formula:



It is only slightly soluble in most solvents, and sublimes partly without undergoing decomposition. In concentrated sulphuric acid it dissolves with a blue colour.

IV. Azins.

The dyes in this group result from the union of one molecule of ortho-diamin with one molecule of ortho-quinol. These two molecules arrange themselves in such a way that the hydrogen atoms of the two amidogen groups (NH_2) of the ortho-diamin unite with the oxygen atoms of the ortho-quinol to form water.



Ortho-diamin + Ortho-quinol = Phenazin.

The two nitrogen atoms then join the remainders of the ortho-diamin and ortho-quinol molecules together, in such a way that a new ring is formed consisting of two nitrogen and four carbon atoms. This process is analogous to that described for anthracene compounds (p. 398).

The chromophore is the group  , which serves to fix the dyes to the tissues.

Phenazin is the simplest type in this group. Its basic character is so feeble that its salts are decomposed by water. On introducing the hydroxyl group, OH, weak acid dyes are formed, while by the introduction of one amido group, NH_2, as in the eurhodins, weak bases result only slightly stronger than phenazin itself. Two or more amidogen groups, however, strengthen the basic character of the chromophore to such an extent that strong dyes are the outcome.

That azins are related to indamins is shown by the conversion of the indamin toluylene-blue (p. 402) into the azin toluylene-red (p. 410) by prolonged boiling, which causes the blue to part with the hydrogen of its amido group, the remaining nitrogen then participating in the formation of the azin chromophore  .

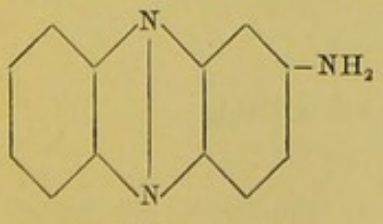
The azins include the following types of dyes:

(1) The amido-azins or eurhodins . . . . p. 409
(2) The oxy-azins or eurhodols . . . . p. 411
(3) The safranins . . . . . p. 412
(4) The safranols . . . . . p. 415

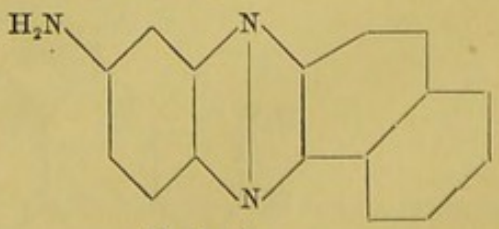
1. Amido-azins or eurhodins.

Both mono- and diamido compounds exist.

(*a*) The mono-amido-azins are feeble basic dyes owing to the group NH_2 and possess a yellow colour. The red mono-acid and the green diacid compounds are both decomposed by water, as is shown by silk which is stained red becoming yellow on washing.



Amido-phenazin.

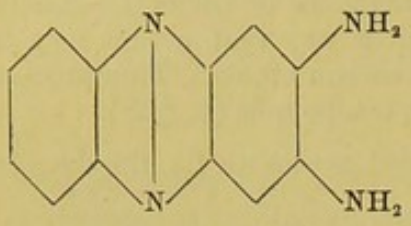


Eurhodin.

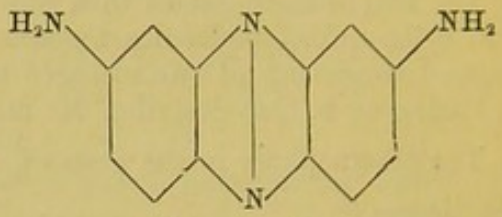
Eurhodin is a yellow base resulting from the action of quinone dichlor-diimide on β-naphthylamin. Its red salts are decomposed by water.

(*b*) The diamido-azins are subdivided into (1) symmetric ones, in which each benzene nucleus contains one amidogen group, and (2) asymmetric compounds, containing both amidogen radicals in one benzene nucleus. In both groups the nitrogen of the benzene rings is in the paraposition to the nitrogen of the central or azin-ring.

Asymmetrical diamido-phenazin was first made by Gries by oxidizing ortho-phenylene-diamin: .



Asymmetrical

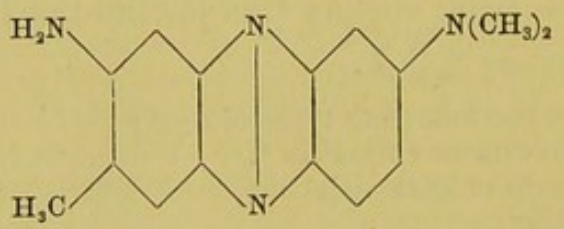


Symmetrical

Diamido-phenazin.

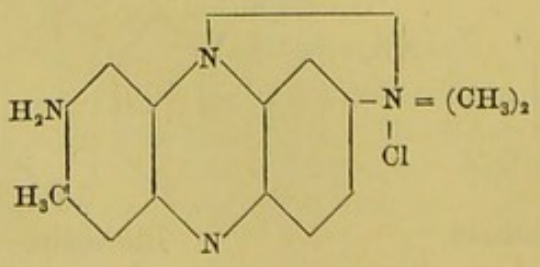
Symmetrical compounds, of which the simplest is obtained by the oxidation of triamido-diphenylamin, are basic in character and form red mono-acids.

Toluylene-red or trimethyl-diamido-azin is obtained by heating the amido-indamin toluylene-blue, when the latter gives off two atoms of hydrogen (see above, p. 402). Its chloride forms neutral red.

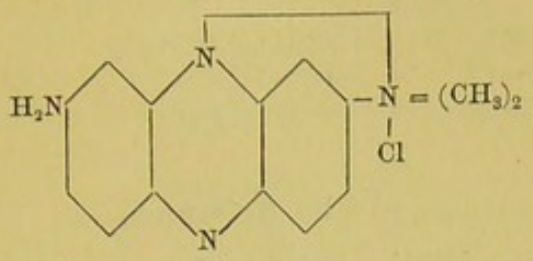


Toluylene-red.

Neutral red is a basic dye, which has received its name from its neutral colour, which is neither red nor yellow. The slightest trace of alkali converts the neutral tint into yellow, while the carbonic acid in ordinary tap-water is sufficient to turn the neutral tint into a pink or fuchsin colour. With strong hydrochloric acid it turns blue. Its solubility in normal saline is less than 1 : 1,000. Its chloride has the formula:



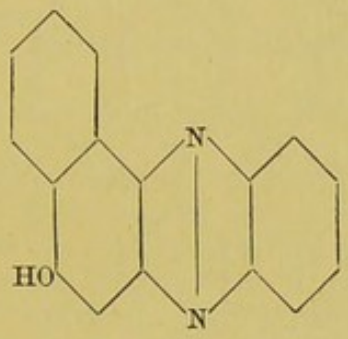
Neutral violet is similar to neutral red in constitution, being also a base. Its chloride has the formula :



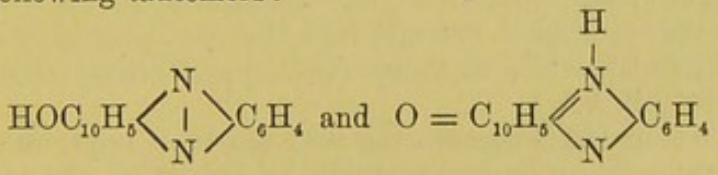
2. The Oxy-azins or eurhodols.

These substances possess both basic and acid characters, and may be obtained by heating the amido-azins with concentrated hydrochloric acid at 180° C. The dioxy-azins may be further symmetrical or asymmetrical.

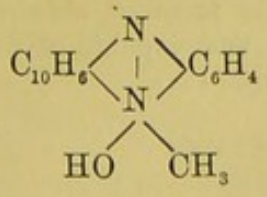
Mono-oxy-azins are represented by oxy-naphtho-phenazin :



When this compound is treated with methyl-iodide (CH_3I) it forms two isomeric methyl-ethers, of which one has the methyl group attached to the oxygen, and the other to the nitrogen. Kehrmann and Messinger (*Ber.* 24, p. 2161) conclude for this reason that oxy-naphtho-phenazin forms the following tautomers :

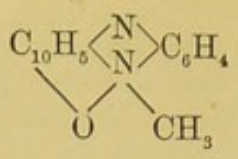


Nietzki believes the mother-substance of this dye to be the ammonium base

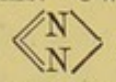


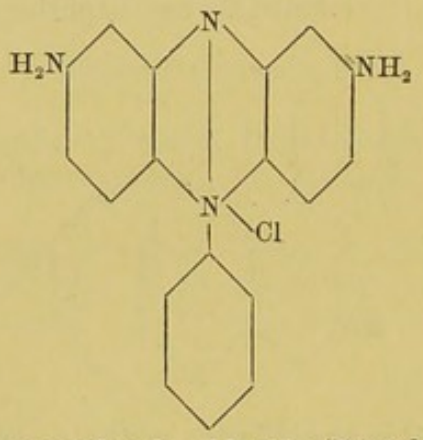
This base, on coming into contact with the acid sulphonic, carboxylic, or hydroxyl groups, forms salts with these acids because it possesses the

strongly basic ammonium group, and therefore it is possible for the dye by anhydration to assume the following constitution:



3. The Safranins.

A great many dyes, differing widely in their constitution and properties, are classed under this heading, but they all contain at least three benzene rings and four nitrogen atoms. Owing to the amidogen groups strengthening the azin chromophore  all safranins resemble in their strongly basic characters the quaternary ammonium bases. They are usually manufactured by the oxidation of one molecule of paradiamin with two



Pheno-safranin chloride (Nietzki).

molecules of monamin. The red safranin of commerce is nearly always an oxidation product of one molecule of para-toluylene-diamin, one molecule of ortho-toluidin, and one molecule of anilin.

If one introduce alcohol radicals into the red safranin, its colour is changed into violet, while methoxyl (OCH_3) and ethoxyl (OC_2H_5) turn its colour into yellow.

If safranin sulphate is decomposed with the exact required quantity of barium hydrate, the red water-soluble base may be obtained, which, however, in the presence of carbonic acid forms the carbonate.

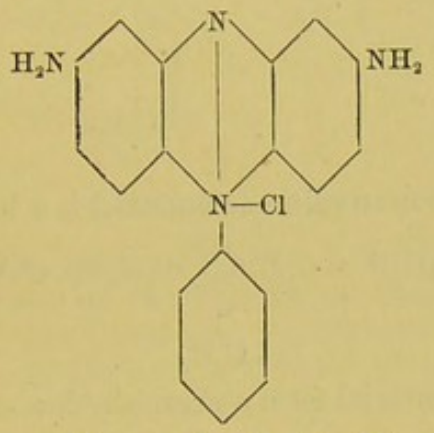
Indoin is a blue basic azo-dye, obtained by combining diazotized safranin with β-naphthol, and capable of fixing itself directly to cotton by means of its azin group.

Safranins may be divided into

(*a*) Benzene compounds . . . . . p. 413
(*b*) Naphthalene compounds . . . . . p. 414
(*c*) Compounds with an aliphatic azonium group . p. 414
(*d*) Apo-safranins . . . . . p. 415

(*a*) *Benzene safranins.*

Pheno-safranin is a phenyl-ammonium compound of the symmetric diamido-phenazin. If the pheno-safranin chloride is treated with glacial acetic acid and sodium acetate, the diacetyl hydrochloride $C_{18}H_{12}N_4(C_2H_3O)_2HCL$ is obtained, which is soluble in alcohol with



Pheno-safranin chloride.

a violet colour and acts as a strong base.—A diazo compound, $C_{18}H_{12}\overline{N_3(HCl)}\,\overline{N}{=}NCl$, is obtained by acting on an acid solution of pheno-safranin chloride with nitrous acid. Its chloride is the commonest salt. It possesses in solution the blue colour of the dibasic salts of safranin, but while the blue colour of the safranin dibasic salt disappears on dilution, that of the diazo compound remains.—If nitrous acid is added to the tribasic green solution of pheno-safranin in H_2SO_4, one obtains a green solution, on dilution with water, which is a tetrazo compound of safranin. (See also p. 444, on combinations of safranin with azo-dyes.)

Dimethyl-pheno-safranin ($C_{18}H_{12}N_4(CH_3)_2$), obtained from one molecule of dimethyl-para-phenylene-diamin and two molecules of anilin, forms a hydrochloride ($C_{20}H_{18}N_4HCl$) and a double zinc salt called **Fuchsia**. By combining nitroso-dimethyl-anilin and xylidin **Giroffle** is formed.

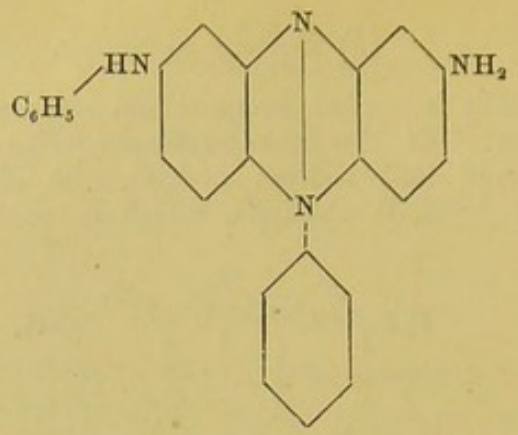
Both diethyl and dimethyl safranins form primary diazo-bodies of a pure blue colour, which combine with phenols to form azo-dyes.

Tetramethyl-pheno-safranin is prepared by the interaction of equimolecular quantities of dimethyl-para-phenylene-diamin, dimethyl-anilin, and anilin.

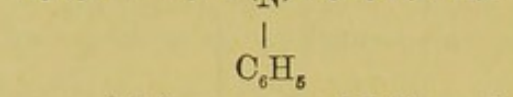
Amethyst-violet is the analogous diethyl double zinc salt and is neither attacked by nitrous acid nor glacial acetic acid.

Mauveine is less basic than safranin. Its mono-acid salts are reddish-violet, and stain wool with the bluish colour of the free base. This is the first coal-tar colour which was manufactured for commercial purposes by Perkin in 1856. That mauveine was a safranin was first shown by A. W. Hofmann and Geyger. Its constitutional formula is this:

Mauveine:



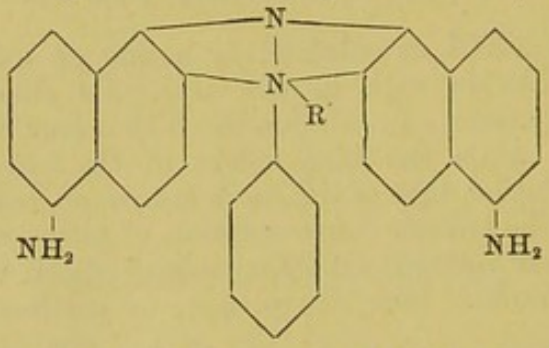
Dimethyl-pseudo-mauveine or **Indazin** is a bluish-violet dye



containing in its commercial form a greenish-blue admixture.

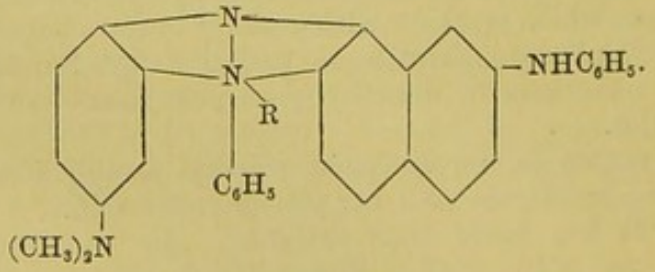
(*b*) *Safranins of the naphthalene series.*

Magdala red or naphtho-safranin (naphthalene red). It is fluorescent



and possesses the strongly basic characters of the safranins.

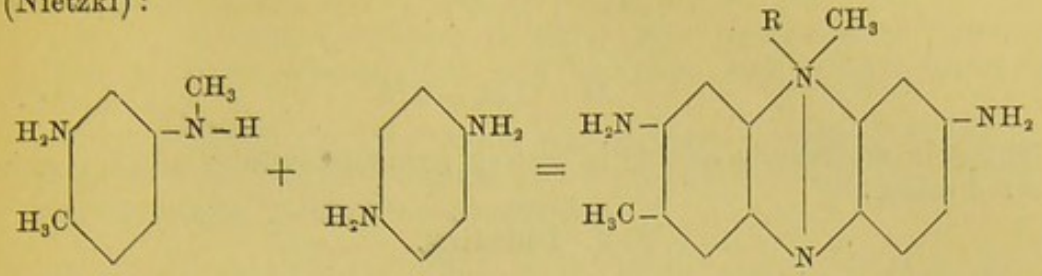
Baseler blue (Armschein). A dimethyl-phenyl derivative of the mixed naphtho-pheno-safranins. It differs from the true safranins in only having one of the auxochrome amidogen groups in the paraposition to the azin ring.



(*c*) *Safranins with an aliphatic azonium group.*

These are derivatives of the methyl- and ethyl-diphenyl azonium chlorides. A safranin is formed, for example, if methyl-toluylene-diamin

is oxidized along with paradiamin according to the following scheme (Nietzki):

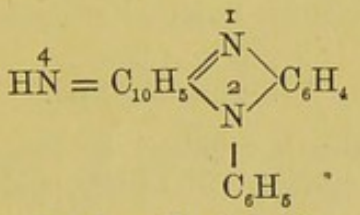


Methyl-toluylene-diamin + Paradiamin = Safranin.

(*d*) *Apo-safranins.*

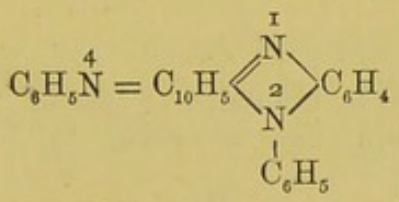
Apo-safranins differ from the true safranins in containing one amidogen group less and are therefore weaker bases. If pheno-safranin is boiled with alcohol one amido group is removed and apo-safranin is formed. Its salts do not fluoresce in alcohol, and are of a fuchsin colour. It forms nitrates and double zinc chlorides, of which the latter are more soluble.

Rosindulin is formed by the interaction of benzene-azo-*α*-naphthylamin + anilin + alcohol under pressure at 170° C. Its chloride forms a red solution. A disulpho acid goes by the same name, therefore care is needed in ordering this dye.



The base Rosindulin.

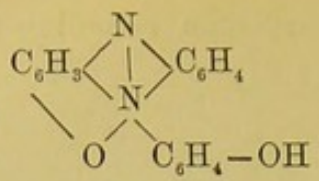
Phenyl-rosindulin (used to be called rosindulin):



Azo-carmin, being a disulphonic derivative of phenyl-rosindulin, is strongly acid.

4. The Safranols.

Safranols are obtained from safranin, apo-safranin, and rosindulin, by treating these with alkalies or concentrated acids, when nitrogen atoms, not belonging to the azin group, are exchanged for oxygen. Thus by prolonged boiling with baryta water or alcoholic caustic potash pheno-safranin is converted into the safranol



Safranols are readily soluble in watery ammonia or fixed alkalies with a red colour.

V. Indulins.

When azo-benzene, amido-azo-benzene, azo-oxy-benzene, and nitro-benzene are heated up with anilin chloride or other aromatic amins, indulins are formed, the molecular structure of which has not as yet been ascertained. All the dyes are basic substances.

Indamin, $C_{24}H_{18}N_4$. Its hydrochloride is water-soluble with a bluish-violet colour.

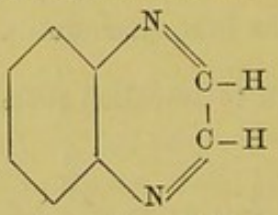
Indulin 3B, $C_{30}H_{23}N_5$, is probably the anilido derivative[1] of indamin, while **Indulin 6B** is phenylated indulin 3B or $C_{36}H_{27}N_5$. All indulins are readily converted into sulpho acids, the ease with which this may be accomplished depending directly on the number of phenol groups present. The greater the number of these the easier the transformation.

Paraphenylene blues are strongly basic substances, owing to the introduction of amido groups into the phenyl radical of the indulins.

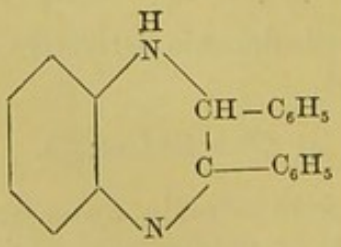
They are obtained by substituting paraphenylene-diamin for anilin in the amido-azo or azo-benzene flux.

VI. Quinoxalins.

The simplest quinoxalin is obtained by the action of *o*-phenylene-diamin $C_6H_4(NH_2)_2$ on glyoxal, CHO·CHO, and has this constitution:



The substance obtained by the union of benzoin, $C_6H_5{\cdot}CHOH{\cdot}CO{\cdot}C_6H_5$, and *o*-phenylene-diamin has, according to Nietzki, probably this formula:



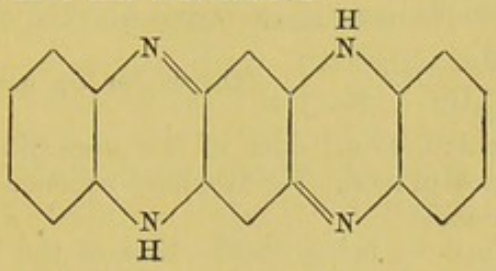
It is of a bright yellow colour, and fluoresces strongly.

[1] Anilides is a short expression for anilin-amides.

7. Fluorindins.

These substances were discovered by Caro and Witt. They are only very slightly soluble, and vary in colour from orange-red to violet-red. Their salts are greenish-blue, and in every case show a brick-red fluorescence.

Fischer and Hepp have found the simplest fluorindin, which they prepared from diamido-phenazin and *o*-phenylene-diamin, the so-called homo-fluorindin, to have this constitution:



H. Phenyl-methane derivatives.

Phenol, carbolic acid or phenyl alcohol, C_6H_5+OH, is benzene in which one of the hydrogen atoms has been replaced by the hydroxyl group OH. The benzene remainder, C_6H_5, is called phenyl, and it may replace in methane or marsh-gas, CH_4, one, two, or three of the hydrogen atoms, when there are formed, respectively, monophenyl-methane or toluene,

$$H_3 \equiv C-C_6H_5$$

diphenyl-methane or benzyl-benzene,

$$H_5C_6-C-C_6H_5$$
$$\|$$
$$H_2$$

and triphenyl-methane,

$$\begin{array}{l} C_6H_5 \\ | \\ H_5C_6-C-C_6H_5 \\ | \\ H \end{array} \quad \text{or} \quad (C_6H_5)_3 \equiv CH$$

On substituting in diphenyl-methane the two hydrogen atoms of the methane remainder by oxygen, benzophenone is formed, which by reduction is changed into benzhydrole:

$$H_5C_6-C-C_6H_5$$
$$\|$$
$$O$$

Benzophenone.

$$H_5C_6-C-C_6H_5$$
$$\diagup\ \diagdown$$
$$H \quad OH$$

Benzhydrole.

If the hydrogen atoms in the phenyl radical are replaced by amidogen NH_2, amido derivatives of phenyl-methane result. Thus diphenyl-methane may become diamido-diphenyl-methane:

$$H_2\overset{4}{N}-H_4C_6-\overset{1}{C}-C_6H_4-\overset{4}{N}H_2$$
$$\diagup\ \diagdown$$
$$H \quad H$$

Diphenyl-methane derivatives[1].

Benzophenone or diphenyl-ketone = $C_6H_5-CO-C_6H_5$.

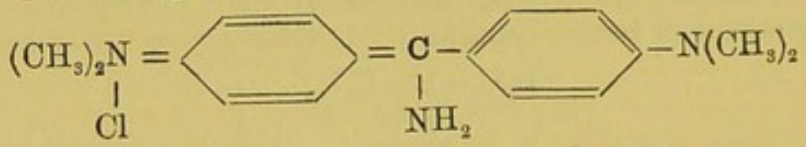
Tetramethyl-diamido-benzophenone, $\begin{matrix}(CH_3)_2NC_6H_4\\(CH_3)_2NC_6H_4\end{matrix}\!\!>\!CO$, or Michler's ketone, is a feeble yellow dye, which by the introduction of sulphur or imide groups [NH] in place of the oxygen of the ketone group, CO, becomes a dark-yellow body. By nascent hydrogen tetramethyl-diamido-benzophenone is converted into the corresponding benzhydrole $\left[\begin{matrix}(CH_3)_2NC_6H_4\\(CH_3)_2NC_6H_4\end{matrix}\!\!>\!C\!<\!\begin{matrix}OH\\H\end{matrix}\right]$. The latter on combining with acids forms blue salts, which, as all dyes of the rosanilin series, become decolourized by an excess of acid. The two most common salts are the hydrochloride and the chloride.

Auramin, obtained by bringing the base of the last-mentioned dye in contact with ammonia, $C_{17}H_{20}N_2O + H_3N = C_{17}H_{21}N_3 + H_2O$, is a yellow basic dye.

Auramin in some respects seems to be an imide of tetramethyl-diamido-benzophenone,

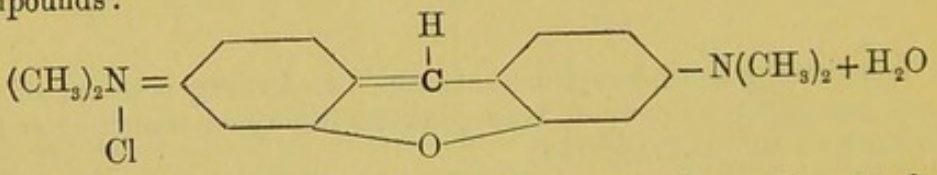
$$\begin{matrix}(CH_3)_2NC_6H_4\\(CH_3)_2NC_6H_4\end{matrix}\!\!>\!C = NH$$

while in other respects it behaves as an amido compound, according to the paraquinone type, thus:



This last constitution allows for the formation of ethyl-, phenyl-, methyl-phenyl-, and diphenyl-auramin (A. Stock).

Pyronin (tetramethyl-diamido-diphenyl-methane) is the only diphenyl-methane dye built after the type of the triphenyl-methane compounds:



In this formula we have, on the left, a paraquinone ring attached to an atom of nitrogen, and also to the carbon of the methane radical thus [$\equiv N = C_6H_4 = C-H$]. As further the two benzene rings are united by an atom of oxygen in the paraposition to the methane-carbon atom, there is formed a central ring consisting of one oxygen, four carbon atoms belonging to the two benzene rings, and one carbon atom derived from the methane.

Pyronin forms a red solution with a yellow fluorescence; by alkalies it

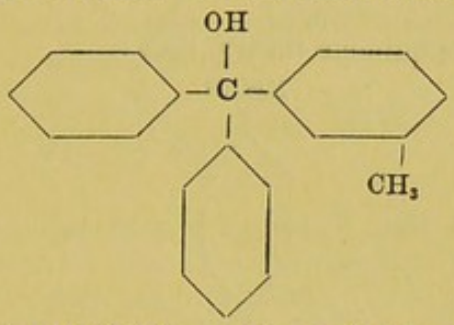
[1] An excellent account of the chemistry of di- and triphenyl-methane compounds is given in Victor Meyer and Paul Jacobson's *Lehrbuch d. organ. Chemie*, 2 [2], 54 and 105, by Jacobson and Arnold Reissert.

becomes converted into the colourless carbinol base, and by acids it is turned yellow.

A substitution product of pyronin, containing a sulphur atom in place of the oxygen atom, has been obtained by Sandmeyer, who acts on tetra-methyl-diamido-diphenyl-methane with the sesquioxide of sulphur (obtained by dissolving sulphur in fuming sulphuric acid). It is a red, strongly fluorescent body.

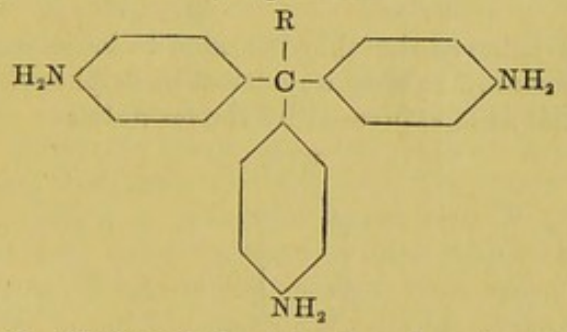
Triphenyl-methanes.

Great difference of opinion exists as to the internal structure of triphenyl-methane compounds and their salts. By oxidizing triphenyl-methane with chromic acid there is formed triphenyl-carbinol $(C_6H_5) \equiv C-OH$, and the chief homologue of the latter is diphenyl-*m*-toluyl-carbinol or 3-methyl-triphenyl-carbinol. This is the mother-substance of rosani-

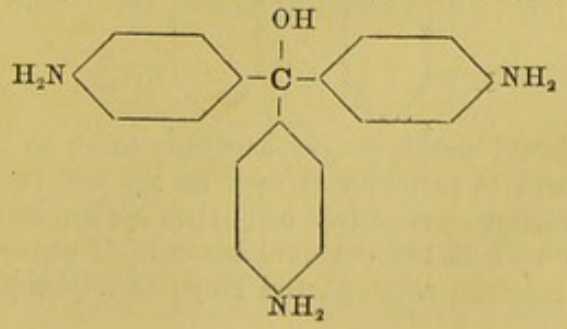


lin, and is readily converted into amido or hydroxyl compounds, which contain the amido group, NH_2, or hydroxyl group always in the paraposition to the methane-carbon.

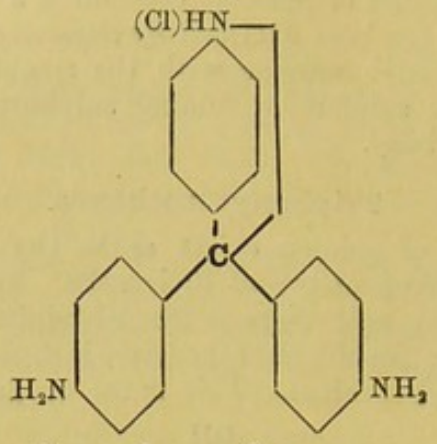
Thus in rosanilin, as regards the three amidogen groups which have been introduced, we find the arrangement:



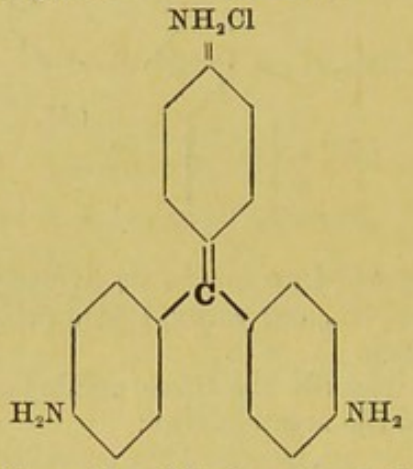
How the fourth affinity of the tetrad carbon is satisfied is doubtful. The so-called rosanilin base (see below), containing the hydroxyl group [OH], has, according to Lefèvre and others, the following formula:



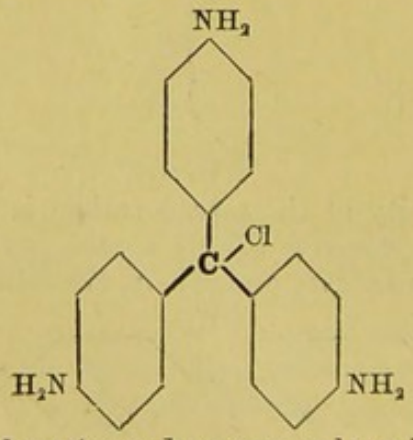
According to E. and O. Fischer, salts of rosanilin—for example, the chloride—are anhydrides, having a bond between the methane-carbon and one nitrogen:



Nietzki upholds the quinone theory, thus:

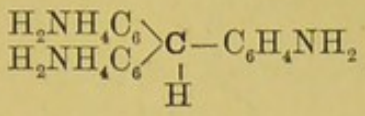


while Rosenstiehl believes the chlorine atom to be joined directly to the methane-carbon, forming in this way triamido-triphenyl-methyl-chloride or a tertiary alcohol as is expressed by the formula:

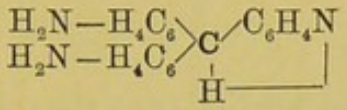


All basic triphenyl-methane dyes can only exist as bases as long as they are in the form of salts, for as soon as the acid radical is removed they become converted at once into colourless carbinols, which latter are erroneously spoken of as the carbinol bases of triphenyl-methane dyes. On introducing into the three phenyl rings of triphenyl-methane three

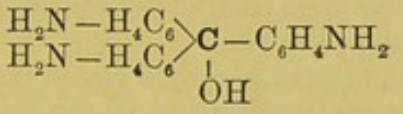
amidogen groups in the paraposition to the methane-carbon ring there is formed a substance called paraleucanilin:



This body by oxidation loses two atoms of hydrogen, and the nitrogen of one amido group undergoes a condensation with the methane-carbon, giving rise to the base pararosanilin,



which only exists in the form of its salts, for as soon as the acid radical is removed it becomes by hydration pararosanilin-carbinol[1], or the so-called carbinol base of pararosanilin:



Hantzsch[2] has isolated triacid dye-stuffs and carbinol salts in the hexamethyl-violet series, of which the hydrobromide salts are stable; thus crystal-violet leuco-hydrate trihydrobromide, $HO{\cdot}C[C_6H_4{\cdot}N(CH_3)_2HBr]_3$, dissolves to form a colourless solution, but becomes on standing or on boiling of a deep violet colour.

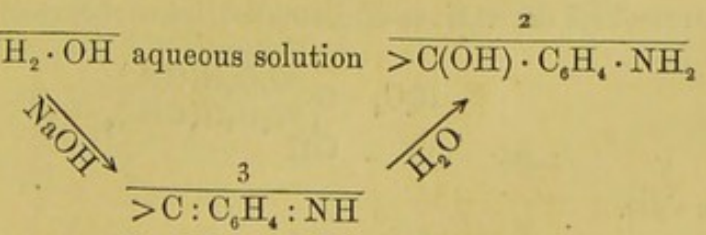
Triphenyl-methane colours may exist in the form of three distinct bases, namely as:

(1) the true coloured ammonium bases, which are insoluble in ether, and exist only in watery solutions, being strong mono-acid bases.

(2) Pseudo-ammonium bases, which are the so-called carbinol bases. They are colourless triacid bases, soluble in ether, and forming colourless salts, which gradually change into the coloured ammonium bases.

(3) Imido or anhydride bases, colourless, soluble in ether, and yielding at once with acids, even with carbonic acid, the coloured dye-salts.

The dye-stuff salt + 1 molecular proportion of caustic soda, gives primarily (1) the true colour ammonium base, which (2) isomerizes in an aqueous solution to the pseudo-ammonium base, and (3) with an excess of the caustic soda is quickly converted into the imido base, which latter (4) by slow hydration forms the pseudo-ammonium base.



[1] Carbinol is a term given to methyl-alcohol, $CH_3 . OH$, which may have one, two, or all three of its methane-hydrogen atoms replaced by alcohol radicals; thus $(CH_3)_3C-OH$ is trimethyl-carbinol.

[2] A. Hantzsch: *Berichte d. deutsch. chem. Gesellsch.* 33, 752–60.

Nietzki, adopting Fischer's view, supposes the chromophore group of all triphenyl-methanes to correspond to this formula:

where R= indicates an imide radical or an atom of oxygen, and he believes the (R=) radical to be the salt-forming group of the basic dyes, because a colourless carbinol base when brought into contact with an animal fibre stains the latter in exactly the same way as if a coloured dye-salt had been used. The fibre therefore reacts towards the imide group as would any acid, such as hydrochloric acid.

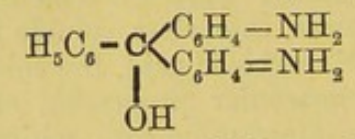
Basic triphenyl-methane dyes readily form sulphonic acids, and then act as acid dyes, without undergoing any colour change.

Triphenyl-methanes.

(1) Rosanilins . . . . . p. 422
(2) Rosolic acids . . . . . p. 426
(3) Phthaleins . . . . . p. 428

1. Rosanilin salts.

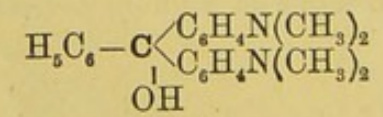
All rosanilins in the form of their so-called bases or carbinol compounds are colourless, and become coloured only by the introduction of salt-forming radicals. The mother-substance from which the simplest rosanilin dyes are obtained is diamido-triphenyl-methane:



which by oxidation may be converted into a violet dye.

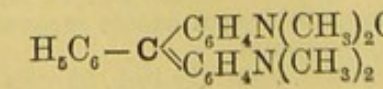
Diamido-triphenyl-carbinols.

Tetramethyl derivatives of diamido-triphenyl-carbinol:



Basic dyes.

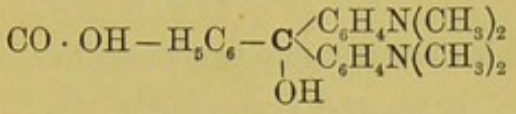
Malachite green. The chloride of this substance has the formula:



The common salts are the hydrochloride ($C_{23}H_{24}N_2HCl$), the sulphate ($C_{23}H_{24}N_2H_2SO_4$), and the double zinc chloride ($C_{23}H_{24}N_2ZnCl_2$).

Brilliant green corresponds to malachite green, but is a tetraethyl compound.

Chrome green is a CO·OH or carboxylic derivative, containing the basic radical:

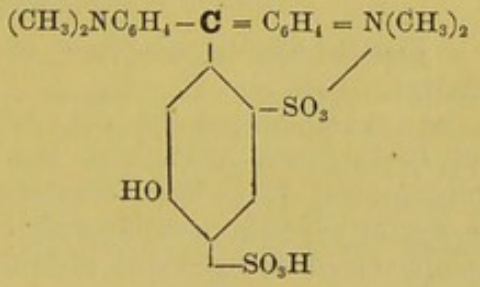


The commercial salt is usually the chloride.

Acid dyes.

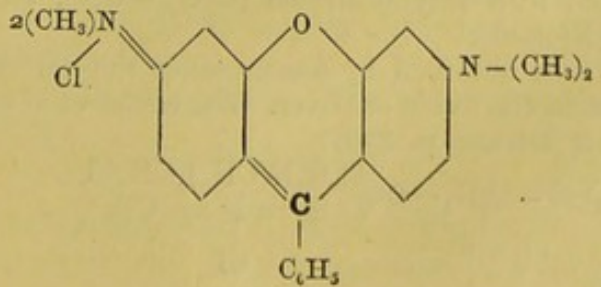
Acid green S is a sulphonic acid compound of diethyl-dibenzyl-diamido-triphenyl-carbinol. The sulphur is contained in the benzene nucleus.

Patent blue contains hydroxyl groups in the non-amidated benzene rings of malachite green, the hydroxyl being in the metaposition to the methane-carbon, and the two sulphonic groups being one in the para- and the other in the ortho-position to the methane-carbon. The staining properties seem to depend on the sulphur groups and not on the carbinol radical, because the salts are not rendered colourless by the addition of alkalies, as in other rosanilin salts. Although this dye contains two sulphonic groups, it behaves as a monobasic acid, and Sandmeyer has obtained from the orthosulphonic acid of the benzaldehyde alkali-proof dyes with hardly any acid properties, which leads Nietzki to assume a bond between the sulphonic group and the dimethyl-ammonium group, thus:



By oxidation with ferric chloride, the dye **cyanin** is obtained.

Rosamin forms bluish-red solutions showing a yellow fluorescence; it is analogous to pyronin (p. 418) as it possesses an oxygen atom which unites the two amidated benzene rings, and in this respect it differs also from the malachite green series:



Triamido-triphenyl-carbinols.

Pararosanilin is formed by heating two molecules of anilin with one molecule of paratoluidin in the presence of oxidizers such as arsenious acid or corrosive sublimate. The pararosanilin base

$$H_2N-C_6H_4-C(OH)\begin{cases}C_6H_4\cdot NH_2\\C_6H_4\cdot NH_2\end{cases}$$

by reducing agents is converted into paraleucanilin,

$$H_2N-C_6H_4-CH\begin{cases}C_6H_4\cdot NH_2\\C_6H_4\cdot NH_2\end{cases}$$

and if the base unites with chlorine, the hydrochloride

$$H_2N-C_6H_4-C\begin{cases}C_6H_4\cdot NH_2\\C_6H_4=NH\cdot HCl\end{cases}$$

is formed, usually known as **Basic rubin.** The acid rubin or rubin S is a sulphonic acid compound, and corresponds to the acid fuchsin prepared from rosanilin (see p. 425).

Methyl-violet is an oxidation product of dimethyl-anilin, and consists of a mixture of tetra-, penta-, and hexamethyl-rosanilin, the bluer the solution the higher the percentage of the hexa-compound:

$$(CH_3)_2N-C_6H_4-C\begin{cases}C_6H_4-N(CH_3)_2\\C_6H_4=NH\end{cases} = \text{tetramethyl-pararosanilin.}$$

$$(CH_3)_2N-C_6H_4-C\begin{cases}C_6H_4-N(CH_3)_2\\C_6H_4=NCH_3\end{cases} = \text{pentamethyl-pararosanilin.}$$

$$[(CH_3)_2N-C_6H_4]_2=C=C_6H_4=N(CH_3)_2\diagdown Cl = \text{hexamethyl-pararosanilin-chloride or crystal violet.}$$

Methyl-violet is a base which, in the form of mono-acids, forms amorphic greenish masses.

Gentian violet, as manufactured by the Aktiengesellschaft für Anilin-fabrikation in Berlin, is a mixture of the chlorides of penta- and hexamethyl-pararosanilin. It is best to substitute the pure hexamethyl compound or crystal violet for gentian-violet.

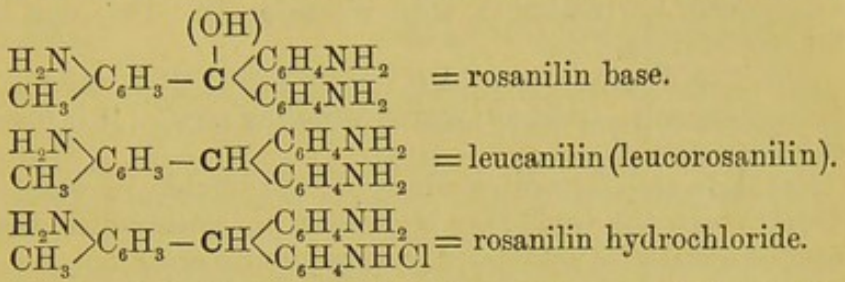
Acid violet is a sulphonated methyl-violet, and acts as an acid, because of the HSO_3 group.

Methyl-green is the chlormethylate of the hexamethyl-pararosanilin-chloride or heptamethyl-pararosanilin, and is obtained by treating methyl-violet with methyl-iodide (CH_3I) or methyl-chloride (CH_3Cl). Because of the method of its preparation and the incomplete removal of the methyl-violet, the commercial salt, if required in a pure state, should be shaken with amylic alcohol ($C_5H_{11}-OH$), which removes the methyl-violet (Nietzki).

The base may be obtained by decomposing the chloride or iodide of methyl-green with the oxide of silver. The commercial salt has probably this constitution (see also p. 459):

$$(CH_3)_2N-C_6H_4-C\begin{cases}C_6H_4N(CH_3)_2CH_3Cl\\C_6H_4=\underset{\displaystyle Cl}{\underset{|}{N}}=(CH_3)_2\end{cases} \quad \text{(Nietzki).}$$

Basic fuchsin or **Rosanilin** (methyl-triamido-triphenyl-carbinol or triamido-diphenyl-toluyl-carbinol) is formed by the oxidation of an equal number of molecules of orthotoluidin, paratoluidin and anilin. Nietzki points out that rosanilins which have been prepared from orthotoluidin and diamido-diphenyl-methane differ from those obtained from anilin and diamido-phenyl-toluyl-methane, as the former contain the quinone-like chromophore attached to the benzene, while the latter have it attached to the toluene remainder. (For New-fuchsin see p. 458.)

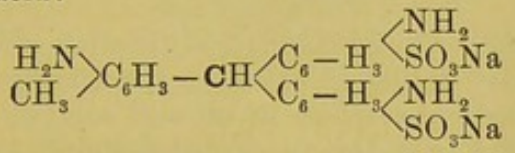


= rosanilin base.

= leucanilin (leucorosanilin).

= rosanilin hydrochloride.

Acid fuchsin, Fuchsin S, is a disulphonic acid prepared from the basic fuchsin by treating the latter at a temperature of 120° C. with fuming sulphuric acid:



Its neutral salts are colourless, while the mono-acid salts are red. As this acid is coloured, an intramolecular salt formation between the sulphonic and the amido groups must take place (Nietzki).

Iodine green or pentamethyl-rosanilin

$$C_{20}H_{17}(CH_3)_4N_3ICH_3I + H_2O$$

is obtained by heating rosanilin with methyl-iodide and methyl-alcohol at 100° C. The best way to purify it from methyl-violet is by amylic alcohol. Its double zinc salt ($C_{25}H_{31}N_3Cl_2ZnCl$) is very soluble. The presence of iodine may be shown by dissolving some of the solid in strong sulphuric acid, and then adding a minute crystal of potassium bichromate (Bolles Lee).

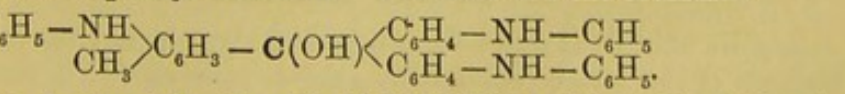
Hoffmann's violet is triethyl-rosanilin ($C_{20}H_{18}(C_2H_5)_3N_3O$). Its iodide ($C_{26}H_{35}N_3I_2$) is more readily soluble in alcohol than in water.

On treating rosanilin at a temperature of 180° C. with a great excess of anilin in the presence of such organic acids as benzoic acid

$$C_6H_5-COOH,$$

which play the part of catalyzers, there are formed mono-, di- and triphenyl-rosanilin compounds, called respectively rosanilin blue, Parma blue (regina violet), and anilin blue.

Anilin blue or triphenyl-rosanilin. The base has the formula:



Anilin-blue hydrochloride ($C_{20}H_{16}N_3(C_6H_5)_3HCl$) is insoluble in water but soluble in alcohol, and is therefore called 'alcohol blue'

(Spritblau). Anilin blue is a base which, because of its insolubility in water, is sulphonated, and thus converted into water-soluble anilin blue.

Sulphonated anilin blues are acid dyes.

'**Methyl blue**' is the trade name for sulphonated anilin blue.

(*a*) The monosulphonic acid of triphenyl-rosanilin, $C_{38}H_{30}N_3(HSO_3)$. **Alkali blue** is its sodium salt, and will dye wool and silk in feebly alkaline media. Nietzki believes the basic radical of the rosanilin to link the dye to the fibre. After dyeing the colour is fixed and brightened by treatment with dilute acids. This dye is chiefly used for wool-dyeing.

(*b*) The disulphonic acid ($C_{38}H_{29}N_3(HSO_3)_2$) is soluble in water, and its sodium salt is sold as '**water blue for silk.**' It is insoluble in dilute sulphuric acid.

(*c*) Tri- and tetrasulphonic acids are soluble in water and in dilute H_2SO_4. In the trade they are known as '**water blue for cotton,**' and are used in baths acidulated with sulphuric acid.

Diphenylamin blue is got by heating diphenylamin with carbon sesquichloride (C_2Cl_6) or oxalic acid at 110–120° C. Similar dyes are obtained by acting on methyl-diphenylamin with oxidizers such as quinone chloride.

Aldehyde green. On treating rosanilin with aldehyde and strong sulphuric acid, a violet dye of unknown constitution is formed, which in the presence of sodium sulphite in an acid solution is converted into aldehyde green, which is soluble in alcohol containing sulphuric acid.

Diphenyl-naphthyl-methanes.

Victoria blue B is prepared from tetramethyl-diamido-diphenyl-methane and phenyl-*a*-naphthylamin:

$$\begin{matrix}(CH_3)_2NC_6H_4\\(CH_3)_2=NC_6H_4\end{matrix}\!\!>C=C_{10}H_6=N-C_6H_5$$

Victoria blue 4R is the pentamethyl-carbidride of the former:

$$\begin{matrix}(CH_3)_2NC_6H_4\\CH_3\!\!>\!\!NC_6H_4\\C_{10}H_7\end{matrix}\!\!>C=C_6H_4=\underset{\underset{Cl}{|}}{N}=(CH_3)_2$$

Night-blue is toluyl-tetraethyl-triamido-*a*-naphthyl-diphenyl-carbinol:

$$\begin{matrix}(C_2H_5)_2NC_6H_4\\CH_4C_6H_4HNC_{10}H_6\end{matrix}\!\!>C=C_6H_4=\underset{\underset{Cl}{|}}{N}=(C_2H_5)_2$$

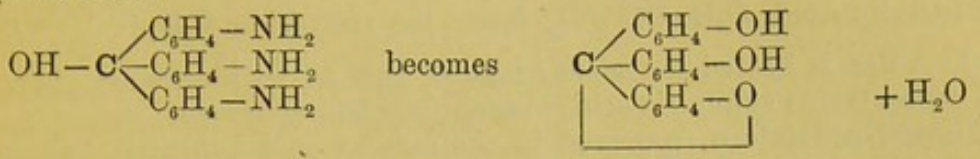
Kresyl-violet has this formula:

$$C-(C_6H_4NH\cdot C_6H_4CH_3)_3$$

2. Rosolic acid derivatives.

Rosolic acid dyes differ from rosanilin dyes in having the nitrogenous radicals of the latter replaced by oxygen radicals. All members of this group are acids, which in the free state are yellow, but in combination, as salts, of a red colour.

Aurin or **Pararosolic acid** is derived from pararosanilin by substitution of the three amidogen groups of the latter by three hydroxyl groups accompanied by a removal of the carbinol-hydroxyl in pararosanilin, thus:

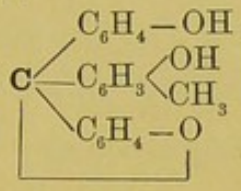


Pararosanilin. Pararosolic acid or aurin.

Yellow corallin is a mixed product, containing about 20 per cent. of aurin. It is practically insoluble in water, but readily soluble in alcohol, with a yellow colour which is turned red by alkalies.

Red corallin is an intermediate product of aurin and pararosanilin, in which one of the hydroxyl groups of aurin is replaced by NH_2.

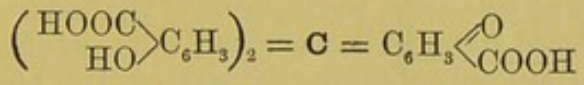
Rosolic acid is formed from rosanilin by a change analogous to that described for pararosolic acid:



Almost insoluble in water and soluble in alcohol, it turns red on the addition of alkalies, and is used extensively for titration.

Eupittonic acid or hexamethoxyl-aurin ($C_{19}H_8(O{\cdot}CH_3)_6O_3$) is a dibasic acid, forming orange-yellow needles insoluble in water but soluble in alcohol and ether. The solution of its salts is coloured blue, and forms with heavy metals, such as lead and tin, blue lakes.

Chrome violet (aurin-tricarbonic acid) and its homologues have this constitution:

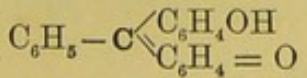


and are obtained by Sandmeyer's process, which consists in condensing salicyclic acid by means of formaldehyde in a sulphuric acid solution and inducing simultaneously an oxidation.

With tissues mordanted in chrome salts reddish-violet tints are obtained.

Benzo-trichloride derivatives.

Benzaurin is obtained by acting on two molecules of phenol with one molecule of benzene trichloride. It has this constitution:



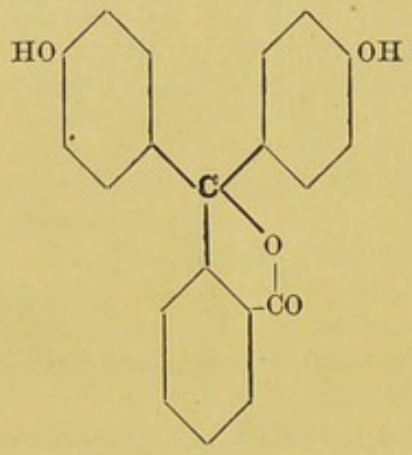
It is insoluble in water, but soluble in alcohol, ether, and glacial acetic acid, with a yellow colour, while alkalies change its colour to violet, acids precipitating it from an alkaline solution in yellow flakes. Alkali bisulphates form, analogously to the rosolic acid series, water-soluble compounds.

Anthracene violet is made from benzene trichloride and pyrogallol, and forms lakes with metallic mordants.

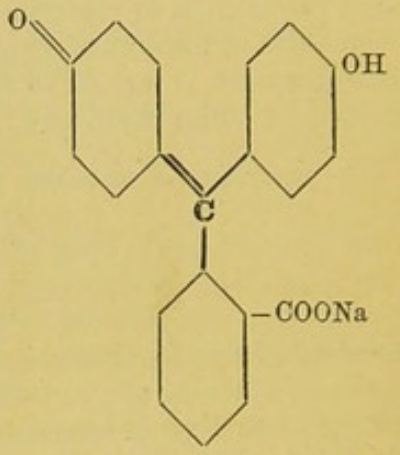
3. Phthaleins.

Phthalic acid, $C_6H_4(COOH)_2$, forms the anhydride $C_6H_4\langle{CO \atop CO}\rangle O$. If the latter is heated for some hours at 120° C. with ordinary phenol and concentrated sulphuric acid, phenol-phthalein is formed.

Phenol-phthalein, prepared in the manner just indicated, has the constitution figured below, showing the existence of a lactone ring. On coming in contact with alkalies, one of the hydroxyl groups is believed by Nietzki to be replaced by O, forming a quinone-like benzene ring, while simultaneously the lactone ring is opened up in the manner indicated :

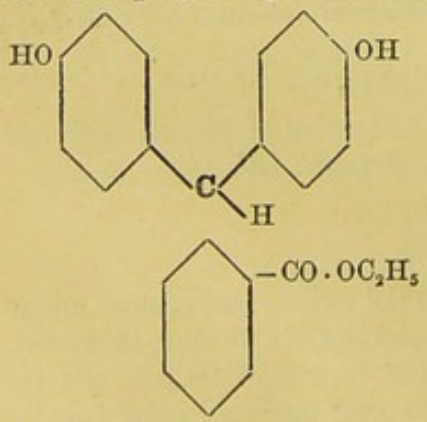


Phenol-phthalein.

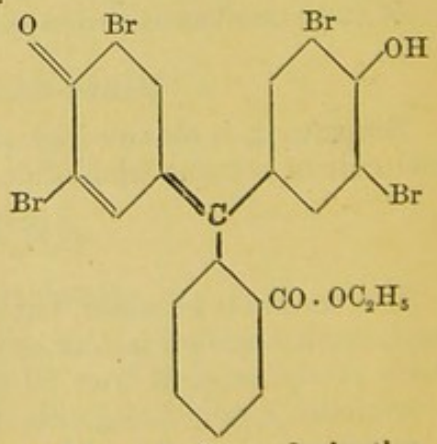


Sodium-phenol-phthalein.

Tetrabrome-phenol-phthalein-ethylester is a derivative of quinonoid phenol-phthalein, and is obtained by converting the ester of phenol-phthalein first into a bromine compound, and subsequently oxidizing this by means of alkaline potassium ferricyanide. The ester is yellow when in its free uncombined state, while the alkali salts are of an intense blue colour. The salts are only slightly soluble in water, but fairly soluble in alcohol, and may readily be decolourized by means of acetic acid. When colourless they stain silk of a deep blue colour, showing that the silk must play the part of a base (Nietzki):

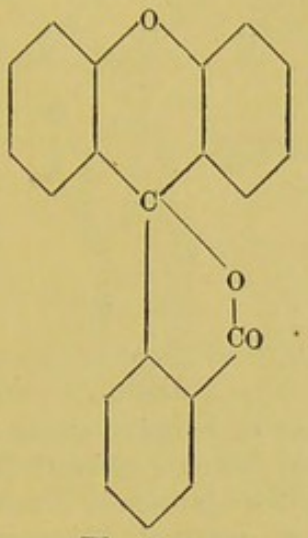


Phenol-phthalein-ethylester



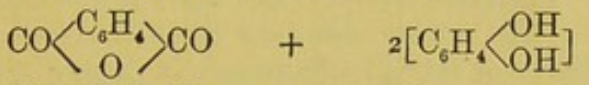
and its tetrabrom derivative.

Fluorane (R. Meyer) is formed in small quantities during the manufacture of phenol-phthalein, owing to the phthalic acid remainder taking up an orthoposition to the hydroxyl groups of the phenols and then undergoing an internal dehydration. It forms the mother-substance of fluorescein :



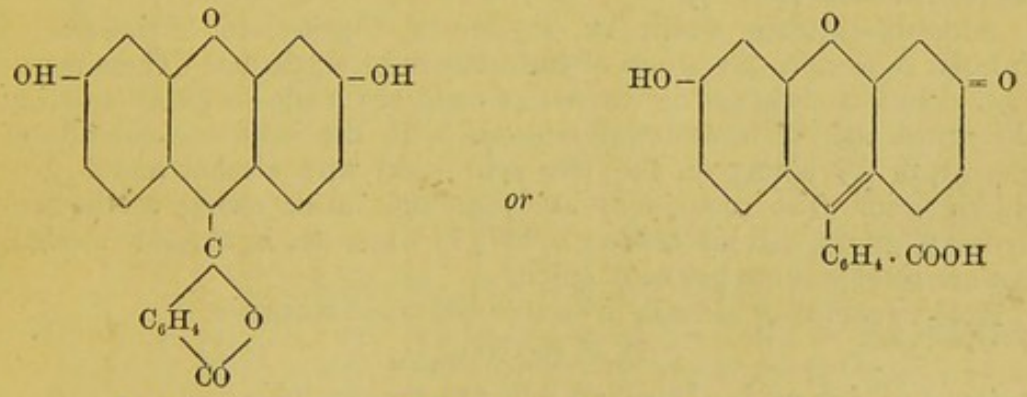
Fluorane.

Fluorescein is prepared from phthalic anhydride and resorcinol :



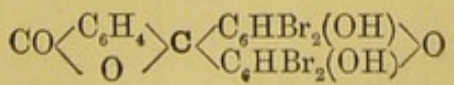
Phthalic anhydride + Resorcinol.

and may be considered as a lactone or a quinonoid compound :

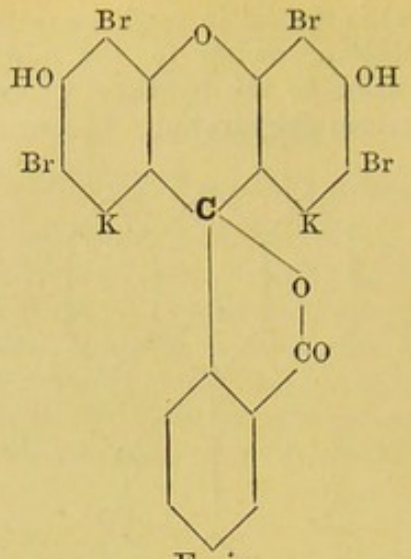


Lactone formula. ← **Fluorescein** → Quinonoid formula.

Uranin is its sodium salt.
Eosin and **Erythrosin** are its two most important derivatives.
Eosin or tetrabrom-fluorescein



is practically insoluble in water, but with potash it forms a brown powder, the **Water-soluble eosin** ($C_{20}H_6Br_4O_5K_2$) :



Eosin.

The greater the number of bromine atoms in eosin the bluer is the shade, while the mono- and dibrome derivatives are yellowish-red.

Being a derivative of the quinonoid fluorescein it contains a free hydroxyl group, and forms insoluble lakes with lead, tin, aluminium and also with chrome compounds.

Erythrosin is the sodium salt of tetriodo-fluorescein, $C_{20}H_8I_4O_5$. The free acids of eosin and erythrosin are readily obtained by adding mineral acids to the watery solutions of their salts, when the water-insoluble tetrabrom- or tetriodo-fluorescein is thrown down. The latter dissolves with a yellow colour in pure alcohol, ether, or chloroform, and in these solutions may be used, according to Mylius' method, as a test for free alkalies (p. 214).

Alcohol-soluble eosin or erythrosin (Sprit-eosin, Primerose à l'alcool) belongs to the group of eosin-ethers or erythrins. These mono-methyl and mono-ethyl derivatives of eosin are made by either treating the potash salt of tetrabrom-fluorescein with the iodide or chloride of methyl, or by acting on the free acid eosin with alcohol and hydrochloric acid. The compounds are monobasic acids owing to the free hydroxyl group, and are neither soluble in water nor in absolute alcohol, but are soluble in 50 per cent. spirit.

Eosin scarlet or safrosin is dinitro-dibrome-fluorescein

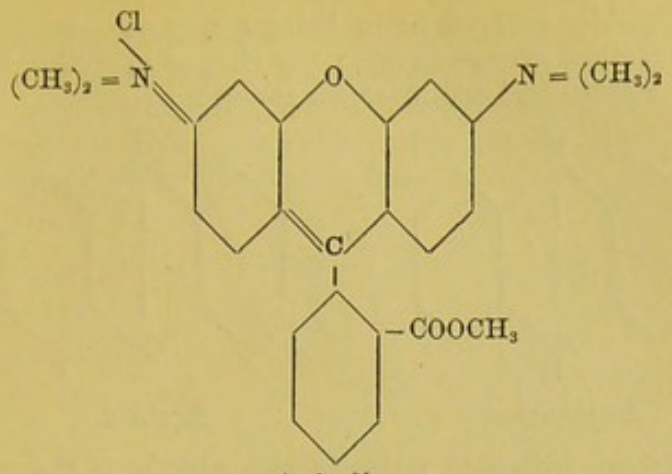
$$C_{20}H_8Br_2(NO_2)_2O_5,$$

a strong dibasic acid. Its alkali salts are water-soluble.

Tetrabrom-tetrachlorfluoresceins are brome compounds of di- and tetrachlorfluorescein. The water-soluble alkali salts are called **Phloxin**, the ethyl ethers are **Cyanosins**, and the tetriodide derivatives form **Rose Bengale**.

By substituting sulphur for the O in the linking ring, dichlorfluorescein is further converted, after combining with bromine, into **Cyclamin**.

Rhodamin (p. 188) is the phthalein of diethyl-meta-amido-phenol and may be considered to be the orthocarbonic acid of rosamin. The penta-alkylated ester having the accompanying formula is a basic substance, forming water-soluble salts, and is called **Anisolin** (Monnet in Geneva).

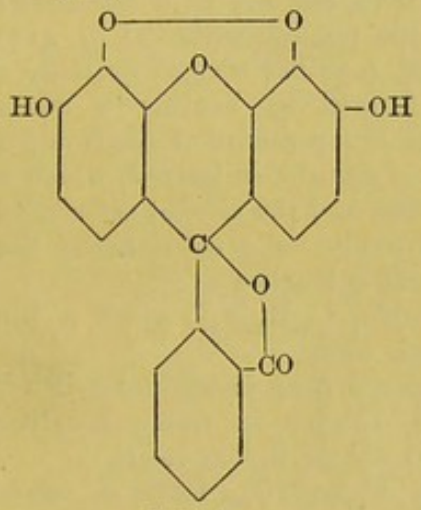


Anisolin.

Rhodamin 6G is a symmetrical diethyl-rhodamin.

Rhodamin S is a succinate of rhodamin, and dyes cotton directly.

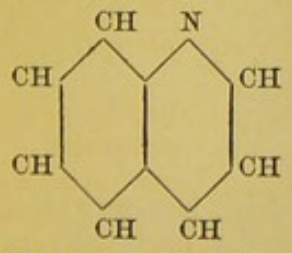
Gallein differs from fluorescein only by the presence of two quinone oxygen atoms which occur in two benzene rings. With alumina and chromium oxide it forms greyish-violet insoluble lakes:



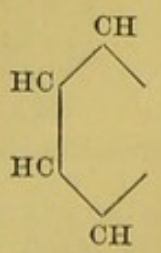
Gallein.

I. Quinolin and Acridin Dyes.

Quinolin, C_9H_7N, may be looked upon as either a naphthalene derivative in which one of the CH or methin groups of naphthalene has been replaced by N, or as a condensation product of one benzene nucleus and a pyridin nucleus, thus:

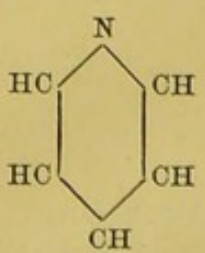


Nitrated naphthalene.



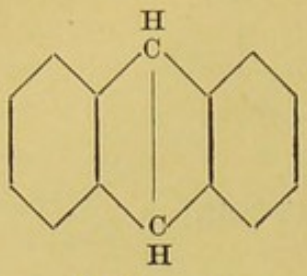
Benzene remainder

\+

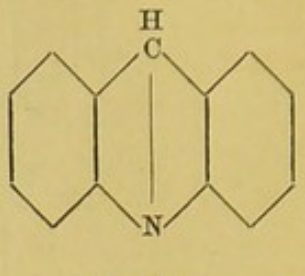


Pyridin.

Acridin resembles anthracene in being a ring compound, but instead of having two methin groups forming the ring as in anthracene, we find in acridin one methin and one nitrogen group :



Anthracene.



Acridin.

The chromophore group in quinolin and acridin is the pyridin ring. According to Nölting, quinolin, containing in the benzene ring an OH group in the orthoposition, reacts with mordants to form lakes, resembling in this respect the oximes (v. Kostanecki).

1. Quinolin Compounds.

Quinolin by itself does not act as a chromogen, and it is probable (Nietzki) that, as in the phenyl-methanes (p. 417), so here, a methane compound serves to link together several quinolin rings.

Cyanin blue is formed by heating a mixture of quinolin and paramethyl-quinolin in the presence of alkali and an alkyl iodide, when the blue mono-iodide of cyanin is formed, which contains one molecule of either of the two bases and the alcohol remainder twice.

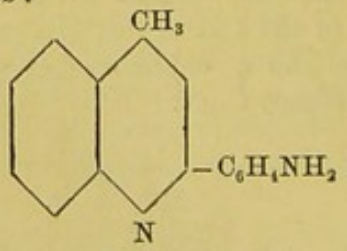
The cyanins are strongly basic substances; the mono-acid salts are blue, and the diacid salts colourless.

Dimethyl-cyanin iodide, $C_{21}H_{19}N_2I$, gives a blue solution which is decolourized by carbonic acid.

Quinolin red prepared from iso-quinolin is a basic dye. Its hydrochloride, $C_{26}H_{18}N_2HCl$, soluble in water, is strongly fluorescent, and stains animal tissues the same tint as eosin.

Quinolin yellow, $C_{18}H_{11}NO_2$, forms a sulpho-acid, which stains similarly to picric acid.

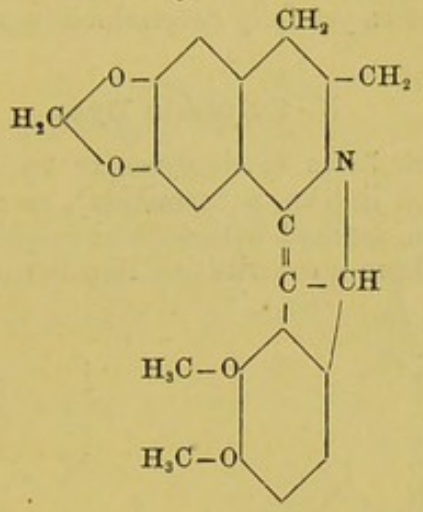
Flavanilin, $C_{16}H_{14}N_2$, is a strong base owing to the presence of an amido group. Its diazo compound flavanol, $C_{16}H_{13}NO$, shows both basic and feebly acid properties :



Flavanilin (Nietzki).

Berberin, $C_{20}H_{17}NO_4$, is the only natural dye which possesses alkaline characters (Nietzki). It is found in the Calumba root (*Jateorhiza Calumba*, Miers) and in the root of the barberry (*Berberis vulgaris*), being

a derivative of iso-quinolin (Perkin, *Chem. Soc. Journ.* 991, 1890). It forms with nitric acid a crystalline compound, $C_{20}H_{17}NO_4HNO_3$, insoluble in an excess of the acid. It stains animal tissues directly, and cotton which has been mordanted with tannin. Its constitution has been determined by H. W. Perkin, jun.

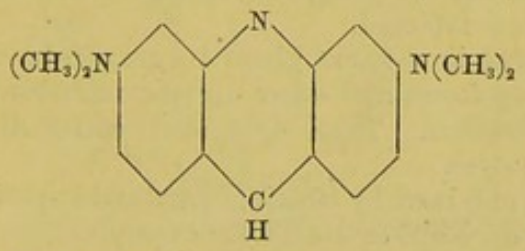


2. Acridins.

Acridin itself is only slightly coloured, but its diamido compounds are strong dyes. As a rule the amido groups take up a paraposition to the carbon atom of the linking ring.

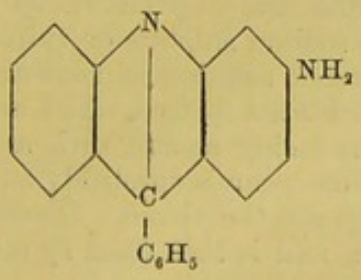
(*a*) Diamido-acridins are sold commercially under the name of acridin yellow or acridin orange.

Acridin yellow or tetramethyl-diamido-acridin has this constitution:



(*b*) Diamido-phenyl-acridins.

Benzo-flavin or symmetrical diamido-phenyl-acridin has this constitution:



It stains wool and silk a yellow colour.

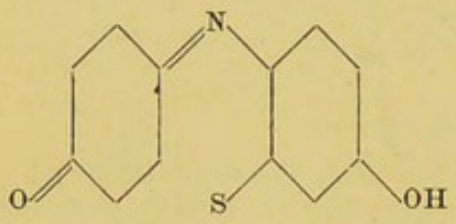
Phosphin is the nitrate or hydrochloride of chrysanilin, which latter is an asymmetrical diamido-phenyl-acridin, as it contains one

amido group in the acridin nucleus, while the other NH_2 group is in the phenol ring, both amido groups occupying the metaposition to the carbon atom of the methane group. Chrysanilin nitrate or phosphin is a basic yellow dye.

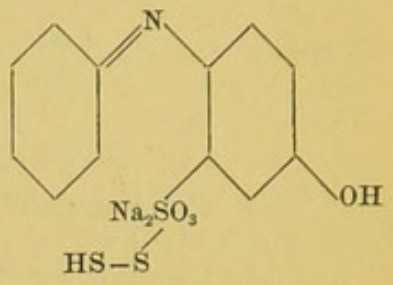
(*c*) **Flaveosin** is a yellow dye with marked fluorescence, the constitution of which is not as yet fully determined (see Nietzki, edition 1901, p. 274).

K. Sulphide Dyes.

According to Jasek these black dyes are formed when a diamin or monamin (substituted in the paraposition), or any compound capable of giving rise to them, is fused with sodium sulphide and sulphur. The dyes are believed to have a constitution like Bernthsen's thionol :



Thionol.



Sulphide black.

The theory of dyeing is as follows: The sulphide dyes are converted into soluble leuco-compounds by the presence of alkaline reducing agents, and as leuco-compounds they readily enter fabrics. On subsequent oxidation with an acid oxidizing agent such as a mixture of potassium chromate and sulphuric acid, the colour is oxidized and fixed as a black insoluble compound.

Cachou de Laval is a name given to greyish-brown or black dyes obtained by treating faeces and other organic matter with a mixture of caustic soda and sulphur. These dyes stain cotton directly, and act as mordants for basic dyes.

Vidal black is obtained by treating para-amido-phenol and quinones with alkali sulphides, Na_2S or NaHS for example.

Immedial black (Cassella & Co.) is prepared on similar principles from dinitro-oxy-diphenylamin (made by the interaction of dinitro-chlorbenzene and *p*-amido-phenol).

Clayton's Fast Blacks (the Clayton Aniline Co., Ltd.) are insoluble in water, but soluble in sodium sulphite or sulphide. Dissolve the dye by boiling in a concentrated solution of neutral sodium sulphite, when probably thiosulphonic acids are formed, which are only slightly coloured and do not stain. By reducing agents, such as glucose and an alkali, there are formed leuco-compounds (probably sulph-hydryl derivatives), which are used to impregnate the tissue. On exposing the latter to air the black colour appears, and is then fixed by a bath of copper sulphate or an alkali bichromate.

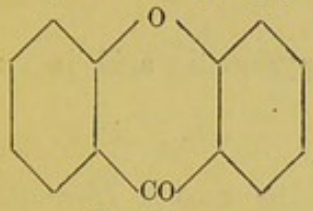
Nitro-sulphide of iron was first used by Z. Roussin in 1860, and has, according to him, the formula $Fe_3S_5K_2(NO)_4$, while O. Pawell gives the formula $Fe_7S_5K_2(NO)_{12}$.

Prud'homme prepares the dye as follows[1]: Dissolve 8 grams of sodium sulphide (Na_2S) and 5 grams of sodium nitrite ($NaNO_2$) in 200 cc. of water. Then dissolve 14 grams of ferrous sulphate in boiling water and add it slowly under constant stirring to the other mixture. Boil for several minutes; filter; wash residue with hot water till the filtrate amounts to 500 cc. This diluted with water forms the dye-bath.

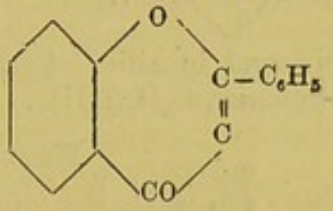
II. Natural Dyes.

Dyes containing the ketone or carbonyl group, CO, as a chromophore.

The ketone group, CO, is most active as a chromophore when it forms with oxygen a closed ring as in xanthone. It also acts in an open chain, especially if several hydroxyl groups (OH) are in the orthoposition to it, but only indirectly, by joining on to metallic oxides:



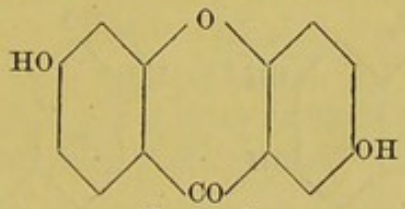
Xanthone.



Flavone.

Euxanthone, $C_{13}H_8O_4$, is obtained from the Purrée or Indian yellow, which contains euxanthic acid, an ester-like compound of euxanthone and glycuronic acid.

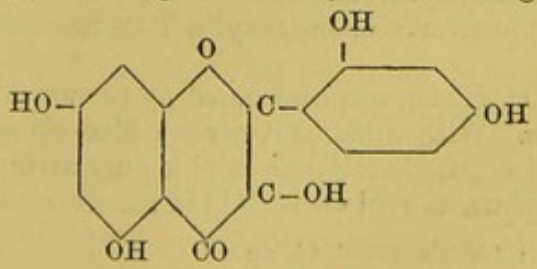
The magnesium salt of euxanthic acid constitutes the yellow paint, Indian yellow:



Euxanthone.

Flavone is a phenyl-phenopyrone, the constitutional formula of which is given above. It forms, according to v. Kostanecki, the chief nucleus in a number of vegetable colouring matters, such as quercetin, rhamnetin, and morin.

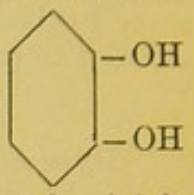
Morin, $C_{15}H_{10}O_7$, is obtained from *Morus tinctoria*, Jacq, or *Maclura tinctoria*, Nettel. According to A. G. Perkin and Bablich it is a tetraoxyl-flavanol, having, according to Nietzki, the following constitution:

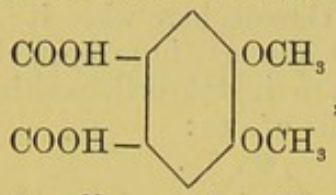


[1] Prud'homme: *Monit. scient.* 251–2 (1901).

Its alkali salts are readily soluble, while the calcium, aluminium, lead, and zinc salts are only slightly soluble.

It is used extensively for dyeing wool.

Related to flavone are also the two pyrocatechin derivatives haematoxylin and brazilin. Pyrocatechin or orthodioxy-benzene 

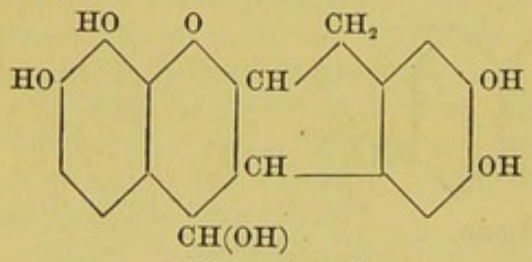
sometimes also called catechol, is the mother-substance of meta-hemipinic acid 

, which latter has been obtained from both haematoxylin and brazilin. Pyrogallol or pyrogallic acid is 1 : 2 : 3 trihydroxy-benzene, $C_6H_3(OH)_3$.

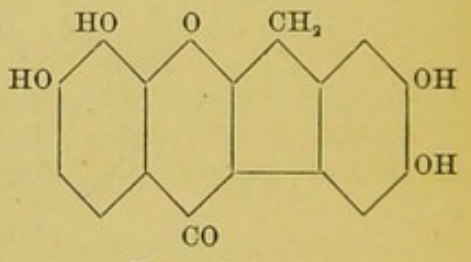
Haematoxylin.

Haematoxylin, $C_{16}H_{14}O_6$, obtained from the wood of *Haematoxylon campechianum* by extraction with ether, is composed of a pyrogallol radical joined to pyrocatechin. Haematoxylin is not a dye in itself, but becomes a dye on being oxidized into haematein, as was first pointed out by Erdmann[1].

The constitution of haematoxylin has been determined by Perkin and Yates[2]:



Haematoxylin.



Haematein.

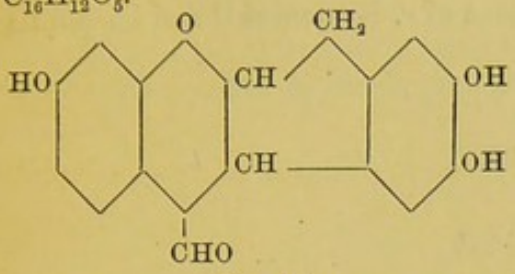
Haematoxylin dissolves in alkalies with a purple colour, and on oxidation into haematein the colour changes first into bluish-violet, and then into a brown. With chrome, iron, and vanadium salts, haematoxylin forms higher oxides, the lakes of which possess a black colour, while the aluminium lake is greyish-violet, and the copper lake a deep blue. The salts of haematoxylin after oxidation are known as haemateates. Nitric acid first converts haematoxylin into haematein, and then into oxalic acid[3].

Haematein, $C_{16}H_{12}O_6$, was discovered by Chevreul in 1810, and called by him haematin. With alkalies it gives a blue-violet colour. The ammonia compound $C_{16}H_{12}O_6(NH_3)_2$ is soluble only with great difficulty. By boiling with sulphurous acid haematein is reconverted into haematoxylin.

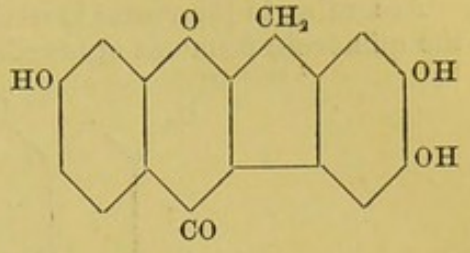
[1] Erdmann: *Liebig's Annal.* 44, 292.
[2] Perkin and Yates: *Proc. Chem. Soc.* 16, 107 (1900).
[3] Pure haematoxylin is only obtainable from Geigy and Co., Basel.

Brazilin.

Brazilin, $C_{16}H_{14}O_5$, is obtained from *Caesalpinia brasiliensis*, Siv., and other species. This substance is the homologue of haematoxylin, and does not act as a dye till it has been oxidized into **Brazilein**, $C_{16}H_{12}O_5$.



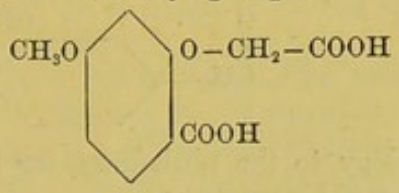
Brazilin.



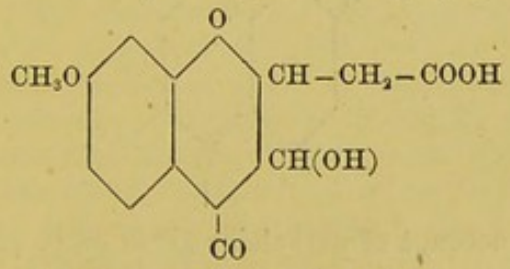
Brazilein.

According to the investigations of Gilbody, Perkin, and Yates [1], on oxidizing trimethyl-brazilin with potassium permanganate there is formed a dibasic acid $C_{10}H_{10}O_6$, melting-point 175° C., containing one methoxyl group. This acid must be derived from the resorcinol portion of the molecule of brazilin, because after fusion with potash it gives with ferric chloride the characteristic violet colour of resorcin.

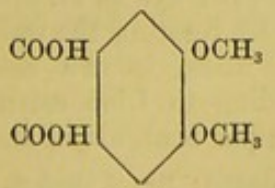
When heated to 200° the dibasic acid gives off CO_2, and forms the monobasic acid: methyl-carboxy-resorcyl-acetic acid, $CH_3O-C_6H_4-O-CH_2-COOH$. The dibasic acid is therefore methyl-carboxy-resorcinol-acetic acid, containing one methoxyl group:



Another monobasic acid, with a melting-point of 129·5° C., was obtained, namely, para-methoxy-salicylic acid $C_{10}H_8O_3-(OCH_3)-COOH$:



Apart from traces of two acid bodies, which on fusion with ferric chloride give the pyrocatechin reaction, the other chief acid is meta-hemipinic acid:

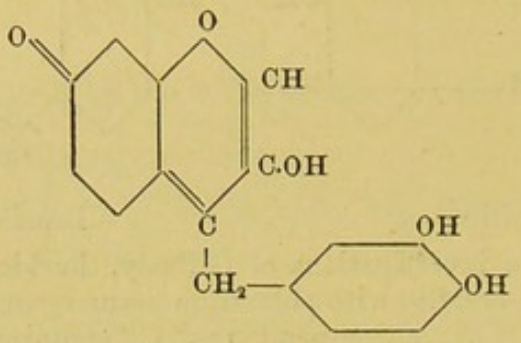


[1] Gilbody, Perkin, and Yates: *Proc. Chem. Soc.* 16, 105–107 (1900).

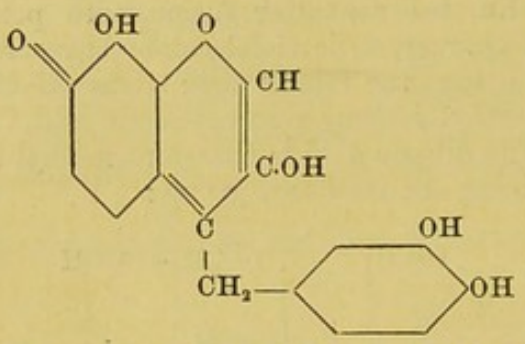
The constitutional formula of brazilin, when compared with that of haematoxylin, is seen to differ only in possessing one hydroxyl group or methoxyl group less.

The alum lake of brazilein resembles alizarin red, while the chrome ake is of a rich brown colour.

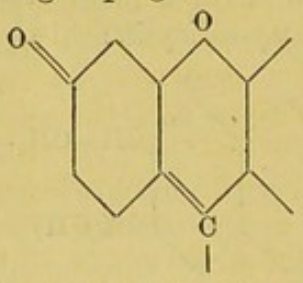
According to the recent investigations of v. Kostanecki[1] and his pupils, the quinonoid formulae of brazilein:



and of haematein:



express best their analogy to the phthaleins, for the latter also possess the chromophoric quinonoid grouping:



Compare with the account of phthaleins given on p. 428.

Carmin.

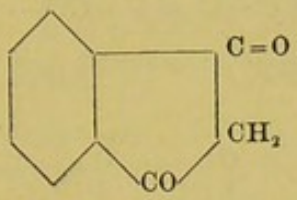
Carmin is obtained from the female *Coccus cacti coccinellifera*, which according to P. Mayer[2] stores the dye as an alkali carminate in the fatty body and yolk of the ova. By treating the watery cochineal extract with alum the amorphous red mass, known commercially as carmin, is obtained, containing, according to Liebermann, 74 per cent. carminic acid, 20 per cent. proteid, 3 per cent. alum, and 3 per cent. lime.

[1] St. v. Kostanecki and V. Lampe: *Ber. d. deutsch. chem. Gesell.* 35, 1667 (1902); E. Bollina, St. v. Kostanecki and J. Tambor: *ibid.* 35, 1675 (1902).

[2] Mayer: *Mittheil. a. d. Zool. Station, Neapel*, 10, 509 (1892).

Carminic acid is obtained from the cochineal insect by extracting the latter with boiling water, and treating the extract with lead acetate or barium hydrate, and finally decomposing the lead or barium carminate with sulphuric acid. When pure the acid forms crystals as first shown by Schunck and Marchlewski [1].

The constitution of carminic acid has not as yet been made out. Nietzki believes it to closely resemble indone dyes, which have a chromophore group called by Wislicenus and Kötzle the 'indandion.'



Carminic acid is a dibasic acid of sufficient strength to decompose calcium carbonate. It combines with the alkali metals to form readily soluble salts, while with the earthy and heavy metals insoluble salts or lakes are formed, which for the most part are violet, but in the case of iron compounds of a greyish black-colour.

Aluminium carminate is soluble in watery or weak alcoholic solutions of acids, and in acid salts, bases, and basic salts such as borax. To obtain aluminium carminate, add aluminium acetate to a solution of carminic acid or ammonium carminate, when an insoluble precipitate is formed. Aluminium chloride is not so good because of the partial solution of the precipitate, while potash alum cannot be used at all, as the precipitate is soluble in the alum solution.

Carmein. It has been known for a long time that carmin kept in an ammoniacal solution, changes in its appearance and in its staining properties. This change is readily induced if carmin is oxidized according to van Wijhe's plans [2]. Dissolve carmin 10 grms. in ammonia 10 cc., and oxidize it by means of peroxide of hydrogen or one per cent. potassium permanganate solution, 20 cc. This mixture is to be boiled for a few minutes. I find that potassium permanganate is apt to oxidize too far, and that for complete oxidation with 10 volume peroxide of hydrogen solution I require at least 30 cc., and that boiling should occupy about 20 minutes. This difference may be due to the fact that I dilute my ammoniacal carmin with 50 cc. of water before I oxidize it. After complete oxidation, for every 25 cc. of carmin solution add 100 cc. of 96 per cent. spirit to precipitate the dye. Half an hour later the oxidized carmin, or as we may call it, 'carmein,' is filtered off and the filter-paper with the precipitate is to be dried in an incubator. When quite dry the precipitate forms a black mass, which is readily broken up into a black powder. It should be preserved in a well-stoppered bottle, as minute traces of ammonia are apt to be given off. This latter fact, however, in no way interferes with the staining properties as far as I am able to see.

[1] C. A. F. Kahlbaum (Berlin, S. O., Schlesische Strasse 35) keeps the crystals in stock.

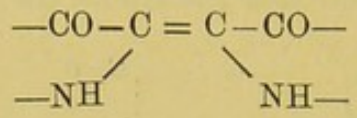
[2] J. W. van Wijhe: *Koninklijke Akademie van Wetenschappen te Amsterdam*, February, 1900.

Indigo.

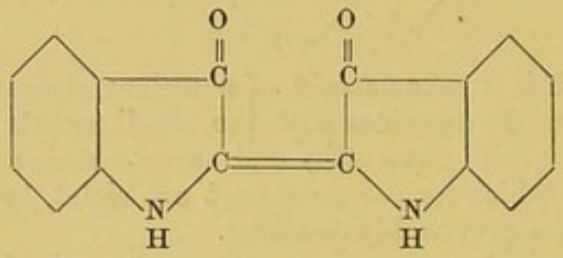
Many plants, in particular *Indigofera tinctoria*, *I. Anil*, *Polygonum tinctorium*, and *Isatis tinctoria*, contain the glucoside indican, which according to Schunck consists of indigo blue and indiglucin, thus:

$$2C_{26}H_{31}NO_{17} + 4H_2O = C_{16}H_{10}N_2O_2 + 6C_6H_{10}O_6.$$

According to Nietzki indigo blue contains the chromophore group:



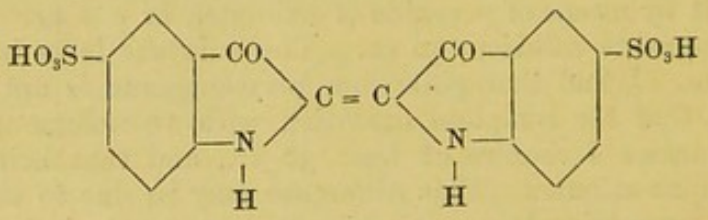
and has the formula:



Alkaline reducers cause indigo blue to take up two atoms of hydrogen and to be converted into indigo white, $C_{16}H_{12}N_2O_2$, which, being a phenol, remains in solution in alkaline media.

Indigo blue contains no salt-forming group, and therefore is not a true dye. It cannot be used directly because of its insolubility in most media, but it is soluble in anilin. Indigo white, however, is attracted, because of its phenol nature, by both wool and cotton.

Sulphindigotate of Sodium. By the action of fuming sulphuric acid on indigo white there is formed the disulpho acid $C_{16}H_8N_2O_2(SO_3H_2)$, having, according to Vorländer and Schubart [1], this constitution:



The sodium salt of this disulpho acid is called sulphindigotate of sodium or indigo-carmin, and acts as an acid dye towards tissues because of the sulphur radicals which it contains.

Murexide.

Murexide, $C_8H_4N_5O_6 \cdot NH_4$, is obtained by the action of ammonia on a mixture of alloxan or mesoxalyl-urea, $CO\langle^{NH \cdot CO}_{NH \cdot CO}\rangle CO + 4H_2O$, and its reduction compound alloxantin. It was used as a dye in 1853 because its red solutions form very insoluble compounds with calcium, barium, tin, lead, and especially mercury salts.

[1] Vorländer and Schubart: *Ber. d. deutsch. chem. Gesellsch.* 34, 1860–63 (1901).

Orcein and Litmus.

The lichens *Lecanora tinctoria* and *Roccella tinctoria* are themselves colourless, but if they are treated with ammonia and are freely exposed to the air, violet or blue colours are produced. These depend on peculiar acids which under the action of alkalies split up into orcin-carbonic acid and orcin, $CH_3 \cdot C_6H_3 \cdot (OH)_2$, and erythroleic acid and azo-erythrin, $C_4H_{10}O_4$. Orcin, acted upon by air and ammonia, is changed into orcein, $C_7H_7NO_3$.

Orcein is a weak acid; it is soluble in alkalies with a violet colour, is precipitated by acids, and forms insoluble lakes with lime and the salts of the heavy metals.

Litmus is obtained from the same lichens which form orcein, if they be treated, in addition to air and ammonia, with lime and potash or soda.

Alcannin.

Alcannin, $C_{17}H_{16}O_{10}$, obtained from the roots of *Anchusa tinctoria*, forms a red paste, acid in character. It is insoluble in water, but soluble in alcohol, ether, ligroin, fatty oils, and, as Pflüger[1] has shown, slightly soluble in glycerin, and fairly so in bile and soap.

Alkalies dissolve it with a blue colour, while acids precipitate it from an alkaline solution as a red amorphous mass.

A diacetate is formed by the action of glacial acetic acid and sodium acetate (Carnelatti and Nassini).

Nothing very definite is known as to the constitution of alcannin. The last account is by Liebermann and Römer[2], who arrive at the conclusion that it is a methyl-dioxyanthraquinone (pp. 398, 399) or a compound richer by two atoms of hydrogen.

III. Compound Dyes.

Under this heading we may group all those dyes which contain an acid chromophore in combination with a basic chromophore, the two together forming a salt.

1. **Eosinate of Methylene-blue.** The idea of uniting the staining principles of acid and basic dyes originated with **Romanowsky**[3], who in 1891 combined equimolecular proportions of methylene-blue and eosin, and thus obtained the eosinate of methylene-blue. He also recognized the formation of a third neutral colour, namely methylene-violet (p. 405), which he believed to have a special affinity for the chromatin of malaria parasites. **Ziemann** (1898)[4] confirmed the observations of Romanowsky, and showed that the eosin-methylene-blue compound is soluble in an excess of either methylene-blue or eosin. **Rosin** (1898)[5] arrived at the same results as Ziemann, but succeeded in

[1] *Arch. f. Physiol.*, August, 1900. See also Hofbauer's paper on 'Fat absorption and Lackroth.' A. Meister, Lucius and Brüning, Höchst a. M.

[2] *Berichte d. deutsch. chem. Gesellsch.* 20, 2428 (1887).

[3] Romanowsky: *Zur Frage d. Parasitol. u. d. Therap. d. Malaria*, St. Petersburg, 1891.

[4] Ziemann: 'Über Malaria u. andere Blutparasiten,' etc., *Centralbl. f. Bakter.* 25 (1898).

[5] Rosin: *Deutsch. Med. Wochenschr.* 39 (1898).

preparing the eosin-methylene-blue in definite crystals, and he was the first to realize the importance of this compound for microchemical reactions. **Zettnow**[1] gives a detailed account of the behaviour of different methylene-blues, the changes produced in them by alkalies, and how to combine these blues with eosin. I do not give his method in full because it is more complicated than that of Laurent and does not give better results. **Nocht** (1899)[2], and independently of him, Rosin[3], discovered that on combining eosin and methylene-blue, there is also formed methylene-azur. The latter seems to be the essential factor in staining the malaria parasite (Nocht, Ziemann, Rosin) and the red granulations in bacteria[4]. **Rosin** in his last paper, January, 1900[5], speaks of five distinct colours formed by uniting eosin and methylene-blue. All colours are soluble in chloroform, but to a different extent; thus, by evaporating the solution, the first substance to separate out is the eosinate of methylene-blue. The five dyes are:

(1) Eosinate of methylene-blue, insoluble in ether, slightly soluble in chloroform, forming a reddish-violet solution with no fluorescence. Readily soluble in alcohol, with a blue-violet colour and green fluorescence.

(2) Methylene-violet (see p. 405), not very soluble in ether, with a carmin colour and brown fluorescence.

(3) Methylene-azur, more soluble in ether than methylene-violet, and with a raspberry colour.

(4) Methylene-orange, very soluble in ether.

(5) Black dye, insoluble in ether, and best obtained by making a chloroform solution of the methylene-violet precipitate, along with which it separates out, and then precipitating the black dye with ether. The methylene-orange seems, however, according to my experiments, to be nothing but tetrabrom-fluorescein, derived from the eosin.

Jenner[6] dissolves the precipitate which is formed by adding eosin to methylene-blue, in Merck's pure methyl-alcohol, in which it is more soluble than in ethyl-alcohol. The use of this solution is explained on p. 219.

Laurent[7] has given the most concise directions for the preparation of methylene-blue eosinate: he uses Grübler's eosin, which is the potash salt of tetrabrom-fluorescein, $C_{20}H_6K_2Br_4O_5$ (see p. 429), and possesses a molecular weight of 724, and Merck's medicinal methylene-blue, $C_{16}H_{18}N_3SCl$ (see p. 404), with a molecular weight of 319·4. As eosin is a dibasic acid, two molecules of methylene-blue to one of eosin must be taken, or 724 eosin $= 2 \times 319{\cdot}4 = 6{,}388$ of methylene-blue,—or to 1,000 cc. of 1 per cent. eosin add 882·3 cc. of 1 per cent. methylene-blue. Let this mixture stand for 2×24 hours to allow the neutral precipitate

[1] Zettnow: 'Romanowsky's Färbung bei Bakterien,' *Zeitsch. f. Hygiene u. Infectionskrankh.* (Koch and Flügge), 1899. See also *Z. f. wiss. Mikr.* for abstract.
[2] Nocht: *Centralbl. f. Bakteriol.* 25 (1899).
[3] Rosin: *Berl. klin. Wochenschr.* 12, 251–252 (1899).
[4] Feinberg: 'Über den Bau d. Bakterien,' *Anat. Anz.* 17, March, 1900.
[5] Rosin: *Centralbl. f. Physiol.* 13, 561–565 (1899).
[6] A. Jenner: *Lancet*, 6 (1899).
[7] Hans Laurent: *Centralbl. f. allg. Pathol. und pathol. Anat.* 9, 86–97 (1898).

to settle. The latter may be obtained in a pure form by washing in water, or it may be shaken up and be kept with the mother liquor, which contains potassium chloride derived from potassium of the eosin and the chlorine of the methylene-blue. Store for future use in well-stoppered bottles. The method of using this neutral precipitate is given in detail on p. 219, and therefore it will suffice now to mention the fact that Laurent has found the precipitate, which is insoluble in water, by prolonged boiling to become soluble and to remain so for 5 to 6 hours.

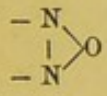
2. **Rosanilin picrate** has been obtained by Ehrlich and Lazarus[1] by combining rosanilin acetate and ammonium picrate.

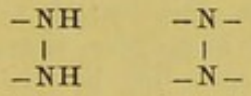
3. **Other compounds** of acid and basic chromophores Rosin described in 1899[2], namely between methylene-blue and erythrosin; methyl-green and methyl-orange; malachite green and rubin S; methylene-blue and picric acid; magenta and picric acid, previously described by Ehrlich and Lazarus.

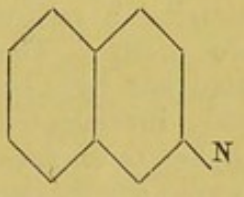
Seyewetz[3], when experimenting with the basic dye auramin, found that aromatic or benzene compounds containing the radicals COOH, OH, SO_3H, NO_2, CO·CO only once do not precipitate auramin, but that they do so if two or more of these radicals be present.

Analogously, compounds containing one group of NH_2, NH, $N\equiv$ or $-N=N-$ radicals do not precipitate picric acid, while those benzene compounds which contain one or more groups of NH_2 combined with the $-N=N-$ group, as in the amido-azo compounds, do precipitate picric acid.

Further, the groups







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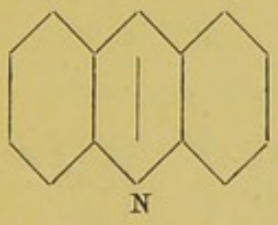


also do not precipitate picric acid.

The general conclusions he arrives at are—1. On introducing in an aromatic substance, not containing a chromophore, the groups OH and NH_2, a substance results which does not precipitate picric acid. 2. There is also no precipitation if COOH groups are present, with or without OH groups in the presence of the radical NH_2. 3. If one or more SO_3H radicals be present beside the NH_2 group then auramin is precipitated.

Seyewetz[4] has continued the researches of Rosin and finds that

[1] Ehrlich and Lazarus: *Die Anaemie*, 1898.

[2] Rosin: 'Über eine neue Gruppe v. Anilinfarbstoffen,' *Berl. Klin. Wochenschr.* 12, 251, 252.

[3] Seyewetz: *Compt. Rend.* 130, 842.

[4] Seyewetz: *ibid.* 1770–1773, abstracted in *Journ. Soc. Chem. Indust.*, Sept. 1900.

rosanilin hydrochloride, representing the basic dye, unites with the sodium salts of the following nitro-dyes: 1 : 2 nitro-phenol; 1 : 2 : 4 dinitro-phenol; picric acid; Martius' yellow; naphthol-yellow S; mono-nitro-resorcinol and hexanitro-diphenylamin, according to the following reaction:

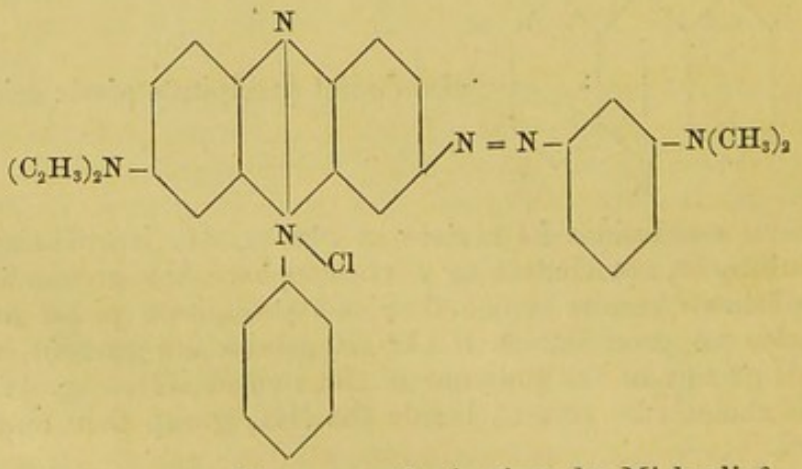
$$C_{10}H_5(NO_2)_2ONa + Cl\cdot C\cdot C_{19}H_{20}N_3 = NaCl + C_{10}H_5(NO_2)_2O\cdot C\cdot C_{19}H_{20}N_3.$$

Martius' yellow + rosanilin hydrochloride = sodium chloride + Martius' yellow-rosanilin.

He further states that dye-stuffs containing only NO_2 and OH groups unite with rosanilin, molecule for molecule, while naphthol-yellow, which also contains a sulphonic group, unites with two molecules of rosanilin. Other dyes unite with rosanilin in these proportions:

1 molecule	diquinone-dioxime	+1	molecule of rosanilin.
	phthalein	+2	
	pararosolic acid	+1	
	chrome violet	+3	
	chrome blue		
	alizarin orange		
	alizarin red	+1	
	alizarin blue		
	amido-alizarin		

4. **Safranin azo-dyes** contain in each molecule an azo group and the azonium chromophore characteristic of safranin. They are made by diazotizing a safranin and subsequently joining it to a phenol or an amido group. Their importance for histological research was first pointed out by Ehrlich[1], and on his suggestion these dyes were thoroughly investigated by Michaelis[2], according to whom the substance diethyl-safranin-azo-dimethyl-anilin, or Janus green, as it is called by the anilin dye manufactory in Höchst, possesses this formula:



A detailed account of its properties is given by Michaelis[2].

[1] Ehrlich: *Verein für innere Medicin*, 1 Dec. 1898.

[2] L. Michaelis: 'Die vitale Färbung, eine Darstellungsmethode d. Zellgranula,' *Arch. f. mikr. Anat.* 55, 558–575 (1900).

ADDITIONAL NOTE I.

MICRO-ANATOMICAL REACTIONS.

Elastic tissue.

THE first to describe a distinct staining method for elastic fibres with anilin red was v. Ebener[1], and Martinotti[2] has collected the older literature up to 1887.

The dyes which have been chiefly used are Picric acid in the form of picro-carmin by Ranvier; Victoria blue in an alcoholic solution by Lustgarten[3]; Safranin (safranin 5 grms., absolute alcohol 100 cc., water 200 cc. for forty-eight hours), after fixation in 0·2 per cent. chromic acid by Martinotti; Fuchsin by Weigert, see below; Orcein by Tänzer and Unna, see below; Kreso-fuchsin in an alcoholic solution by Röthig[4], and Methyl-violet by myself in a watery solution after alcohol fixation. The latter stains elastic fibres deeply, but alcohol discharges the colour, and therefore the method of W. H. Welch (1876) should be employed.

Welch dries sections partially with filter-paper (I use cigarette or thin toilet-paper), then covers them with xylene and alternately applies xylene and blotting-paper till the sections are quite clear. This is a very good method if we desire to preserve the actual staining effect of watery stains.

The best method for elastic fibres I consider to be Weigert's, as only elastic fibres are stained. The orcein methods are the next best, but the nuclei are also stained.

Weigert's method for elastic fibres.

The stain is prepared thus: Dissolve in a porcelain basin basic fuchsin or parafuchsin or new-fuchsin 2 grms. and resorcin 4 grms. in 200 cc. of water by boiling; add to the boiling mixture 25 cc. of the liquor ferri sesquichlor. Pharm. Germ. III, and continue to boil for some minutes.—I add sufficient of a saturated ferric chloride solution to precipitate the whole of the dye and to have a distinct yellow colouration of the supernatant solution. A black very sticky sediment forms in the basin; pour off as much of the solution as will flow off readily; add 200 cc. of 95 per cent. alcohol and dissolve the sediment by boiling; filter while hot into a bottle and add to the alcoholic solution, after it has

[1] v. Ebener: *Rollet's Unters. a. d. Inst. f. Phys. u. Histol. in Graz* (Leipzig, 1870), p. 32.

[2] G. Martinotti: *Zeitsch. f. wiss. Mikr.* 4, 31 (1887).

[3] Lustgarten: *Med. Jahrb. d. Kaiserl. Königl. Ges. d. Aerzte z. Wein*, p. 285 (1886).

[4] Röthig: *Arch. f. mikr. Anat.* 56, 354 (1900). This dye stains elastic fibres blue, mucus, cartilage, and keratin red.

cooled, 4 cc. of hydrochloric acid.—I have added with advantage up to 10 cc. of hydrochloric acid. Traces of ferric chloride in the stain are an advantage, as shown by Weigert, Paul Mayer, and my own observations.

Stain for one hour; rinse in 95 per cent. alcohol, clear in xylene, and mount in balsam. Weigert employs for clearing 95 per cent. alcohol followed by the method of Welch given above.

The elastic fibres stain a bluish-black colour. To bring out the nuclei it is best to stain the sections in carmin or to counterstain in Bordeaux red.

Michaelis[1] has made a special study of the three components in Weigert's staining solution, namely of the fuchsin, the resorcin, and the ferric chloride. The ferric chloride seems to act as an oxidizing medium for its place may be taken by ammonium persulphate. The resorcin, $C_6H_4(OH)_2$ [1 : 13], may be replaced by other phenols. Weigert himself had stated that phenol, C_6H_5OH, may be substituted for resorcin, and Michaelis has obtained positive results with orcin (p. 441), pyrogallol (p. 436), and orthocresol (p. 92). Instead of fuchsin the following basic dyes may be used: dimethyl-safranin (methylene-violet, Farbwerk Mühlheim), ordinary safranin, methyl-violet, kresol-violet RR (Mühlheim), thionin and toluidin-blue. The latter is not very good for elastic fibres and methylene-blue does not stain at all[2]. In addition to the coloured bases mentioned above, uncoloured aromatic bases were also found to give positive results, as do for example the chlorides of anilin, dimethyl-anilin, and paratoluidin.

The following colour effects are obtained with these dyes:

Fuchsin + resorcin	= blue-black.
Fuchsin + orcin	= red-violet.
Fuchsin + pyrogallol	= dark-red.
Dimethyl-safranin + resorcin	= red-violet.
Safranin + resorcin	= red.
Methyl-violet + resorcin	= green.
Dimethyl-anilin + resorcin	= bluish-black.
Paratoluidin + resorcin	= brown-black.
Anilin + resorcin	= grey.

Nothing is known about the chemical constitution of these dyes or that of the elastic tissue, to throw any light on the chemical interaction. It seems necessary to always use these dyes in strongly acid solutions, the hydrochloric acid producing probably a partial double dissociation of the dye radical and the elastin radical.

The Orcein method of Tänzer.

Tänzer[3] introduced in 1891 the orcein method for staining elastic fibres, and I like his original formula better than that published by Unna in 1894[4].

[1] L. Michaelis: *Deutsche med. Wochensch.* 27, No. 14, p. 219 (1901).
[2] The chemical constitution of these dyes is given on p. 404.
[3] Tänzer: *Monatshefte f. prakt. Derm.* 12, 394 (1891).
[4] Unna: *ibid.* 19, 398 (1894), and *Zeitsch. f. wiss. Mikr.* 12, 240 (1895).

	Tänzer's Solution.	*Unna's Solution.*
Orcein	1 grm.	1 grm.
Absolute alcohol	80 cc.	100 cc.
Distilled water	40 „	
Hydrochloric acid	40 drops	1 cc.

Tänzer fixes in alcohol (or Müller's solution), stains section for 6 to 24 hours, and differentiates in a solution of

Hydrochloric acid	0·1
95 per cent. spirit	23·0
Distilled water	5

The chemistry of orcein is discussed on p. 441.

The Haematoxylin method of Harris.

Harris[1] states that he accidentally discovered haematein solutions to have a remarkable affinity for elastin, if they be prepared in a certain way. I have known about this property for a considerable time, because my haematein solution (p. 239), if old, will readily stain the elastic fibres in the mesentery of the frog, and I have used this method for class purposes for several years.

Harris makes his 'elasthaematein' in the following way:

Haematoxylin	0·2 grm.
Aluminium chloride	0·1 „
50 per cent. alcohol	100 cc.

'Dissolve the haematoxylin and aluminium chloride and then carefully bring the solution to a boil: 0·6 grm. of mercuric oxide is now slowly added, and as soon as the mixture assumes a dark purple color it is removed from the flame and cooled rapidly.' Filter, cool, add one drop of hydrochloric acid, and set aside for some weeks to let the solution ripen. The ripening does not depend on oxidation, and Harris was unable to find out the reason why his solution, when freshly prepared, as a rule, does not stain. To stain elastic tissue proceed thus: Immerse sections in the stain for 5 to 10 minutes, wash the tissue for about one minute in a 1 per cent. solution of nitric acid in alcohol, wash in neutral alcohol to remove the acid, clear and mount in balsam.

The elasthaematein of Harris closely resembles the muc-haematein of Mayer (p. 238). If sections be stained in Mayer's muci-carmin (p. 246), and then be differentiated in nitric acid alcohol, 'any elastin present stains much more brilliantly than do the other structures.' Mucin also stains by the elasthaematein, but there is no possibility of confusing it with elastin.

The best method for fixing elastic tissues for micro-chemical research is of course alcohol, but for micro-anatomical research any of the ordinary methods may be used. Gardner[2] has studied the histogenesis of elastic

[1] H. F. Harris: *Zeitsch. f. wiss. Mikr.* 18, 290 (1902).

[2] Gardner: *Le Physiologiste Russe*, 1, 3 (1898-9), abstracted in *Zeitsch. f. wiss. Mikr.* 18, 63 (1901).

fibres in the amnion of different animals. The amnion is fixed for two to three days in Müller's solution, rinsed in distilled water; the chrome salts extracted in the dark with 60 per cent. alcohol and the amnion preserved till needed in 75 per cent. alcohol. After removing the epithelium by shaking it with a pair of forceps the amnion is divided into lamellae and stained in Tänzer's modification of Unna's fuchsin method:

Stain as a preliminary in Bismarck-brown, rinse in water to remove the excess of this dye, then stain for twenty-four hours or longer in Tänzer's solution:

Fuchsin	0·5
Alcohol	25·0
Distilled water	25·0
Nitric acid, 25 per cent. . .	10·0

Transfer lamella for one second into 25 per cent. caustic potash, and rapidly wash out the potash in a series of dishes containing distilled water, and examine in very dilute glycerin or water containing thymol. The elastic fibres are dark blue, the plasm pink, and the nuclei dark red.

For the study of the general arrangement of elastic fibres to the rest of the tissue, Gardner fixed the amnion in

Copper acetate, saturated watery solution	4 parts
Osmium tetroxide, 1 per cent. . .	1 part

This mixture is said not to cause the same amount of shrinkage as does sublimate or osmium.

Fibrin method of Weigert.

This method resembles that used by Weigert for staining bacteria, but differs from the latter in the method of differentiation.

Fix tissues in alcohol; stain sections for five to fifteen minutes in Weigert's methyl-violet oxalic acid stain (p. 213) or in Ehrlich's gentian-violet anilin water stain (p. 213); rinse in 0·6 per cent. salt solution; dry with filter-paper or cigarette-paper; treat with an iodine-iodide of potassium solution[1] for two to three minutes; dry with filter-paper. Decolourize and differentiate in a mixture of anilin oil two parts and xylene one part; remove the anilin with pure xylene and mount in balsam. Everything is decolourized except fibrin and bacteria.

Kromayer[2] uses a slight modification of Weigert's method, a saturated watery solution of methyl-violet 6B and of anilin water equal parts, and for differentiating the sections, anilin oil one part and xylene two, three, or four parts, to obtain a sharp staining of Ranvier's fibrils in stratified epithelia.

Beneke[3] by using two parts of anilin oil to three parts of xylol demonstrates by Weigert's fibrin method mitoses; connective-tissue fibres (bluish- or reddish-violet); elastic fibres (brilliant red); fibrils in bone; Sharpey's fibres, Bowmann's segments in striped muscle and neuroglia.

[1] Iodine 1 grm., iodide of potassium 2 grms., water 100 cc.
[2] *Arch. f. mikr. Anat.* 39, 141 (1892).
[3] *Zeitsch. f. wiss. Mikr.* 11, 79 (1894).

Nerve fibrils.

The methods of Apáthy (p. 240) and of v. Kupffer (p. 224) have already been given, while that of Bethe is given below. I have used for the tracing of bundles of fibrils tissues fixed in osmo-sublimate, in formol, or in chromo-sublimate. It is quite unnecessary to stain sections to see the fibrils, but they may be readily stained by mordanting sections in chromic acid, copper, or other reagents giving deeply coloured lakes, and staining with very dilute haematoxylin, or staining in one per cent. haematoxylin and then differentiating very slowly.

Bethe's method for nerve fibrils and Golgi's pericellular network.

According to Bethe's own account his method leaves much to be desired as it is impossible to make absolutely sure of success, but notwithstanding this drawback it is a good method. His full account[1] should be consulted by all who want to stain nerve fibrils.

Fixation: Cut fresh material into as thin slices as possible (4 to 10 mm.); place the slices on blotting-paper and cover with 3 to 7·5 per cent. nitric acid[2]. Turn the slices several times in the course of twenty-four hours to ensure equal fixation. The temperature of the fixing solution must never rise above 20° C., the best temperature being 12 to 15° C.

The nitric acid serves to fix the tissues and to diminish the normal affinity for basic dyes which is shown by the granules of Nissl and the nuclei. The tissues should be sufficiently nitrated to turn a pale yellow colour, but on no account should they be of a deep yellow colour. If on treatment with ammoniacal alcohol (see below) they turn brown, they should be discarded.

Hardening: Transfer the slices directly, for twelve to twenty-four hours or even longer, to 96 per cent. alcohol.

Extraction: To partially remove the nucleo-proteid radical of Nissl's granules, extract the slices for twelve to twenty-four hours first with a mixture of

Ammonia, sp. gravity 0·95 to 0·96	1 part
Water	3 parts
Alcohol, 96 per cent.	8 „

The presence of water facilitates the extraction and the alcohol prevents the tissues from swelling. Remove the ammonia by leaving the slices for six to twelve hours in pure 96 per cent. alcohol.

Then extract with a mixture of

Hydrochloric acid (sp. gr. 1·18 = 37 per cent.)	1 part
Water	3 parts
Alcohol, 96 per cent.	8–12 „

[1] Bethe: *Zeitsch. f. wiss. Mikr.* 17, 13-35 (1900).

[2] The strong commercial nitric acid has a specific gravity of 1·40, and is equal to a 65 per cent. nitric acid (see p. 71). It is this ordinary nitric acid which is used for making the 3 per cent. solution.

Keep the slices in this mixture for twenty-four hours, below 20° C., then wash out the acid in 96 per cent. alcohol for ten to twenty-four hours.

Removal of alcohol: Leave slices in distilled water for two to six hours, but not longer.

Mordanting: Use a 4 per cent. solution of ammonium molybdate (large white crystals) for twenty-four hours at 10 to 15° C. for nerve fibrils and at 18 to 30° C. for Golgi's pericellular network. Rinse slices in distilled water.

Imbedding: Dehydrate for ten to twenty-four hours in 96 per cent. alcohol, and for the same length of time in absolute alcohol. Then take through xylene, toluene, or chloroform and the paraffin process. Do not use the celloidin method.

Sectioning and mounting: Sections should be 10 μ thick and be fixed to the slide by Mayer's albumin method, as floating the sections on warm water leads to a differentiation of the ammonium molybdate. Water at ordinary temperature has no action.

As the slices of the nervous system are not mordanted equally through and through, it is necessary to keep all the sections and to mount for example sections one and twenty on the same slide to see at what depth the best results are obtained. Remove the paraffin with xylene and alcohol.

Differentiating and staining: Cover the slides containing the sections with 1 to 1·5 cc. of distilled water, so that the latter forms a layer about 1·5 to 2 mm. thick; place the sections in an incubator heated up from 55 to 60° C., and leave the slides for two to ten minutes (at the most twelve minutes); the cerebrum and cerebellum require two to six minutes, the medulla three to seven minutes, the spinal cord five to ten minutes.

Now pour off the water and rinse the slide carefully three or four times with distilled water; dry the slide with a clean duster and cover the sections with 1:3,000 of toluidin-blue; put the slide into the incubator once more and leave it for ten minutes. Rinse the sections for three-quarters to two minutes in distilled water so as to wash off all the dye which is not bound chemically to the molybdenum. Dehydrate thoroughly, clear in chemically pure benzene and mount in neutral balsam (p. 377).

I believe Bethe's method to depend on the principle that under certain conditions, namely, those where the fibrils stain deeply, the ammonium molybdate has certain affinities unsatisfied by the fibrils, and that these unsatisfied valencies are satisfied by the dye. The ammonium molybdate acts as the chemical link between the dye and the fibril (p. 228).

At the time when the fibril and molybdenum compound has certain free valencies, owing to an incipient decomposition or dissociation of this compound, the other cell constituents are as yet able to satisfy the molybdenum completely and therefore the latter will have no affinity for the basic dye. This explanation is based on Bethe's observation that after differentiating the nerve fibrils stain first, then are stained Golgi's pericellular networks, and finally the ordinary cell-plasm and Nissl's bodies. Bethe explains his results on the coefficient of dis-

tribution theory of v. Georgievics (p. 331). He believes his staining effects to depend on what amount of differentiation is brought about by the equilibrium established between the tissue constituents and the warm water; these dividing the ammonium molybdate between themselves according to Berthelot and Jungfleisch's law of coefficient of distribution. It must be remembered that the coefficient of distribution theory excludes all chemical interaction, and I hold that ammonium molybdate unites chemically with the base toluidin-blue.

ADDITIONAL NOTE II.

Knecht[1], in 1888 and 1889, whilst working with dyes which stain silk and wool directly, arrived originally at the conclusion that staining did not depend on mechanical, but on chemical processes (see pp. 343, 361). Then Witt published his theory of solid solutions (p. 330), and explained the extraction of dyes from fabrics by means of absolute alcohol as due to the passage of the dyes into a solution in which they were more soluble, namely, alcohol.

As Knecht found that animal fibres only took up the dye radical from the dye-bath, while the hydrochloric acid radical remained in the bath, he arrived at the conclusion that the dye radical was united chemically in the fibre to the lanuginic acid of wool or the sericin of silk, and that this union was after the principle of Witt's solid solution[2].

In his most recent communication Knecht has shown that chemical combinations after the principle of salt formation must take place in the fibre, for the reasons stated below, and he no longer supports Witt's theory.

Both lanuginic and sericinic acid form intensely coloured water-insoluble precipitates with those basic dyes which stain directly. The precipitates or lakes are, however, soluble in alcohol and become re-precipitated on diluting the alcohol with water. On extracting with alcohol silk or wool which have been dyed, for example, in fuchsin and diluting the alcohol with water, an insoluble precipitate is also obtained.

That the latter is not merely a coloured rosanilin base or a strongly basic rosanilin hydrochloride was shown by dyeing silk (and wool) with night-blue (B. A. S. F.[3]); washing and drying the silk and finally extracting it in a Soxhlet apparatus with alcohol till it became colourless; inspissating the alcoholic extract to a small bulk and then pouring it slowly into a warm solution of barium hydrate to precipitate the night-blue base in an insoluble form. After filtering off the dye-base, treating the filtrate with carbon dioxide, boiling, filtering again and inspissating the filtrate, the latter produces intensely coloured precipitates, both with night-blue and with fuchsin.

[1] Edmund Knecht: *Ber. d. deutsch. chem. Ges.* 21, 1557, 2804 (1888); 22, 1120 (1889), and 35, 1022 (1902).

[2] Knecht: *Journal of the Soc. of Dyers and Colourists*, 5, 77.

[3] B. A. S. F. means Badische Anilin u. Soda Fabrik.

The lake precipitate is soluble in alcohol, and therefore the substance which produces lakes with the dye-bases behaves in every respect like sericinic (or lanuginic) acid.

The fact that the lakes are not quite insoluble in water will readily explain why tissues do not exhaust a dye-bath completely. As the result of his recent researches, Knecht has no hesitation in explaining the dyeing of animal fibres as due to purely chemical action.

The power of 'precipitating dyes from their watery solutions,' or as I should like to put it, the formation of water-insoluble salts (dye + tissue constituent), is also shown, in addition to sericin and lanuginic acid, by 'somatose' and albumin digested with pepsin.

Histologically the observations just recorded are of the greatest importance, because they show that the ready removal of dyes from sections does not speak against a chemical explanation of staining, and they also emphasize a point to which I have repeatedly drawn attention, namely, the absolute necessity of examining tissues, after staining, in water, before using alcohol.

The readiness with which monovalent alcohols extract colour seems to depend on their molecular weight: thus **Kisskalt**[1] has found, after staining with Gram's method, that the methyl-violet is extracted most by methyl-alcohol, and gradually to a less and less extent by ethyl-, propyl-, butyl-, and amyl-alcohol (see p. 84). Thus certain bacteria, which according to Gram's method remain stained if ethyl-alcohol is used, become decolorized with methyl-alcohol, while on the other hand certain organisms which become decolorized by Gram's method after the use of ethyl-alcohol do not part with their colour if propyl-, butyl-, and amyl-alcohols are used.

Martin Heidenhain[2] has realized both the chemical and the physical aspects of staining. Not being aware of the work of Mathews (p. 348), he has confirmed the observations of the latter in many essential points, and further brought to light new facts.

Heidenhain finds that feeble aromatic sulphonic acids precipitate only the more acid proteids, while the strong sulphonic acids also precipitate the essentially basic albumins, there being formed acid albumins. As regards the precipitability by dyes there is not much difference between neutral and acid solutions of albumins. Thus:

metanilic acid, $C_6H_4(NH_2)(SO_3H)$: 1 : 3
sulphonilic acid, $C_6H_4(NH_2)(SO_3H)$: 1 : 4

decompose congo-red, but they do not precipitate 1 per cent. watery serum-albumin or casein dissolved in 10 per cent. acetic acid. With half per cent. serum-albumin and casein dissolved in 0·2 per cent. caustic soda they give a (neutralization ?) precipitate. With 0·5 per cent. yeast nuclein dissolved in 0·2 per cent. NaOH a delicate precipitate insoluble in an excess of the colour acid is obtained, while half per cent. nucleic acid shows a marked turbidity.

On the other hand all the proteids just mentioned are precipitated by:

[1] C. Kisskalt: *Centralbl. f. Bacteriol.* 30, 281 (1901).
[2] Heidenhain: *Arch. f. d. ges. Physiol.* 90, 115 (1902).

resorcin-disulphonic acid $C_6H_2\begin{cases}(OH)_2\\(SO_3H)_2\end{cases}$ (2 per cent.)

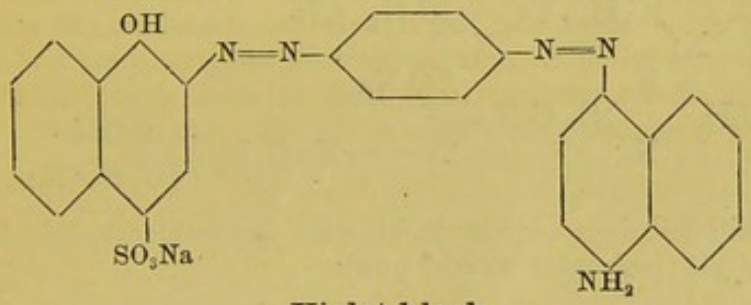
naphthalene-β-monosulphonic acid $C_{10}H_7(SO_3H)$ (1 per cent.)

2 : 4 dinitro-1 naphthol-7 sulphonic acid, $C_{10}H_4(NO_2)_2(SO_3H)OH$ (1 per cent.)

indigo-blue monosulphonic acid (phoenicin-sulphonic acid) } $C_{16}H_9N_2O_2SO_3H$.

Heidenhain suggests that cyclic and acyclic colour acids, which vary in their acidity, should be employed, as was picric acid in the past, for a simultaneous staining and fixing of tissues[1]. He states that for over ten years he has been in the habit of acidulating all acid dyes with a few drops of 0·4 per cent. acetic acid to help the tissues to dissociate those colour acids which are employed in the form of their salts.

The next point discussed is why feeble acid dyes have strong precipitating powers. Thus the sodium salt of *p*-phenylene-diamin-azo-α-naphthylamin-azo-1 naphthol-4 sulphonic acid, called violet-black by the Badische Anilin und Soda Fabrik, possesses a comparatively large molecule (molecular weight 519) in which the acid tendencies of the OH and SO_3H radicals are weakened by the basic NH_2-group.



Violet-black.

Of this dye Heidenhain says that it possesses a power of precipitating albumin as probably no other known substance. In 16 cc. of water

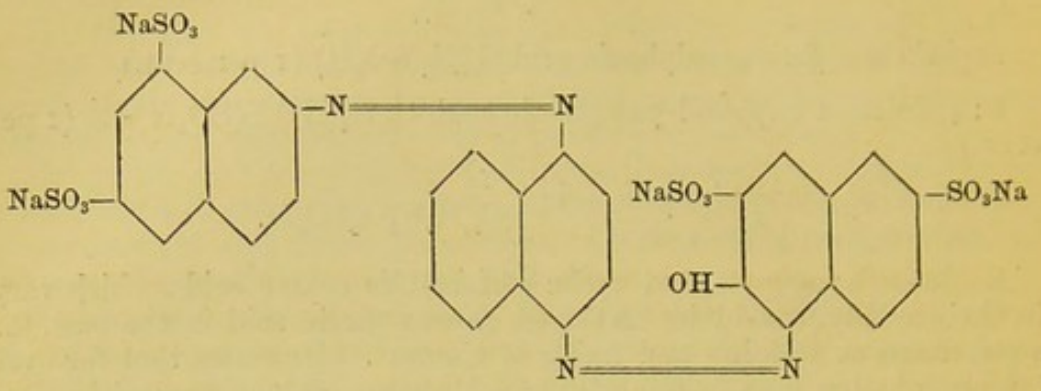
0·0008 grm. of violet-black precipitates
0·00025 grm. of egg-albumin in the presence of
0·00495 grm. of acetic acid.

The explanation offered is, that the colour acid liberated by the acetic acid is so insoluble in water that it combines chemically with the smallest amounts of albumin present in the solution; and that the albumin compound after chemical union has taken place is secondarily denaturalized and coagulated (l. c., p. 139[2]). The chromotropes behave analogously to violet-black. They are characterized by showing greater affinity for casein than for albumin.

Blue-black B with 1 (OH) and 3 (SO_3H) groups (molecular weight

[1] The disadvantages of picric acid as a fixative are pointed out on pp. 119, 120.
[2] For a possible explanation of this behaviour see p. 459.

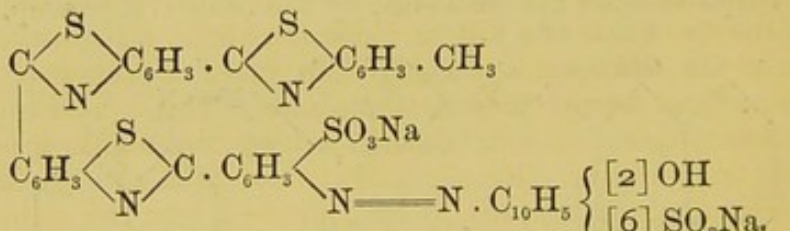
757) and brilliant black 3B with 1 (OH) and 4 (SO_3H) groups (molecular weight 860):



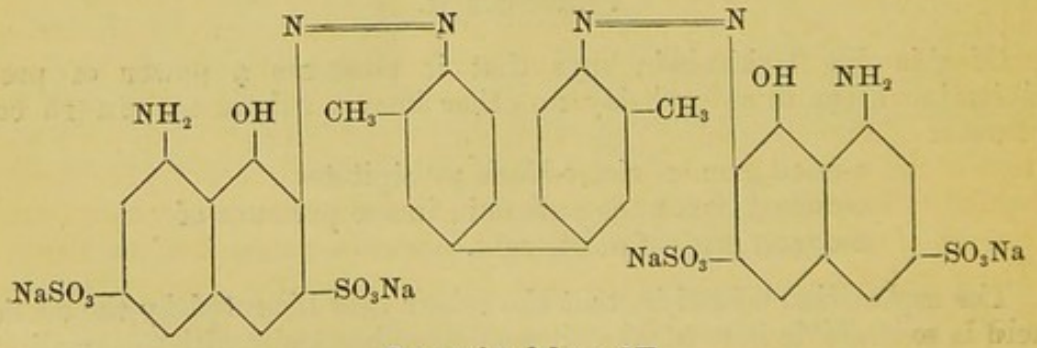
Brilliant black 3B.

do not coagulate albumin as well as does, for example, ponceau 2R with 1 (OH) and 2 (SO_3H) groups (molecular weight 480) or new-coccin with 1 (OH) and 3 (SO_3H) (molecular weight 604).

The following large moleculed dyes also do not coagulate albumin: Thiazin-red with a molecular weight of 865:



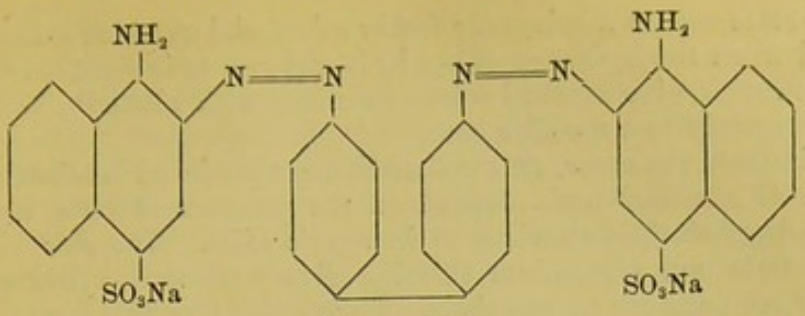
and such amidated dyes as diamin blue BX (molecular weight 843) and oxamin blue 3R (molecular weight 960):



Oxamin blue 3R.

The reason given why large moleculed dyes do not coagulate albumin, is that the acid radicals, being distributed over a large molecule, are unable to concentrate their coagulating tendencies on the albumin.

The amido-azo-sulphonic dyes are especially suitable for throwing light on the chemistry of staining, because their free colour acids have an entirely different tint than their salts, as already mentioned (p. 387).



Congo-red.

Congo-red contains two amido and two sulphonic acid groups. On being brought into contact with acids, for example acetic acid, the free, blue congo acid is liberated. If now two test-tubes be taken, one of which contains a certain amount of distilled water, while the other contains the same amount of 1 per cent. serum-albumin solution; if to either test-tube three to four drops of 1 per cent. congo-red solution are added; and if finally 10 per cent. acetic acid is added to the two test-tubes, then the congo-red solution in distilled water is turned blue at once, while the other test-tube containing the albumin solution retains its red colour, even if sufficient strong acetic acid is added to allow of its detection by smell.

Heidenhain believes that the addition of congo-red to albumin solution leads to the formation of a salt in which the albumin plays the part of an acid while the undissociated colour salt takes on the part of a base, there being formed congo-sodium albuminate. This is held to be possible because feeble acids such as alizarin act on the amido groups of the amido-azo sulphonates. By the addition of acid the unstable congo-sodium albuminate is believed to be converted into the stable albumin-congo sulphonate. The reason for this assumption is the fact that addition of acid to a congo-red-albumin mixture produces a distinct darkening in the red colour.

By arranging a series of test-tubes of 1 per cent. serum-albumin, and adding 1, 2, 3 up to 15 small drops of 1 per cent. congo-red solution; mixing the solutions thoroughly and then adding cautiously 0·4 per cent. acetic acid, the first few test-tubes will show no change, the next few show the 'after-darkening' just referred to, and depending on the formation of the stable albumin-congo sulphonate. Simultaneously the limit of solubility is reached, for in the next few tubes the albumin is precipitated. Excess of acid will cause the coloured albumin precipitate to dissolve. These experiments are comparable to neutralization precipitates (see p. 55).

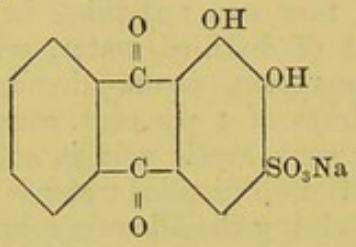
The union between albumin and congo-red is so firm that even 5 per cent. sulphuric acid does not always liberate the free, blue congo acid. It follows therefore that albumin-congo sulphonates are relatively more stable than are the sodium salts of this colour acid.

The yellow, free acids of methyleosin (dibromdinitro-fluorescein) and eosin when brought into contact with albumin regain their red colour at once. (Compare with the effect of the Mylius reaction, p. 214.)

Alizarins (see p. 399) containing two hydroxyl groups (OH) in the orthoposition to the carbonyl group ═C═O, are feeble acids which in

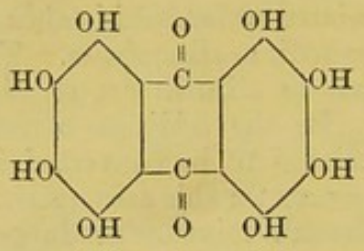
the free state are, as a rule, only feebly coloured, but which become deeply coloured when the hydrogen of the hydroxyl groups is replaced by metals. These alizarins give marked colour reactions with albumin, as the latter acts analogously to a metallic base.

To eliminate the error, that colour-changes produced in alizarins by the addition of serum-albumin depend on the presence of salts, Heidenhain has investigated the behaviour of inorganic salts. The chlorides of the alkali metals and ammonium chloride give with most alizarins at the ordinary temperature no reactions. Carbonates (for example $(NH_4)CO_3$) in very small amounts produce colour-changes very readily, alizarin red S, containing the sulphonic group in the orthoposition to the dioxy group, being especially sensitive.



= alizarin red S.

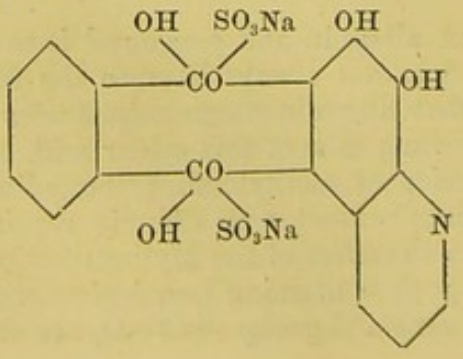
Sodium and potassium carbonate give the same colour as does albumin with alizarin-cyanin 3R, anthrapurpurin, and alizarin red S, but on the whole albumins give quite different colour reactions; thus anthracene blue WR



with potassium or sodium carbonates forms steel-blue solutions, and with albumin a Bordeaux-red solution which on heating becomes violet.

Ammonium carbonate greatly resembles albumin in its reaction, but the nearest approach to proteids is obtained with aromatic amins, the active factor in each case being the amidogen radical. Biuret is too feeble a base to change alizarin, but urea, when heated, produces colour-changes in the alizarin solutions. The indol radical of proteids has probably no effect on alizarins, as pure indol does not change the colour of alizarin solutions.

One very characteristic reaction with albumins, however, is obtained by employing alizarin blue S:



for with potassium, sodium, and ammonium chlorides, this salt gives a reddish precipitate; with potassium and sodium carbonates, on heating, a blue colour which disappears on cooling; with ammonium carbonate, naphthylamin or anilin, on heating, blue solutions which on cooling separate out dark precipitates. Serum-albumin gives, however, a brilliant cyanblue solution which is not affected by cooling the solution or by keeping it. On adding acetic acid to the coloured solutions of the salts and amins, the precipitate is increased, but on carefully adding acetic acid to the blue albumin solution, there is formed at first a precipitate, owing to the action of the sulphonic radical of the alizarin blue S, but then the precipitate dissolves again completely on the further addition of a few drops of glacial acetic acid, and a beautiful blue solution is obtained.

The action on albumin of colour bases used in the form of salts may be summarized thus:

1. If the base is very feeble, then the acid an-ion radical of the dissociated salt is taken up by the albumin, while the kat-ion colour base remains in the mixture with that colour which is characteristic of the free base. Two examples of very feeble basic dyes are:

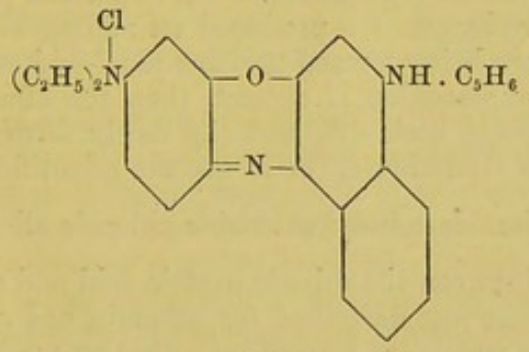
Dimethyl-amido-azo-benzene, $C_6H_5—N=\overset{1}{\overline{N}}—C_6H_4\overset{4}{\overline{N}}(CH_3)_2$

and

Chrysoidin (the hydrochloride of diamido-azo-benzene), $C_6H_5—N=N.C_6H_3\begin{cases}{}^2NH_2\\ {}^4NH_2\cdot HCl\end{cases}$

Neutral red and neutral violet (see p. 410) being stronger bases, have their an-ions removed only by strongly basic albumins, such as Schuchardt's commercial serum-albumin.

A still stronger base is Nile-blue hydrochloride:



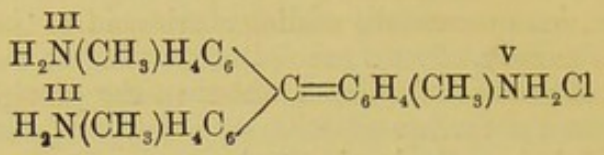
but it also behaves as a comparatively feeble base and may serve as the type of this group.

On adding the blue solution of Nile-blue hydrochloride to a solution of Schuchardt's serum-albumin, the an-ion chlorine radical unites with the albumin to form an albumin chloride, while the solution becomes red owing to the liberation of the basic Nile-blue, which in its free or uncombined state is red. On heating this mixture, the Nile-blue becomes a kat-ion and the albumin chloride an an-ion, and the solution becomes blue again, owing to the formation of the salt Nile-blue-albumin chloride. [Nile-blue base] + [albumin chloride] = [Nile-blue-albumin chloride] [1].

[1] I have couched Heidenhain's results in the language of physical chemistry.

The albumin is therefore able to link on both ions of the dye-salt Nile-blue hydrochloride. Heidenhain believes his observation to support Pauli's view on globulin coagulation (see pp. 56 and 57).

2. Strong colour bases, as used technically, possess several and usually three amidogen radicals, but as a rule only one acid radical joined to one of the NH_2 groups. Thus new-fuchsin:



contains two free amidogen groups with trivalent N-atoms, while the third NH_2 group has linked on the acid chlorine, owing to the N-atom having become pentavalent.

New-fuchsin and rosanilin acetate produce at the ordinary temperature a coagulation of serum-albumin, while malachite-green oxalate does the same on gently warming the mixture. Using five drops of a 0·5 per cent. new-fuchsin solution is sufficient to cause a permanent, thick, and voluminous precipitate of five cc. of 1 per cent. serum-albumin.

The acid radical is believed not to be the cause of this coagulation, because acids such as acetic (in rosanilin acetate) or oxalic acid (in malachite green) do not coagulate albumin. The active factors are, on the other hand, the free amidogen groups of the dye molecule.

The colour-changes accompanying the coagulation make a purely physical precipitation also unlikely[1]; thus very dilute rosanilin nitrate, acetate, and picrate solutions are yellowish-red, but they turn a pure rose-red on the addition of albumin, and analogously the bluish-red tint of methyl-violet is changed into a pure blue. The important point, however, is that the same colour-changes are produced on adding acetic acid to solutions of the dyes, there being formed perhaps pluri-acid salts having a different tint. If this explanation is correct, then the albumin acts as an acid towards the strong basic dye, and the newly formed compound is new-fuchsin-chloride albuminate, 'or to use the scientific term:

Triamido-tritolylcarbidride-chloride albuminate.'

Compounds between the dibasic nucleic acid and colour bases are quite analogous to those just described for albumin, and are also analogous to the precipitates which are formed by acting on nucleic acid with the salts of copper, zinc, iron, lead, and silver.

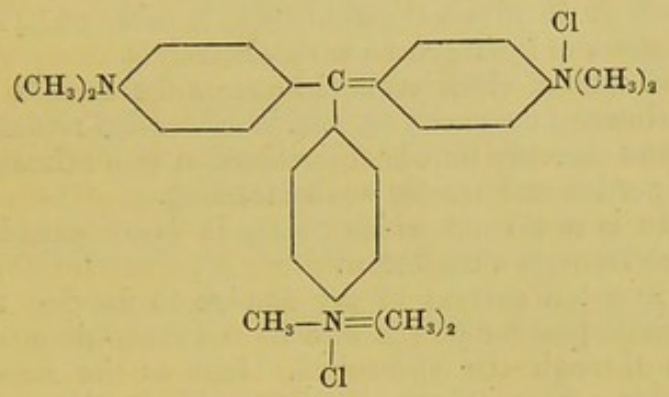
The nuclear 'chromatin' of authors consists, according to M. Heidenhain, of two different substances, namely the basichromatin which has an affinity for basic dyes, and oxychromatin which unites with acid dyes. As far as I can see such a division is not justified: we are not dealing with two distinct substances, but with nucleins, the nucleic acid radical of

[1] Heidenhain is very positive: 'Salting out or precipitation by physical means is quite impossible. How could we explain by physical laws that five drops of a 0·5 per cent. solution of new-fuchsin can precipitate 5 cc. of a 1 per cent. serum-albumin solution?' The reader, after having consulted the chapter on coagulation, will probably be less dogmatic.

which stains with basic dyes, while the protamin, histone, or globulin radical combines with acid dyes.

If it is desired to stain only the 'basichromatin,' such dyes should be chosen, the amido-groups of which are not readily attacked chemically. In this case the comparatively feeble, acid cell-plasm will not be able to act on the dye, while the strongly acid nucleins of the nuclei will either have some affinity for those amidogen groups which are not accessible to the cell-plasm, or even liberate the amidogen groups from the acid radicals to which they are united.

A dye the amido groups of which are not readily attacked is methyl-green :



Methyl-green has three amidogen groups, of which two contain already a pentavalent N-atom attached to the acid chlorine, while the third amido group has a trivalent N-atom.

It is this trivalent N which gives a feeble basicity to the whole dye molecule, and we have to assume, when nuclein compounds are stained by methyl-green, that the trivalent N attaches itself to the nuclein compounds after having become pentavalent, or that the nucleic acid radical of the nuclein displaces in the dye molecule one or both of the chlorine atoms.

Whether the explanation offered by Heidenhain as regards the behaviour of violet-black (see p. 453) is correct seems to me doubtful. I believe what happens in bringing feeble acid dyes in contact with albumin is to precipitate the dye in a colloidal form, and this dye in setting carries the albumin with it by a purely mechanical process.

The possibility of a mutual colloidal precipitation owing to a slight alteration in the molecules of the dye and the albumin does also not seem quite impossible.

MEASURES AND WEIGHTS.

Electrical Measures.

A **dyne** is that unit of force which, acting on a mass of 1 gram in a state of rest, gives it an acceleration of one centimetre per second per second.

An **erg** is the unit of work done by propelling a body through a distance of one centimetre against the force of one dyne.

As the force of gravity on one gram = 981 dynes, the work of lifting one gram weight through a height of one centimetre = 981 ergs.

A **coulomb** is that unit quantity of electricity which can deposit under appropriate conditions 1·118 milligrams of silver from a solution of a silver salt, or to give a chemical unit, it takes 96,540 coulombs to liberate 1·01 gram of hydrogen from an electrolyte.

The **ohm** or unit of electrical resistance is the amount of resistance offered by a column of mercury 1 sq. mm. in section and 1·0626 metres long.

The **Siemens** mercury unit is the resistance of a column of mercury 1 sq. mm. in section and exactly one metre long.

An **ampère** is a current which passes in every second at the rate of one coulomb through a conductor.

Therefore to get a current of one ampère to develop 1·01 grm. of hydrogen, it must pass for 96,540 seconds or twenty-six hours and forty-nine minutes through the electrolyte. Just as the same amount of water, but falling from different heights, will do different amounts of work according to the impetus it has acquired, so will an electrical current, according to its tension, electro-motive force, or potential difference, produce different effects. The electro-motive force is measured by taking as a unit the volt or the difference of potential found at the two ends of a resistance of one ohm, when an ampère, that is one coulomb of electricity per second, is passing through the resistance.

The **watt** or **volt-coulomb** is the product of the volt into the coulomb, and is equivalent per second to 10^7 ergs, 10,204 gravitation units, or 0·241 calories.

A **macrocallory** is that amount of heat which is required to raise one kilogram of water from 0° C. to 1° C.

A **microcallory** is the $\frac{1}{1000}$ part of a macrocallory, and therefore corresponds to the amount of heat which will raise 1 gram of water from 0° C. to 1° C.

The Board of Trade unit of electric power is 1,000 watt hours.

$$\text{Ohm's Law} = C\,\frac{E}{R} = \text{current}\,\frac{\text{electro-motive force}}{\text{resistance}} = \text{ampère}\,\frac{\text{volt}}{\text{ohm}}.$$

[**C.G.S.** means centimetre, gram, and second units, chosen in 1881 by the international congress of electricians at Paris.]

English and French Measures and Weights.

English avoirdupois weights.

1 pound (lb.)	= 16 ounces	= 453·5925	grams.
1 ounce (oz.)	= 16 drachms	= 28·3495	grams.
1 drachm (dr.)	= 27·34375 grains	= 0·772	gram.
1 grain (gr.)		= 0·06479	gram.

French weights (Metric System).

One gram is the weight of a cubic centimetre of distilled water at 4° C.

1 milligram	= 0·001 gram	= 0·01543 grain.
10 milligrams (mg.)	= 1 centigram (cg.)	= 0·15432 grain.
10 centigrams	= 1 decigram (dg.)	= 1·5432 grain.
10 decigrams	= 1 gram (g. or grm.)	= $15\frac{2}{5}$ grains.
10 grams	= 1 decagram (Dg.)	= 2 drachms 34 grains.
10 decagrams	= 1 hectogram (Hg.)	= 25 drachms 40 grains.
10 hectograms	= 1 kilogram (Kg.)	= 2 pounds 3 ounces 119·8 grains avoirdupois.
10 kilograms	= 1 myriagram (Mg.)	

The Conversion of French into English Weights.

In this table the gram, which equals 15·4346 grains, is taken as being equal to $15\frac{2}{5}$ grains.

1 gram	=	$15\frac{2}{5}$ grains.				
2 grams	=	$30\frac{4}{5}$,,				
3 ,,	=	$46\frac{1}{5}$,,				
4 ,,	=	$61\frac{3}{5}$,,	..... or	1 drachm	$1\frac{3}{5}$	grain.
5 ,,	=	77 ,,	..... ,,	1 ,,	17	grains.
6 ,,	=	$92\frac{2}{5}$,,	..... ,,	1 ,,	$33\frac{2}{5}$	,,
7 ,,	=	$107\frac{4}{5}$,,	..... ,,	1 ,,	$47\frac{4}{5}$	,,
8 ,,	=	$123\frac{1}{5}$,,	..... ,,	2 drachms	$3\frac{1}{5}$	,,
9 ,,	=	$138\frac{3}{5}$,,	..... ,,	2 ,,	$18\frac{3}{5}$	,,
10 ,,	=	154 ,,	..... ,,	2 ,,	34	,,
11 ,,	=	$169\frac{2}{5}$,,	..... ,,	2 ,,	$49\frac{2}{5}$	,,
12 ,,	=	$184\frac{4}{5}$,,	..... ,,	3 ,,	$4\frac{4}{5}$	,,
13 ,,	=	$200\frac{1}{5}$,,	..... ,,	3 ,,	$20\frac{1}{5}$	,,
14 ,,	=	$215\frac{3}{5}$,,	..... ,,	3 ,,	$35\frac{3}{5}$	,,
15 ,,	=	231 ,,	..... ,,	3 ,,	51	,,
16 ,,	=	$246\frac{2}{5}$,,	..... ,,	4 ,,	$6\frac{2}{5}$	,,
17 ,,	=	$261\frac{4}{5}$,,	..... ,,	4 ,,	$21\frac{4}{5}$	,,
18 ,,	=	$277\frac{1}{5}$,,	..... ,,	4 ,,	$37\frac{1}{5}$	,,
19 ,,	=	$292\frac{3}{5}$,,	..... ,,	4 ,,	$52\frac{3}{5}$	,,
20 ,,	=	308 ,,	..... ,,	5 ,,	8	,,
30 ,,	=	462 ,,	..... ,,	7 ,,	42	,,
40 ,,	=	616 ,,	..... ,,	10 ,,	16	,,
50 ,,	=	770 ,,	..... ,,	12 ,,	50	,,
60 ,,	=	924 ,,	..... ,,	15 ,,	24	,,
70 ,,	=	1078 ,,	..... ,,	17 ,,	58	,,
80 ,,	=	1232 ,,	..... ,,	20 ,,	32	,,
90 ,,	=	1386 ,,	..... ,,	23 ,,	6	,,
100 ,,	=	1540 ,,	..... ,,	25 ,,	40	,,

The Conversion of Grains and Ounces into Grams.

	Grains to Grams.	*Ounces to Grams.*	*Grains to the Ounce = Grams to 100 cc.*
1	0·06479	28·3495	0·22817
2	0·12958	56·9660	0·45635
3	0·19437	85·0485	0·68452
4	0·25916	113·3980	0·91269
5	0·32395	141·7475	1·14086
6	0·38874	170·0970	1·36904
7	0·45353	198·4465	1·59721
8	0·51832	226·7960	1·82538
9	0·58311	255·1455	2·05356
10	0·6479	283·495	2·2817

English measures of capacity.

1 gallon (C) = 8 pints = 4543·487 cc.
1 pint (O) = 20 fluid ounces = 567·936 cc.
1 fluid ounce = 8 fluid drachms = 28·396 cc.
1 fluid drachm = 60 minims = 3·5495 cc.
1 minim = 0·05916 cc.

French measures of capacity.

One cubic centimetre (cc.) equals 16·896 minims and in weight is equivalent to 1 gram. In the following table the cubic centimetre is taken as equal to 17 minims.

1 cubic centimetre	=	17	minims.					
2 cubic centimetres	=	34	,,					
3 ,,	=	51	,,					
4 ,,	=	68	,,	or	1	drachm	8	minims.
5 ,,	=	85	,,	,,	1	,,	25	,,
6 ,,	=	102	,,	,,	1	,,	42	,,
7 ,,	=	119	,,	,,	1	,,	59	,,
8 ,,	=	136	,,	,,	2	drachms	16	,,
9 ,,	=	153	,,	,,	2	,,	33	,,
10 ,,	=	170	,,	,,	2	,,	50	,,
20 ,,	=	340	,,	,,	5	,,	40	,,
30 ,,	=	510	,,	,,	1	ounce	0 drachm	30 minims.
40 ,,	=	680	,,	,,	1	,,	3 drachms	20 ,,
50 ,,	=	850	,,	,,	1	,,	6 ,,	10 ,,
60 ,,	=	1020	,,	,,	2	ounces	1 drachm	0 ,,
70 ,,	=	1190	,,	,,	2	,,	3 drachms	50 ,,
80 ,,	=	1360	,,	,,	2	,,	6 ,,	40 ,,
90 ,,	=	1530	,,	,,	3	,,	1 drachm	30 ,,
100 ,,	=	1700	,,	,,	3	,,	4 drachms	20 ,,
1000 ,,	=	1·760773	pints.					

Conversion of minims, drachms, ounces, and pints to cubic centimetres and litres.

	Minims to cc.	*Drachms to cc.*	*Ounces to cc.*	*Pints to Litres.*
1	0·05916	3·5495	28·396	0·56792
2	0·11832	7·0990	56·792	1·13584
3	0·17748	10·6485	85·188	1·70376
4	0·23664	14·1980	113·584	2·27168
5	0·29580	17·7475	141·980	2·83960
6	0·35496	21·2970	170·376	3·40752
7	0·41412	24·8465	198·772	3·97544
8	0·47328	28·3960	227·168	4·54336
9	0·53244	31·9455	255·564	5·11128
10	0·59160	35·495	283·96	5·6792

French measures of length.

1 millimetre (mm.)	=	0·03937 inch.
1 centimetre (cm.)	=	0·3937 inch.
1 metre (m.)	=	39·37079 inches.
1 kilometre (km.)	=	1093·6331 yards.

Comparison of the English inch with the millimetre.

Inch.	*mm.*	*Inch.*	*mm.*
$\frac{1}{25000}$	0·001016	$\frac{1}{16}$	1·587486
$\frac{1}{10000}$	0·002534	$\frac{1}{12}$	2·116648
$\frac{1}{1000}$	0·025340	$\frac{1}{8}$	3·174972
$\frac{1}{800}$	0·031750	$\frac{1}{4}$	6·349943
$\frac{1}{500}$	0·050800	$\frac{1}{2}$	12·69989
$\frac{1}{250}$	0·101599	$\frac{3}{4}$	19·04983
$\frac{1}{100}$	0·253998	1	25·39977
$\frac{1}{50}$	0·507995	12	304·8
$\frac{1}{25}$	1·015991		

MEASURES OF TEMPERATURE.

Formulae for converting degrees of Celsius, Fahrenheit, and Réaumur into one another (after 'Behrens' Tabellen').

$$n^\circ C. = \frac{4}{5} n^\circ R. = \frac{9}{5} n + 32^\circ F.$$

$$n^\circ F. = \frac{5}{9} (n - 32)^\circ C. = \frac{4}{9} (n - 32)^\circ R.$$

$$n^\circ R. = \frac{5}{4} n^\circ C. = \frac{9}{4} n + 32^\circ F.$$

$1^\circ C. = 0{\cdot}8^\circ R. = 33{\cdot}8^\circ F.$
$100^\circ C. = 80^\circ R. = 212^\circ F.$

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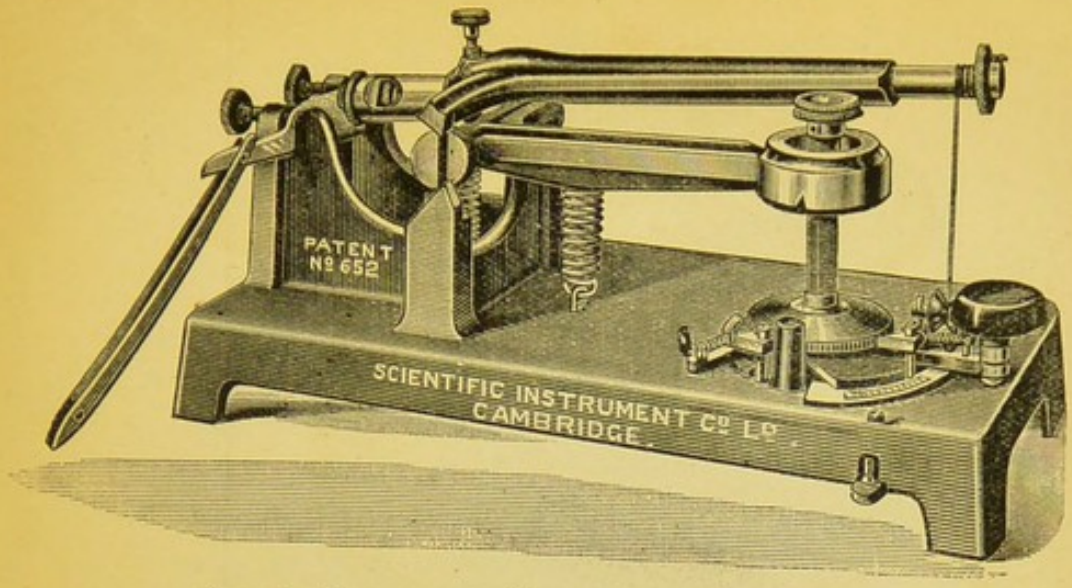


FIG. 6. THE SMALL CAMBRIDGE MICROTOME.

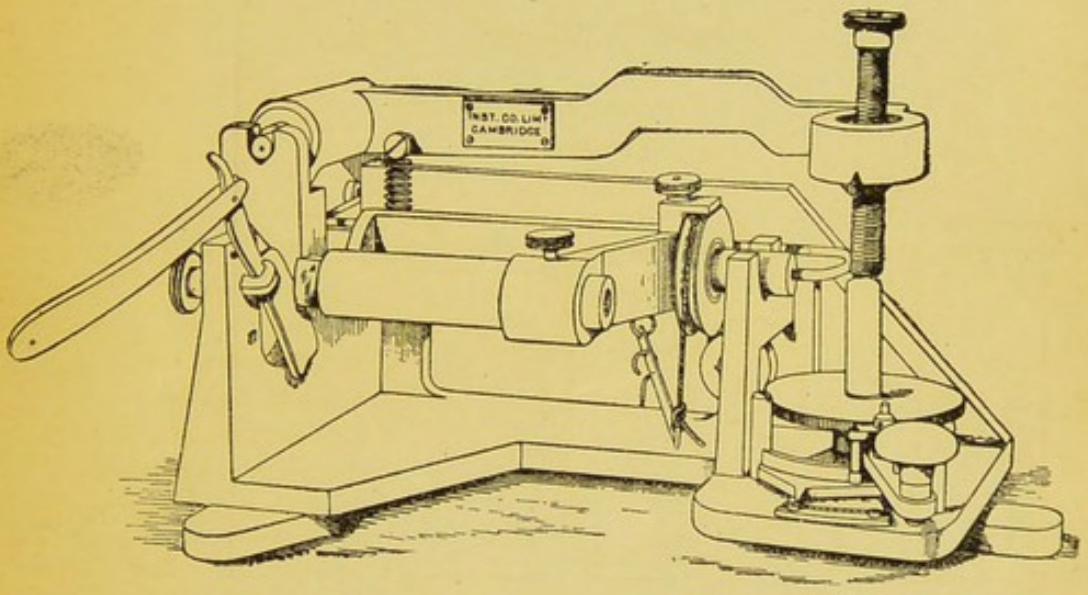


FIG. 7. THE LARGE CAMBRIDGE MICROTOME.

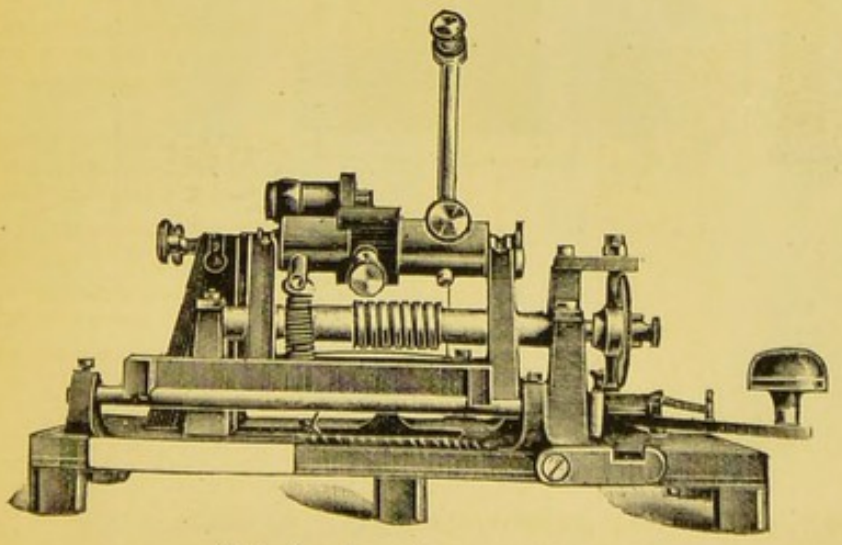


FIG. 8. LEEK'S MICROTOME.

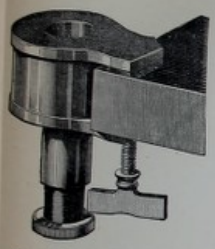


Fig. 1. Stirling's microtome.
(*W. Stirling.*)

Fig. 2. Ranvier's microtome.
(*W. Stirling.*)

A. Well for holding the tissue and the imbedding mass.
B. Brass plate acting as a support for the knife.
C. Mother-screw for holding D.
D. Screw for raising the tissue in the well A.
E. Spring-gauge for determining the thickness of sections.
F. Screw for fixing the microtome to the table.
G. Box for holding the freezing mixture.
H. Exit tube for the water.

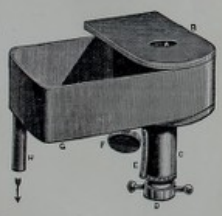
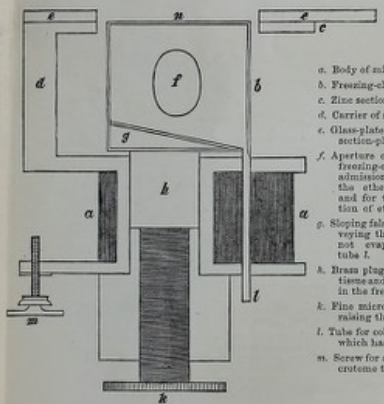


Fig. 3. Rutherford's microtome.
(*W. Stirling.*)

Fig. 4. Quecket's microtome.
(*W. Stirling.*)



a. Body of microtome.
b. Freezing-chamber.
c. Zinc section-plate.
d. Carrier of section-plate.
e. Glass-plate covering the zinc section-plate.
f. Aperture on either side of freezing-chamber for the admission of the nozzle of the ether-spray-producer, and for the free evaporation of ether.
g. Sloping false bottom for conveying the ether which is not evaporated to the tube *l*.
h. Brass plug for carrying the tissue and moving upwards in the freezing-chamber *b*.
k. Fine micrometer screw for raising the brass plug *h*.
l. Tube for collecting the ether which has not evaporated.
m. Screw for attaching the microtome to the table.

Fig. 5. Bevan Lewis' microtome.
(*W. Stirling.*)

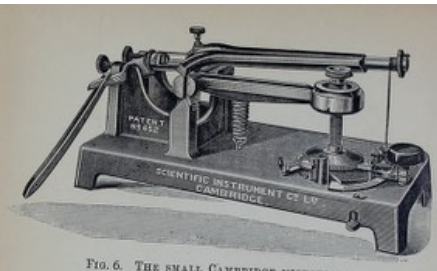


Fig. 6. The small Cambridge microtome.

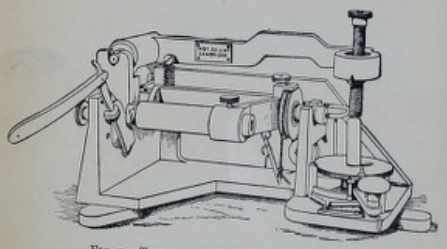


Fig. 7. The large Cambridge microtome.

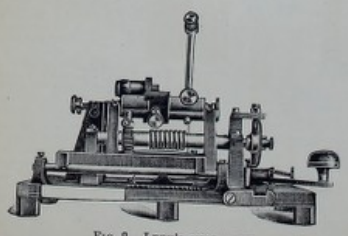


Fig. 8. Leek's microtome.

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