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THE APPLICATION OF THE PRINCIPLES OF PHYSICAL CHEMISTRY TO THE STUDY OF THE BIOLOGICAL ANTIBODIES

BY

SVANTE ARRHENIUS

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PREFACE

THE following pages contain a summary of six lectures
on the Immunity Reactions delivered at the University of
California, in Berkeley, California, during the summer
session of 1904. The object of the lectures was to illus-
trate the application of the methods of physical chemistry
to the study of the theory of toxins and antitoxins. The
idea that the reciprocal action of toxin and antitoxin is
of the same nature as a chemical reaction is nearly as old
as the study of these phenomena, which was inaugurated
by the discovery of the diphtheria antitoxin by Behring
and Kitasato in 1890. The German school, led by Ehrlich,
the renowned Director of the Prussian Serum Institute in
Frankfort-on-the-Main, has in particular done much work
in support of the opinion that the interaction of toxin and
antitoxin is of the nature of a chemical reaction; whereas
the French school, led by Metschnikoff, tried to show that
the effect of an antitoxin is chiefly of physiological order,
an antitoxin was supposed to stimulate in some way the
organic tissues in their struggle against the attack of the
poison.[1]

When Ehrlich succeeded in showing that the agglutinat-
ing action of ricin upon red corpuscles (erythrocytes) sus-
pended in a physiological salt-solution (0.9% NaCl) is
inhibited by the antibody, — the antiricin, — the notion
that a physiological effect is executed by the antibody was

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[1] The first studies in this direction were carried out by Buchner (1893)
and Roux and Vaillard (1894). Cf. Chapter II.

abandoned by most scientists. Although I adhere to the chemical school, I cannot say that the proof of Ehrlich is quite convincing, since the erythrocytes may well be regarded as "living" even after their separation from the blood of an animal. The further fact stated by Ehrlich, namely, that approximately the n-fold quantity of a toxin requires the n-fold quantity of antitoxin for its neutralisation, may be regarded as a more convincing proof against the physiological hypothesis. The chemical hypothesis is now generally accepted, and has been adopted recently by Bordet, who originally expressed ideas similar to those held by Metschnikoff.

Nevertheless, many difficulties to the chemical hypothesis remained. Nothing was more natural, therefore, than that the further elucidation of the problem should be sought through the aid of the modern theories of solution. To this end Madsen and Ehrlich invited me to join in their work. My work with Madsen in the Copenhagen Institute enabled us to fix upon a simple explanation of the chief difficulty exhibited by the so-called phenomenon of Ehrlich. The Chief of the Frankfort Institute was so deeply interested in the progress of these studies that he invited me to work in his Institute on the chemical behaviour of compound hæmolysis. In this case, also, it was determined that the laws of equilibrium found their application. It would seem, therefore, that the adherents of the chemical hypothesis should have felt wholly satisfied with the results. However, one of the strange incidents with which the history of science is replete occurred. In our explanation of the investigated phenomena, especially regarding the diphtheria toxin, Madsen and I, in accordance with the usual rule in the exact sciences, tried to employ as few hypotheses as possible, and in this we fol-

lowed the example of Bordet. We tried to show that the phenomena observed might be explained on the supposition that diphtheria toxin is a simple substance which slowly decomposes into an innocuous material that still neutralises antitoxin. In his explanation Ehrlich had previously assumed the presence in the diphtheria poison of a large number of poisonous substances of different strength. Now Ehrlich did not wish to yield this explanation, which he regards as the principal point in his doctrine; and therefore he and his numerous pupils raised a number of objections to the treatment of this branch of science in accordance with the modern theories of chemistry. Thereupon Biltz, encouraged by Ehrlich, took up and elaborated the old idea of Bordet, which had been abandoned by this prominent savant in favour of the chemical hypothesis, and suggested that antitoxin does not react chemically with toxin, but behaves about in the same manner as a dye when it becomes fixed in a fibre.

Some of these recent objections to the ideas brought forward in the lectures have been taken here into consideration, notwithstanding that they have appeared since the lectures were delivered. In the same way, much recent work, especially by Madsen and his pupils (in large part as yet unpublished), bearing upon the velocity of reactions, has been given consideration in the following pages. And the recent work of Hamburger on precipitins has been made use of in the final chapter.

I have given to these lectures the title " Immuno-chemistry," and wish with this word to indicate that the chemical reactions of the substances that are produced by the injection of foreign substances into the blood of animals, *i.e.* by immunisation, are under discussion in these pages. From this it follows also that the substances with which these

products react, as proteins and ferments, are to be here considered with respect to their chemical properties. And for the purpose of a clarification of ideas, other substances that behave in an analogous manner will be given a consideration in the discussion.

It is evident that the objection recently raised by Ehrlich and Sachs to this manner of investigation, namely, that it does not enter upon the mode by which the living body produces these so-called antibodies, is quite true. An investigation of the chemical relations of toxin and antitoxin need not carry with it an elucidation of the synthesis of the antitoxin. But I fancy that there are many who are so deeply interested in the chemical behaviour of these substances that they will find an investigation of this question well worthy of study. And for myself, furthermore, I believe that the physiological side of the problem, alluded to by Ehrlich, will not find a satisfactory solution until the more simple chemical aspect is elucidated.

The chief purpose of theoretical considerations is to afford clear and concise ideas of the observed facts. They thereby stimulate scientific research to a high degree. I venture to hope that the reader of the following pages will find that the theoretical views advanced by myself in this branch of science have fulfilled their rôle in a most satisfactory manner during the few years that they have been employed. I am glad to say that during these few years a large experimental material has been produced, which shows that the chief lines pointed out by theory are closely congruent with the facts; and this material has been produced almost exclusively in order to verify the said theoretical considerations. These have, therefore, already been of great scientific use, and yet this is only a small part of

what may be expected. The reader will have frequent opportunities to compare the different scientific points of view that are connected with the theoretical views here exposed with those founded on other theoretical considerations.

I am deeply indebted to my friend Professor Alonzo Engelbert Taylor of Berkeley, California, for his great kindness in the revision of the manuscript and for the correction of some errors.

To my many friends in California, these lines will, I hope, recall the pleasant period of time during which I had the good fortune to live with them in their agreeable country.

SVANTE ARRHENIUS.

STOCKHOLM, SWEDEN,
October, 1906.

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CHAPTER I

INTRODUCTION

RECENTLY the so-called antibodies that are produced in animals after the injection of certain more or less poisonous substances have acquired a very great importance, and the chemical behaviour of these antibodies has been the object of a large number of investigations. Some of these have led to the idea that the reactions of these substances are incomplete and follow the law of Guldberg and Waage. It is of these researches, carried out chiefly by the Director of the Danish State Serum Institute, Dr. Thorwald Madsen, and myself, that I wish to give a short review in the following lectures.

For only a few poisons have the corresponding antibodies been prepared. These poisons are termed toxins. They are all of organic derivation. Bashford[1] and Besredka[2] have tried in vain to prepare antitoxins to solanin and saponin by the injection of these substances into rabbits and guinea-pigs. And the so-called antimorphine has proved to be a failure.[3] Solanin, saponin, and morphin

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[1] Bashford: "Über Blutimmunität," *Arch. intern. de pharmacodynamie et de thérapie*, T. **8**. 101 et **9**. 451 (1901).

[2] Metschnikoff: "L'immunité," Paris, 1901, p. 410.

[3] Morgenroth: *Berl. klin. Wochenschrift*, 1903, No. 21.

are therefore not toxins. On the other hand, extracts from the seeds of *Ricinus communis* contain a toxin called ricin, against which we possess an antitoxin called antiricin. In the same way antitoxins are known corresponding to abrin and robin, extracted from the seeds of *Abrus præcatorius* and of *Robinia pseudacacia*. It is not only against poisons, but also against wholly or nearly inoffensive bodies, that animals produce antibodies. Furthermore it seems that if we introduce nearly any type of cell into the veins of an animal, its blood will contain after a time an antibody which destroys the particular variety of cell. Even after the injection of rennet, the ferment of the coagulation of milk, we obtain an antibody called antirennet, which hinders the coagulative power of rennet.[1] It is very difficult to draw a distinction between enzymes or ferments and toxins. Like rennet, many others of these substances are found to yield antibodies after injection into the blood of different animals. Thus, for instance, v. Dungern[2] prepared in this way antibodies against proteolytic enzymes from pathogenic microbes. Hildebrand[3] obtained in a similar way an antibody against the ferment emulsin. Gessard[4] found it possible to prepare an antibody against tyrosinase, an oxydase extracted from mushrooms; and Sachs[5] showed that the serum from a goose which had been injected with pepsin contained antipepsin.

A. Schütze prepared "antilactase" by injection of "lactase from kefir" into rabbits or hens; other antibodies

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[1] Morgenroth: *Centralblatt f. Bakteriologie*, Abt. I, Vols. **26** and **27**.

[2] v. Dungern: *Centralblatt f. Bakteriologie*, Thl. I, Vol. **24** (1898).

[3] Hildebrand: *Virchow's Archiv*, Vol. **131** (1893).

[4] Gessard: *Annales de l'institut Pasteur*, **15**. 607 (1901).

[5] H. Sachs: *Fortschritte d. medicin*, **20**. 425 (1902).

have been prepared against cynarase, fibrin ferment, pancreatic ferment, zymase, and urease.[1]

It therefore seems to be only a question of time when we shall be able to prepare antibodies against ferments and enzymes in general.

An antibody to rennet is contained in the so-called normal serum of the horse; that is, horse blood freed of fibrin by being shaken with small solid bodies such as glass beads or pieces of iron wire, and separated from the red blood corpuscles by centrifugation. (This was first shown by Hammarsten and Röden.[2]) In the same manner fresh serum and even egg-white contain antibodies against many other substances, as for instance against trypsin and tetanolysin. By natural or normal blood serum is meant that obtained from fresh animals that have not been inoculated in any manner. If before the preparation of the blood serum foreign bodies have been injected into the veins of the animal, we obtain generally not normal serum but serum containing an antibody, which is "specific" to the injected body (*i.e.* immune-serum).

In order to render this peculiar subject clearer I will give a short review of the mode in which these antibodies appear in the serum and how they afterwards disappear from it.

The next curve shows the concentration of cholera-agglutinin in the blood of a goat, which had some time before the experiment been immunised with cultures of *Vibrio choleræ*, so that the concentration of agglutinin in it

[1] Compare A. Schütze: *Zeitschr. f. Hygiene*, **48** (1904).
[2] Röden: *Upsala Läkareförenings Förhandlingar*, **22**. 546 (1887); *Maly's Jahresbericht*, **17**. 160 (1887).

was at the commencement four arbitrary units. If we inject a culture of the bacillus (*e.g. Vibrio choleræ*) into the blood of a goat, it produces antibodies specific to just the injected bacilli. Amongst these antibodies is one called agglutinin, because it causes cholera bacilli to clump together and sink to the bottom of the liquid in which they are suspended. We may measure the concentration of agglutinin in a liquid by a method to be described below.

The experiments, the results of which are given in the curve, were carried out by Madsen and Jörgensen,[1] following the subcutaneous injection of 40 c.c. of a culture of cholera bacilli into a goat. From the jugular vein of the goat small amounts of blood were taken every day and examined as to their content of agglutinin. In the first two days no increase in the quantity of agglutinin in the blood serum was observed, but later on it rose rapidly till it reached a maximum, "acme," on the eighth day, after which it sank at first rapidly, then more slowly.

The continuation of the curve illustrates an experiment with the same goat, in which an injection of 5 c.c. of a culture of cholera bacilli was made daily during the whole period of research. Here the first period, in which no agglutinin was produced, lasted four days, and the second period, where the agglutinin increased, eleven days.

Madsen has found that the decrease after the maximum follows closely the equation for the velocity of a chemical reaction :

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[1] A. Jörgensen and Thorwald Madsen : "The Fate of Typhoid and Cholera Agglutinins," *Festskrift oed indvielsen af statens serum-institut*, VI, 12, 1902, Copenhagen.
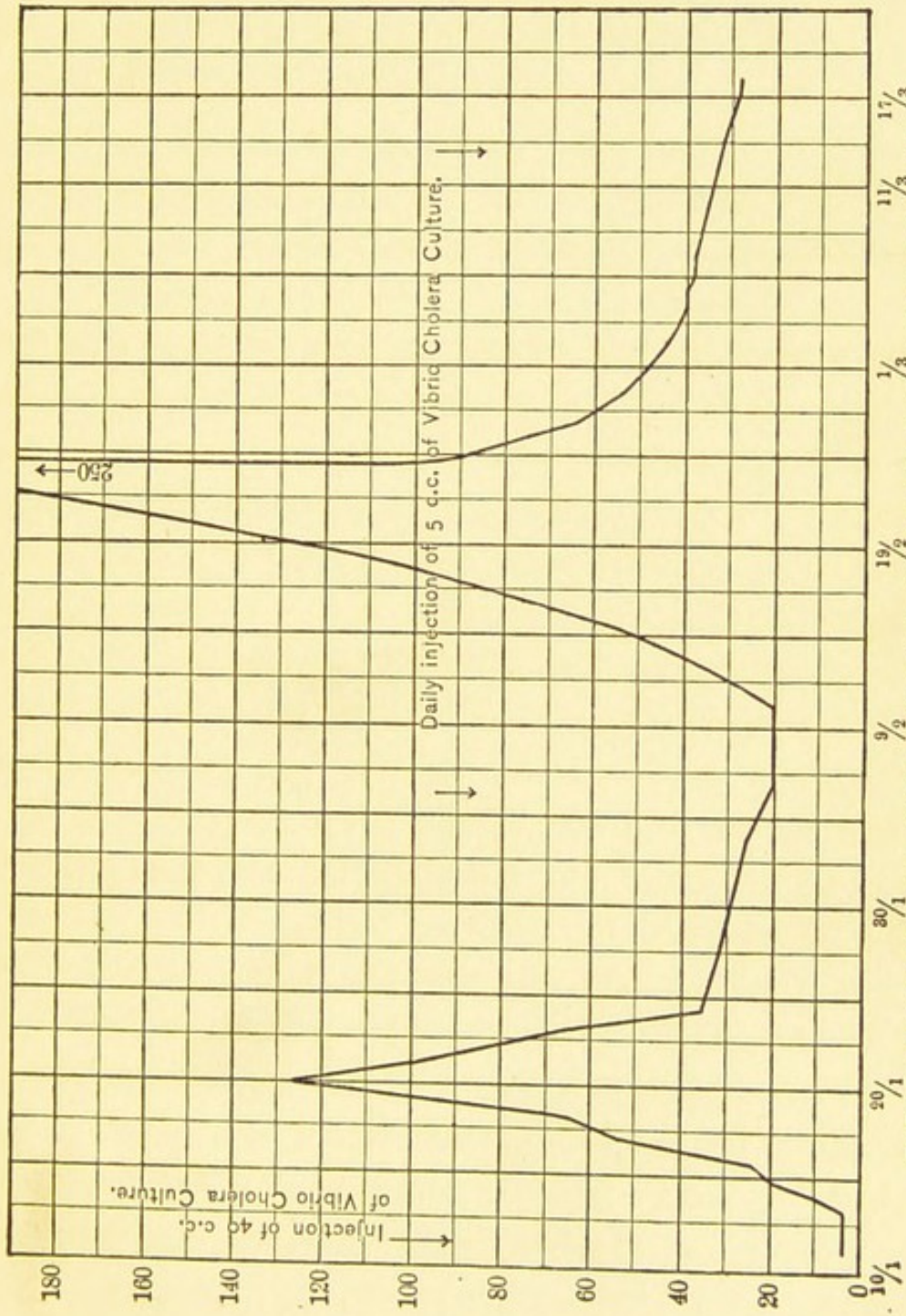
CURVE, INDICATING THE VARIATION OF THE QUANTITY OF CHOLERA-AGGLUTININ
IN A GOAT INJECTED WITH CULTURES OF CHOLERA-VIBRIONS. (Madsen and Jörgensen)
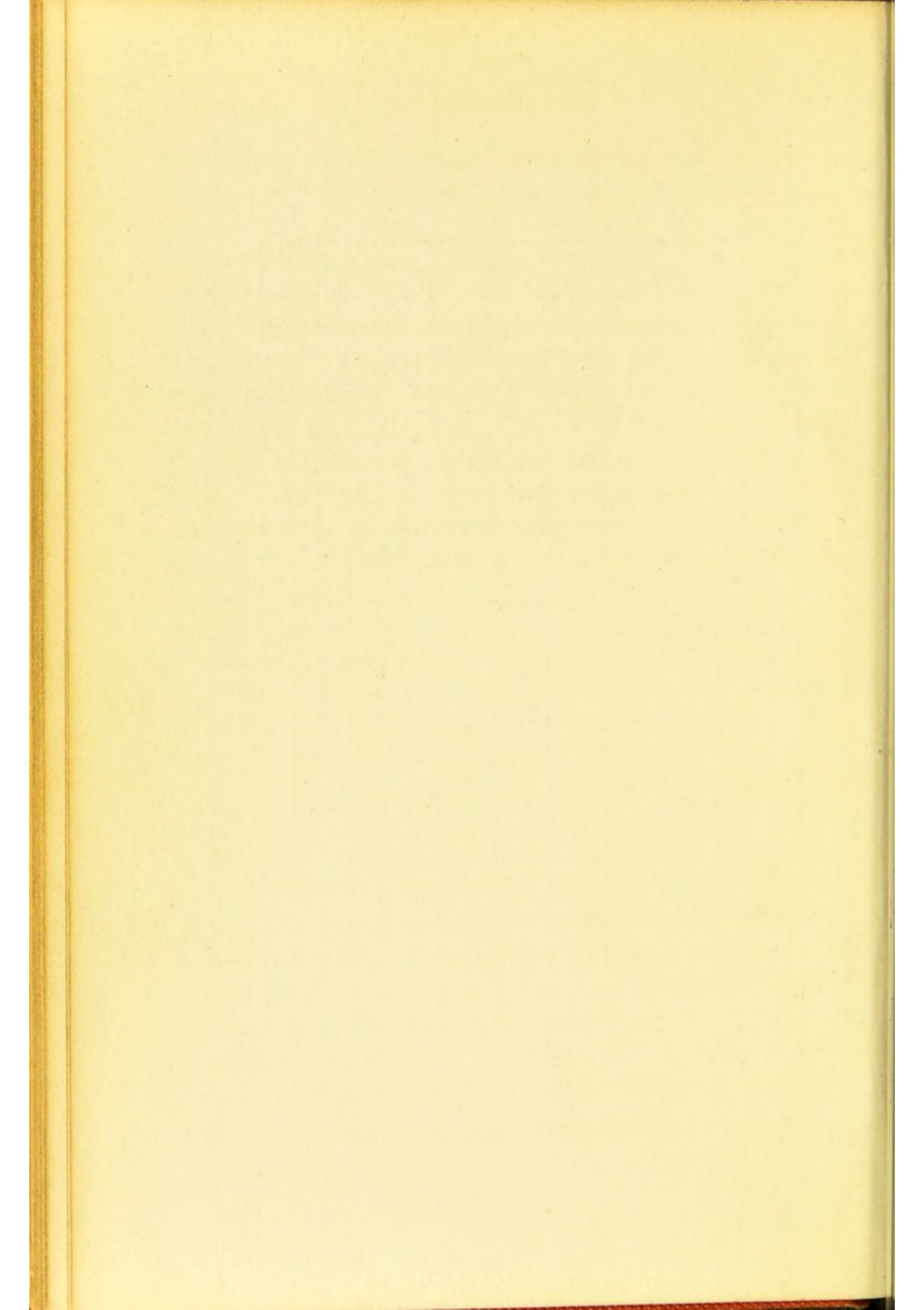
Rate of decrease = const. (concentration),

which gives the integrated formula

$$\frac{1}{(\text{concentration})^{n-1}} = \text{const.}_1 + \text{const.}_2\, t,$$

where t represents the time (after the maximum) that corresponds to the concentration in the formula.

For the case with cholera agglutinin produced by goats, Madsen found $n = 3.5$, const.$_1$ = 0.082 and 0.32 resp., and const.$_2$ = 0.13 and 0.032 resp. for the first and the second part of the curve represented above.

As example we give the figures of the second part, together with the results of the calculation.

T (Days)	Conc.$_{OBS.}$	Conc.$_{CALC.}$	T (Days)	Conc.$_{OBS.}$	Conc.$_{CALC.}$
0	250	250	8	43	43
1	91	96	10	40	40
2	77	74	12	38	37
3	63	63	14	36	35
4	56	57	16	34	33
5	52	52	18	32	31
6	49	48	20	30	30
7	46	45	22	28	29

In this case the exponent n was rather high, higher than in other cases examined by Madsen.[1] For goats with typhoid agglutinin he found $n = 1.3$ and $n = 1.5$. This last figure was also found for typhoid agglutinin in the blood of rabbits. The figures of Bomstein for the quantity of diphtheria-antitoxin in the blood of dogs and guinea-

[1] Th. Madsen: "The Decrease of Antibodies in the Organism," *Festskrift*, VIII, Copenhagen, 1902.

pigs after direct injection [1] of this antibody in the blood of these animals gave $n = 1.2$. In this case the constant const.$_2$ is nearly of the same magnitude for different dogs, as one might expect (it is in three cases 0.15, 0.18, and 0.19). For the destruction of antibodies in the human body, Madsen has found $n = 2$ in many cases. Of course this formula is only an empirical one. Its physical meaning is easily understood. The examples calculated by Madsen show a very good agreement with the experiment.

As further instances may be cited the following figures for the decomposition of typhoid agglutinin in the blood of a passively immunised goat (according to Jörgensen and Madsen) and in that of a man who had suffered from typhoid fever (according to Jörgensen).[2] $x =$ concentration.

DESTRUCTION OF TYPHOID AGGLUTININ IN A PASSIVELY IMMUNISED GOAT			DESTRUCTION OF TYPHOID AGGLUTININ IN THE BLOOD OF A MAN AFTER TYPHOID FEVER		
T (Days)	$10^4 \times \left(\frac{1}{x}\right)$ obs.	$10^4 \times \left(\frac{1}{x}\right)$ calc.	T (Days)	$10^3 \times \left(\frac{1}{x}\right)$ obs.	$10^3 \times \left(\frac{1}{x}\right)$ calc.
0	11	11	0	17	17
0.3	18	19	2	20	20
1	30	30	6	28	28
3	48	50	10	40	37
5	60	64	15	60	53
8	80	81	20	85	74
11	100	94	27	100	113
15	110	110	35	150	168
			42	250	255

$n = 3.$ const.$_2 = 8 \times 10^{-6}.$ $n = 1.2.$ const.$_2 = 0.0302.$

[1] So-called passive immunisation: "Active immunisation" is produced by injection of the toxins or cells themselves, as is described above.

[2] Madsen: "Lecture Held at the Meeting of Brit. Med. Ass., Oxford, July, 1904," *British Medical Journal*, Sept. 10, 1904.

The use of formulæ represents a great step forwards in the study of this portion of the science of immunisation. Formerly investigators were content to state that the quantity of antitoxin in the blood decreases gradually, and in the first stages more rapidly than later on. The application of the formula of Madsen teaches us much more. It shows that the phenomenon is a regular one, and we are impelled to seek for a cause for the differences of the values of the constants n and $const_2$. For instance, the different values of $const._2$ for the three days in the experiments of Bomstein — are they really different or do the observed differences depend only upon experimental errors? This and other questions suggest themselves after the use of such an equation, and they lead to improvement in the experimental methods, and to very sharp and well-defined ideas of the natural phenomena themselves. With the help of formulæ, which may be empiric or rational, scientific progress will be much more rapid than without them ; and as the experimental material increases, the empiric formulæ will probably be converted into rational ones, _i.e._ we shall detect new laws of nature. It is therefore very much to be regretted that efforts have been made, especially recently, to reject the use of formulæ in the treatment of questions of serum-therapy. These efforts may be regarded as a last desperate struggle against the stringent conclusions that may be reached by means of the application of mathematical treatment — a struggle that cannot be greatly prolonged.

The injection of toxins or cells into the blood of an animal can be done in different ways. Perhaps the most used of them is the "intravenous injection" directly into the

veins of the animal; as a special case may be regarded the "intracardial injection" directly into the heart of the animal. More slowly acts the "intraperitoneal injection" into the peritoneum, and still more so the "intramuscular injection," or the "subcutaneous injection," under the skin of the animal. This last method is very much used. In these cases the injected body reaches the blood very slowly, in which fluid it produces antibodies. The antibodies may be divided into two great groups according to the nature of the injected fluid, whether it is a homogeneous solution or an emulsion of cells, *e.g.* bacteria or red blood corpuscles. These suspensions of, *e.g.*, erythrocytes are secured by centrifugating blood freed of fibrin. Between the centrifugalized blood corpuscles there still remains a noticeable quantity of normal serum. This serum contains a very effective reagent for most of the reactions which we wish to study. It is therefore necessary to wash it away. For this purpose we use a so-called physiological solution, in most cases of sodium chloride (0.8–0.9 %). Suspended in this solution blood corpuscles and most bacilli remain unaltered. In stronger solutions the corpuscles or bacilli contract; in weaker solutions the erythrocytes give up their red colouring matter and remain colourless ("stromata"). The bacilli are injured by plasmolysis or imbibition of water. To be washed, the blood corpuscles are shaken with the physiological solution, centrifugalized, separated, and this operation repeated until the serum is washed away as much as necessary. Usually two or three washings with five times the amount of solution are sufficient.

The antibodies produced after the injection of a homo-

geneous solution of a substance, *e.g.* a toxin, combine in most cases with the injected body to form more innocuous compounds. If these compounds are soluble in the mixture, we call the antibodies antitoxins (at least if the injected body is poisonous). If, on the contrary, the compound is insoluble, the antibody is called a precipitin. Such precipitins are produced after the injection of different albuminoid substances.

If the injection is a suspension of cells, the antibody formed sometimes dissolves these cells; in this case it is called a lysin. Thus after the injection of bacteria there are formed bacteriolysins, which under proper conditions dissolve the bacteria of the injected variety. After the injection of erythrocytes so-called hæmolysins are formed, which produce hæmolysis, *i.e.* cause the red colouring matter (the hæmoglobin) of the injected erythrocytes to leave them and to diffuse into the surrounding liquid.

In other cases, other antibodies are formed, often simultaneously with lysins, so-called agglutinins, which produce an agglutination of the injected cells. In this case the presence of salts plays an important rôle.

The normal serum often contains a certain quantity of antibodies. Thus, for instance, in the normal serum of horses re markable quantities of diphtheria-antitoxin and of antirennet are often found. This peculiarity is so frequently observed, that Ehrlich supposes that all possible antibodies exist in normal sera, though in most cases the amount is not sufficient to be shown experimentally. In the blood of many animals we find two organic substances, cholesterin and lecithin, which react with many of the injected poisons and therefore play an important rôle as antitoxins or even as

constituents of toxins. Thus, for example, the hæmolytic poison tetanolysin that is produced by the *Bacillus tetani* may be interfered with in its hæmolytic power by cholesterin. The researches of Madsen and Walbum seem to prove that the compound of these two bodies is quite innocuous, so that the cholesterin may be used as an antibody against tetanolysin. Now it is not specific against tetanolysin, as is the antitetanolysin, but it is effective also against other lysins; namely, saponin, cobralysin, cobralecithid, and olive oil, but not against staphylolysin and arachnolysin.[1]

The lecithin, on the other hand, combines with the poison of cobra, so that a hæmolysin cobralecithid is formed, that also in exceedingly minute quantities exerts an hæmolytic influence upon erythrocytes. The cobra-poison itself displays such a property, but to a much less degree.[2]

These different bodies are often rather unstable, so that they are spontaneously decomposed. This decomposition proceeds much more rapidly at higher than at lower temperatures. They are therefore in most cases conserved at very low temperatures, sometimes in a frozen form.

Ransom[3] has made a very interesting observation regard-

[1] Kyes and Sachs: *Berl. klin. Wochenschrift*, 1903, Nos. 2–4.

[2] Overton has expressed the opinion that the action of lecithin and of cholesterin are associated to their lipoidal properties. With respect to lecithin, it seems that this idea may be applied to much of the experimental material. The lecithin seems, namely, to become dissolved in the membranes of the cells (*e.g.* the erythrocytes) and thereby facilitates the passage of some poisonous substances (*e.g.* mercuric chloride) into the cell or of hæmoglobin outwards through the cell membrane (cf. p. 158). The action of cholesterin seems to be quite different, and of a neutralizing character (cf. Chap. VIII). On the interesting investigations of Overton regarding the permeability of cellular membranes cf. Rud. Hoeber: "Physikalische Chemie der Zelle und Gewebe," 2d ed., Leipzig, 1906, pp. 163–197.

[3] *Deutsche med. Wochenschrift*, 1901, No. 13.

ing the action of cholesterin. Saponin is a powerful hæmolytic agent. Its action is conditional upon the presence of cholesterin in the blood corpuscles. On the other hand, cholesterin in the blood serum protects the blood corpuscles from being attacked by saponin. Weigert[1] compared the cholesterin with a lightning conductor, which has its proper place on the outside of the protected house. From a chemical point of view we explain the observation in the following manner. The membranes of the red blood corpuscles are permeable to saponin, but not to cholesterin or to the hæmolytic compound of this substance with saponin. Therefore the saponin is divided between the blood corpuscles and the serum practically in proportion to their content of cholesterin. If the content of saponin-cholesterin in the blood corpuscles reaches a certain amount, these are hæmolysed, otherwise not. It is the poison dissolved in the blood corpuscles that exerts an action; the poison on their outside is without effect on them.

The first step in the development of sero-therapy into an exact science consisted in devising methods for measuring the quantities of the different substances employed. In this regard Ehrlich enjoys great distinction in having been the first who with sufficient exactitude measured the strength of diphtheria-poison. To estimate justly the great progress that was due to the introduction of measurement methods by Ehrlich, it must be borne in mind that, at the time that Ehrlich did his work, nearly all of the leading men believed it impossible to measure toxins and anti-

[1] C. Weigert : " Einige neuere Arbeiten zur Theorie der Antitoxinimmunität," Wiesbaden, 1899.

toxins. This belief was due to the very different effects which the same quantity of poison exerted on two different individuals of the same species, *e.g.*, diphtheria-poison on guinea-pigs. It was only the practical necessity of having measurements of the force of poisons that led Ehrlich to overcome the great difficulties.[1]

The chief investigations of Ehrlich concern the diphtheria-poison and its antitoxin. He wished to determine the lethal dose of this poison for guinea-pigs. For this purpose he injected a *great number* of guinea-pigs with different doses; they lay in the neighbourhood of the lethal doses. It may be here mentioned that large guinea-pigs in general endure a greater quantity of poison than do small ones. Ehrlich supposed that the resistency of different individuals was proportional to their weight, and on this assumption he calculated the dose corresponding to a "normal" guinea-pig of 250 grammes weight. To obtain uniform results he found it necessary to use animals of nearly the same weight, age, and race. In the summer the animals are more resistent and give more uniform results than at other seasons, when they evidently suffer from the changes of temperature and other climatic conditions.

The lethal dose of diphtheria-toxin was defined by Ehrlich as the quantity which injected subcutaneously into a great number of guinea-pigs causes the death of the larger fraction of the animals in three to four days, the rest of the animals dying about as many before as after this time. According to this method of measurement a great deal of

[1] P. Ehrlich: "Wertbestimmung des Diphtheriheilserums," Jena, 1897; "Constitution des Diphtheriegiftes," *Deutsche med. Wochenschrift*, 1898, No. 38; Ehrlich, Kossel, and Wassermann : *Deutsche med. Wochenschrift*, 1894.

the material (namely, the animals killed in shorter or longer times) is left unconsidered. As now the exactness of the measurements increases sensibly with the material, it is of importance to use the whole material. This may be done in the following manner. The observations contain many cases, in which doses larger or smaller than the lethal dose have been injected. These observations offer a statistical material of how many days "normal" guinea-pigs live after injection of 1.5, 1.4, 1.3, 1.2, 1.1, 0.9, 0.8, 0.7, etc., lethal doses. By means of this statistical material the reduction table given below was constructed, by the aid of which all the data regarding the killed guinea-pigs may be taken into consideration.[1]

Death after Days	Injected Lethal Doses	Death after Days	Injected Lethal Doses
1	1.6	5	0.80
1.5	1.4	6	0.71
2	1.25	7	0.64
2.5	1.15	8	0.59
3	1.05	9	0.55
3.3	1.0	10	0.51
3.5	0.97	12	0.45
4	0.91	14	0.4

In order to increase the statistical material, Madsen proposed to consider the decrease of weight of the animals in determining the lethal dose.[2] In this manner an independent determination was given for each series of measurements.

[1] Arrhenius and Madsen: "Le poison diphtérique," *Oversigt danske Vid-selsk forh.*, Copenhagen, p. 269 (1904).
[2] Arrhenius and Madsen: *l.c.*, p. 274.

By the aid of all these different measurements it is possible to determine the probable error of every measurement. This was omitted in all previous considerations of this question. Under these circumstances an overestimation of the exactitude of the measurements resulted, which often led to conclusions without any solid foundation.

The lethal dose of the toxin having been determined, the next step was to standardise the solution of antitoxin. For this purpose Ehrlich determined the quantities of the solution in question that were necessary to neutralise a certain number (*e.g.*, 100) of lethal doses of the poison. As experience has taught that the antitoxin is destroyed much more slowly than the toxin, especially if it is conserved with certain precautions, a given preparation of diphtheria-antitoxin is assumed as "standard serum," and the properties of all poisons and antitoxins to be examined are compared with this standard unit.

Ehrlich in his measurements used the assumption that injections of the same quantities of free poison have the same effect, independently of the quantities of "neutralized poison" present. The correctness of this assumption has recently been subjected to question, to which we will return later.

The diphtheria-poison can be measured as far as we know only by the aid of experiments on living animals. But there are many other poisons which may be studied outside of the living body, as for instance the hæmolysins, the precipitins, and the agglutinins. Experiments "in vitro" have already long been used in physiological researches, *e.g.* in digestion, to study processes outside of the living body. Ehrlich adopted this method to study

the agglutinating power of ricin on erythrocytes in the presence of its antibody.[1] In this case it is possible to discriminate between different degrees of agglutination and thereby to measure the quantity of free ricin present. This method has been developed chiefly in the Danish serum institute.[2]

The most fruitful application of these researches "in vitro" has been in the study of the hæmolysins.[3] The lysin to be investigated is added in different quantities to circa 8 c.c. of a suspension of 2 c.c. erythrocytes in 98 c.c. of physiological salt solution. Before the admixture of lysin, water was added to the lysin-solution until its total quantity became 10 c.c. The best method is to add the suspension in an energetic manner so that a well-mixed fluid immediately results. Otherwise the lysin is concentrated in some parts and is absorbed by the erythrocytes in the proximity, and other erythrocytes remain nearly intact if the shaking takes place even a few seconds (30 to 60) after the mixing. Therefore the usual result is that the hæmolysis is less the longer the time intervening between mixing and shaking. (Variations of 50 per cent frequently occur for this reason.) To develop the hæmolysis, the test-tubes are placed during a certain time, generally one or two hours, in a water-bath or other thermostat at the desired temperature (in most cases 37° C.). The hæmolysis increases with time and seems to tend to a limit which is reached the more rapidly the more active

[1] Ehrlich: *Fortschritte der Medicin*, 1897, No. 2.

[2] Compare Jörgensen and Madsen: *Festskrift*, VI, 6, Copenhagen, 1902.

[3] Madsen: "Über Tetanolysin," *Zeitschr. f. Hygiene*, **32.** p. 214 (1899); Arrhenius, Madsen: *Festskrift*, III, 9, Copenhagen, 1902.

the poison (at 37° C. it is reached in about 40 minutes with sodium hydrate, in about 100 minutes with ammonia, and not fully in 200 minutes with tetanolysin as the hæmolytic agent). After this time the tubes are placed on ice during about twenty-four hours, when the unattacked erythrocytes fall to the bottom, leaving over them a clear hæmoglobin-coloured fluid. Generally the intensity of the colour is proportional to the quantity of poison added. The strength of the hæmolysis and the colour proportional to it is measured by common colorimetric methods. It is supposed that the hæmolysis is dependent (under similar external conditions) only on the quantity of free lysin present, and not to a sensible degree on the quantity of bound lysin or antitoxin present in the mixture. By an operation analogous to that described under the measurement of diphtheria-toxin, it is possible when the hæmolysis lies between certain limits (below total hæmolysis and above a certain observable minimum), to use not only a certain strength of colour, but the whole material of research, for the comparison.

The experiments "in vitro" may be carried out through quite definite intervals of time and at any fixed temperature (between 0° and 100° C.). Further, to the fluid examined, we may add large quantities of any foreign body and, for instance, investigate the influence of different salts by using a physiological solution of cane-sugar (about 7.2 per cent) for the suspension. It is evident how much more diversified knowledge we may obtain by these researches "in vitro" than by those on living animals, which are also expensive, and hence can only be carried out in a relatively small number and at the uncommon institutions equipped with well-filled quarters for animals.

The strengths of the agglutinins are measured by the addition of physiological salt solution until the mixture is unable to produce a certain sharply observable agglutination of a given suspension of cells in a given time and at a definite temperature. The dilution of this mixture is a measure of its content of agglutinin as displayed toward the given type of cells.[1] Madsen and Jörgensen[2] prefer to measure the agglutinins according to their power of clarifying a bacterial suspension.

Even for precipitins (*e.g.* rennet) such a degree of precipitation or coagulation may be found which is rather sharply defined from higher or lower degrees. By inclining the tubes containing the solutions to be investigated we get an impression of the consistency of the contents after treatment during a given time at a definite temperature. In a similar way as for the agglutinins it is possible to determine the strength of the precipitating or coagulating preparations.

The bacteriolysins have not been examined quantitatively. Their effects are studied in mixtures of suspensions of bacilli which are injected intraperitoneally into animals (*e.g.* guinea-pigs) and examined in specimens taken out after a time. The method of observation makes it very difficult to collect a material fit for quantitative treatment.

[1] Eisenberg and Volk: *Zeitschr. f. Hygiene,* **40.** 155 (1902).
[2] Jörgensen and Madsen: *Festskrift, l.c.*

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CHAPTER II

REVERSIBILITY OF REACTIONS BETWEEN ANTIBODIES

As has been stated, many of the substances with which we deal in sero-therapy are rather unstable. This instability is very different in different cases. Sometimes (*e.g.* for snake-venom) the toxin is more stable than the antitoxin; in other cases (*e.g.* for diphtheria and tetanus poison) the converse holds true. The snake-venom resists, as Calmette showed, an elevation of the temperature to 68° C., which, however, destroys its antitoxin, the antivenin, in aqueous solution. This circumstance was used by Calmette [1] to separate the poison from the antivenin; after heating a mixture of the two a poisonous solution remained. According to more recent investigations of Martin and Cherry [2] this experiment is not successful if the mixture is held at the temperature of the room for more than thirty minutes before the heating is done.

As Martin and Cherry intimate, the simplest explanation of this behaviour is that the snake-poison and the antivenin require a certain time to react with each other. Hence if the mixture is heated to 68° for ten minutes before the reaction has practically reached the end-value, the free antivenin is destroyed and after the heating the mixture contains some free poison.

[1] Calmette : *Ann. de l'Inst. Pasteur*, **8.** 275 (1894); *Compt. Rend. de l'Ac. de Sc.* 134, No. 24 (1902).

[2] Martin and Cherry : Proc. Roy. Soc., **63.** 420 (1898).

The experiments of Calmette yield therefore no evidence for his conclusion that the binding of snake-venom by antivenin is a reversible process, whereby as soon as the free antivenin is destroyed, its compound with the poison is dissociated with the production of new quantities of poison and antivenin. The same comment may be made regarding the decomposition, by boiling, of the innocuous mixture of a poison generated by the *Bacillus pyocyaneus* and its antitoxin. After the boiling, the poison, which is stable at that temperature, remains in the solution, while the more unstable antitoxin is destroyed, as Wassermann [1] has shown.

But there are many other cases that demonstrate reversible processes between analogous substances against which no such objections can be upheld. One of the most remarkable of these processes is used for the production of the so-called immune bodies. (Ehrlich terms them "amboceptors.") If we inject a suspension of the erythrocytes of an ox into the vein of a rabbit, this animal after a certain time presents in its blood a hæmolytic substance, which hæmolyses the erythrocytes of the blood employed; *i.e.* erythrocytes of the ox and perhaps of some nearly related species.

If we heat blood-serum containing this hæmolysin to 55° C. for about thirty minutes, we find that it loses its hæmolytic power. It is said to be "inactivated." The hæmolysin is evidently decomposed, but a fraction of it remains intact, as may be shown by adding the normal serum of a guinea-pig. After the addition of this in-

[1] Wassermann : *Zeitschr. f. Hygiene u. Infectionskrankheiten,* **22.** 263 (1896).

nocuous serum, the inactivated serum has regained its power of hæmolysing bovine erythrocytes. (The guinea-pig serum has in reality a slight hæmolytic action on foreign erythrocytes, but in the experiment it need be used in such small dosage as to provoke in itself no visible hæmolytic action.)

Bordet,[1] who discovered this interesting phenomenon, concluded from his investigations that the agglutinating and hæmolytic power, on erythrocytes from a rabbit, of the serum from a guinea-pig which had been treated with five or six injections of erythrocytes (10 c.c. of defibrinated blood) of a rabbit, is due to the presence of two substances, of which the one, the "immune-body," resists an elevation of the temperature for thirty minutes to 55° C., whereas the other, the "alexin" (Ehrlich's "complement"), is destroyed at that temperature, but may be replaced by normal serum from a guinea-pig or even from a rabbit. Bordet expressed the opinion that the "alexin" serves as a sensibilitator, and renders the erythrocytes susceptible to the "immune-body," which they are supposed not to be in their natural state.

Ehrlich,[2] on the other hand, tried to demonstrate that the hæmolysin is a compound of the "immune-body" and the alexin, which compound is partially dissociated. The "immune-body" is stable at higher temperatures, the alexin not. The alexin may be replaced by a similar substance, an alexin contained in many normal sera. As will be seen in the following pages (Chapter VIII), the added alexin is really consumed in the formation of the hæmolysin, and

[1] Bordet : *Amun. de l'Inst. Pasteur*, **12.** 692 (1898).
[2] Ehrlich and Morgenroth : *Berl. klin. Wochenschrift*, No. 1 (1899).

acts therefore not as a sensibilitator, but is chemically bound, as Ehrlich insists. This is certainly true for the alexin and "immune-body" absorbed by the erythrocytes; but the phenomenon of Neisser and Wechsberg indicates that these two substances are combined to a certain degree even outside the erythrocytes (compare Chapter VIII).

Here we have evidently before us a reversible chemical process. Similar reversible processes are found for all the different hæmolysins which are formed after the injection of a suspension of erythrocytes from one animal into the veins of an animal of another species. In a similar manner, according to Ehrlich, behave the bacteriolysins, which are formed analogously to the hæmolysins. A similar experiment was made by Madsen, Famulener, and Walbum on innocuous mixtures of the hæmolytic agent produced by staphylococcus, called staphylolysin, and its antitoxin. Here the innocuousness of the mixture shows that the hæmolysin is really bound, for during the time of the reaction, as long as there is some hæmolysin free, it is, on being mixed with a suspension of erythrocytes, rapidly absorbed by these, which thereafter lose their hæmoglobin. The staphylolysin behaves in a very peculiar manner. If it is heated to 70° C., it loses a great deal of its hæmolytic power, which, curiously enough, returns almost completely after heating for five minutes to 100° C. Its antitoxin is destroyed by such a heating. On heating the innocuous mixture of staphylolysin and its antitoxin for five minutes to 100° C., the mixture gains hæmolytic properties. The binding of staphylolysin with its antitoxin is therefore a reversible chemical process.

Bordet injected the milk of one animal into another

animal of another species and found that the serum from this second animal contained a substance, called lacto-serum, which gave a precipitate with the casein of the injected milk.   P. T. Müller,[1] who investigated the prop-erties of this lactoserum, found that it entered into a com-pound with the casein of the milk, which compound was precipitated in the presence of calcium-salts.   Now the lactoserum is decomposed by heating to a temperature of 70 to 71° for thirty minutes.   By heating the precipitate which contained the lactoserum combined with casein, in a solution of NaCl, to 100° C., Müller succeeded in recov-ering the casein without loss.   Evidently the precipitate is a little soluble, and the dissolved precipitate is partially dissociated into its components.   Through the high tem-perature the free lactoserum is destroyed, then new quantities of the precipitate are dissolved, and the process goes on until all the lactoserum is decomposed and the casein dissolved and recovered.

There are other methods of destroying one of the com-ponents in an innocuous mixture of a toxin and its anti-body.   Different chemical agents may destroy the one of these substances more rapidly than the other.   Thus ricin is digested by proteolytic ferment, but to a much smaller extent than its antibody.   This behaviour was used by Danysz[2] for restoring the poisonous effect of ricin which had been mixed with so much antiricin that the resulting fluid was innocuous.   This fluid was mixed with a solution of ferment and left for a sufficient time (e.g. twenty-four

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1 P. T. Müller: *Archiv f. Hygiene*, **44**. 150 (1902).
2 Danysz: "Mélanges des toxines avec les antitoxines," *Ann. de l'Inst. Pas-teur*, **16**. 311 (1902).

hours) and then found to behave like a solution of ricin. In this case the process goes on so slowly that the ricin and antiricin will have had sufficient time to combine before their destruction has reached a noticeable degree.

It is possible to separate partially the two neutralised substances by much less vigorous means; namely, by shaking their solutions with other solvents. Thus, for instance, Madsen and Noguchi treated an innocuous mixture of saponin and cholesterin, which unite so rapidly that their velocity of reaction can scarcely be measured, in the following manner. The mixture was evaporated to dryness and thereafter ground to a powder, which was extracted with chloroform or ethyl ether. These fluids possess a great dissolving power for cholesterin. The residue of the powder was dissolved in 0.9 per cent solution of sodium chloride. The solution so prepared displayed the properties of a solution of saponin, especially in regard to its hæmolytic activity.[1]

Another experiment of the same nature was carried on by Madsen and Walbum.[2] They prepared a mixture of ricin and antiricin, which, after having been exposed for two hours to 37° C., showed no toxic effect when injected into guinea-pigs. This mixture was shaken for a time at 37° C. with an equal quantity of a 10 per cent suspension of erythrocytes from a rabbit in physiological salt solution, and then centrifugated. The liquid was then shown to contain an excess of antiricin by its attenuation of the aggluti-

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[1] Madsen and Noguchi: *Oversigt Ac. of Sc. of Copenhagen*, No. 6, p. 461 (1904).

[2] Madsen and Walbum: "De la ricine et de l'antitoxin," *Centralblatt f. Bakteriologie*, **36**. 253 (1904).

nating power of ricin on erythrocytes. On the other hand, the centrifugated rabbit-erythrocytes contained an excess of the nerve poison in ricin; for when they were hæmolysed by the addition of pure water they gave a poisonous solution, which injected into guinea-pigs produced death. In this case we have two different poisons,—the hæmolytic and the nerve poison in the ricin and the corresponding two antibodies in the antiricin. The experiment shows us that both of these poisons are bound to their respective antibodies by means of reversible chemical processes.

Another experiment of Wassermann and Bruck [1] may be explained in a similar way. They injected an innocuous mixture of the nerve poison tetanospasmin (which together with the hæmolytic tetanolysin is produced by the *Bacillus tetani* in bouillon medium) and its antitoxin into the hind limb of a guinea-pig. This animal showed no symptoms of tetanus. But if the animal had previously received a local injection of adrenalin, it was killed by the injection. As H. Meyer and Ransom have shown, the antitoxin is chiefly absorbed by the vascular system, the tetanospasmin by the nerves. The adrenalin contracts the vessels and thus hinders the absorption of the antitoxin, but it has no effect on the nervous system, so that the tetanospasmin may execute its disastrous effect. Hence the experiment showed that the innocuous mixture contained free toxin, but we are not quite certain in this case that the poison and its antidote had had sufficient time to fully combine.

The simplest way to separate toxins from antitoxins in mixtures is by the aid of diffusion. Toxins as well as antitoxins diffuse in water or in gel, but the toxins generally

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[1] Wassermann and Bruck: *Deutsche med. Wochenschrift*, No. 2 (1904).

much more rapidly than the antitoxins, which are often said not to diffuse. Madsen and I have made an investigation of this phenomenon. In common test-tubes a 5 per cent solution of gelatine was poured to a height of about 10 cm. This solution solidified in a refrigerator. After this a solution of toxin or antitoxin was added to a height of 1.3 cm. above the column of gel, and the test-tube placed on ice (mean temperature 6° C.), where it remained for some time, (from one to four or more weeks) according to the diffusibility of the substance. After this the fluid solution and the different layers of the column of gel were analysed for their content of the different substances. By the aid of these determinations it is possible to calculate the diffusion constant of the substances examined. In this way we found the following constants, valid for 12° C. and expressed in days and centimeters : —

Sodium chloride 0.94
Diphtheria-toxin 0.014
Diphtheria-antitoxin 0.0015
Tetanolysin 0.037
Antitetanolysin 0.0021

To the theoretical meaning of these figures we shall return later.

The very slow diffusion of the other substances as compared with that of sodium chloride is evidently connected with their high molecular weight. This is probably of the same order of magnitude as that found by E. W. Reid[1]

[1] E. W. Reid: *Journ. of Physiology*, **33**. 13 (1905). The molecular weight was calculated from the osmotic pressure of a 1 per cent solution. This pressure was found to be 3.85 mm. at 15° C.

for hæmoglobin, viz. 48,000. For the antitoxins it may be still some ten or one hundred times higher.

As the antitoxins diffuse about ten times more slowly than the toxins, it seems theoretically possible to separate them by diffusion. The first experiment in this direction was done by Martin and Cherry.[1] They prepared 60 c.c. of an innocuous mixture of diphtheria-toxin, containing three hundred lethal doses and its antitoxin, and heated it for two hours at 30° C. This mixture was allowed to filter under pressure (50 atm.) through a film of gelatine supported by a Pasteur-Chamberland filter. The filtrate contained chiefly water, which passes through the filtrum very much more rapidly than the toxin or the antitoxin. This filtrate was examined and found to contain per cubic centimeter less than 3 and 5 per cent respectively of the poison in one cubic centimeter of the original mixture, the poison being supposed to be entirely free. Now water passes more rapidly than sodium chloride through gelatine, and sodium chloride about sixty-seven times more rapidly than diphtheria-toxin. Hence we should expect that even if no poison at all were bound, the first filtrate would contain per cubic centimeter only 1.5 per cent of the quantity of poison in the original mixture. Later on as the original mixture by the extraction of water became more concentrated, as seems to have been the case in these experiments, a higher percentage might have been expected. But the conclusion which has been drawn from them, that but a small part, say 5 per cent, of the toxin was actually free, may not be regarded as warranted by the facts.

A similar experiment has been carried out by Craw in

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[1] Martin and Cherry: *Proc. Roy. Soc.*, **63**. 420 (1898).

the Lister Institute on mixtures of a lysin from the *Bacillus megatherium* and its antitoxin.[1] He found that the poison from innocuous mixtures was concentrated in the gelatin-film, whereas the residue of the fluid exhibited antitoxic properties. He therefore concludes that the binding of this poison to its antibody is due to a "partially" reversible process. This proof seems the more conclusive, as Craw evidently worked under theoretical premises which led him to seek for proof against the reversibility of the process.

The experiments resulted in the same manner, even if a great excess of antilysin, one to three times the "neutralising" quantity, were added. Craw had taken the precaution to heat his mixtures for three hours at 37° C. and then to let them stand one hour at 10° C., so that there is every reason to believe that the reaction, which probably is very similar to that of tetanolysin, had practically reached its end. In the filtrate, Craw did not find a trace of the poison when he worked with "neutral" or "over-neutralised" solutions.

Let us for a moment consider the ideas which led Craw to suppose that the processes of binding between toxin and antitoxin are not reversible. Toxins, and specially antitoxins, are said to be colloids. Craw therefore supposes the so-called solutions of antitoxins and the products of their reactions with toxins to be in reality fine suspensions. For this statement no evidence is adduced, but it seems as if Craw regarded the lack of diffusibility as characteristic of suspensions, and this may be conceded as being correct. But since Craw supposes antitoxins to be non-diffusible, he

[1] T. A. Craw: *Proc. Roy. Soc.*, **76**. 179 (1905).

seems not to have known the results of Madsen's and my investigations on this point; instead he drew untenable conclusions from Brodie's [1] experiments, which indicate that diphtheria-antitoxin does not in an appreciable degree pass through a gelatin filter in a very short time. We may therefore leave the theoretical considerations of Craw, which he furthermore himself considered not to be applicable to certain of his own experiments.

Others say, with Nernst,[2] that the laws of van 't Hoff are not applicable to colloids, *i.e.* to toxins and antitoxins. Nernst has himself shown that diffusion is caused by and is proportional to the osmotic pressure. As now toxins and antitoxins diffuse just as other known substances, we may conclude that they obey van 't Hoff's law of osmotic pressure. In Madsen's and my experiments these substances showed themselves to be distributed in the different layers of gel just as these laws demand. And if van 't Hoff's law is found to be valid for the osmotic pressure of these substances, then also the laws of chemical mass-action (Guldberg and Waage's law) must hold good for their reactions. And even if we did not know that these substances behaved in this regard as do other substances, we would be entitled to work with this quite natural hypothesis, until it was shown with great accuracy that the hypothesis was wrong. Otherwise we would proceed in a manner wholly different from that used in the other disciplines of science.

There has been very much discussion of this question

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[1] T. G. Brodie: *Journ. of Pathology and Bacteriology*, **97**. 460–464 (1896).
[2] Nernst : "Über die Anwendbarkeit der Gesetze des chemischen Gleichgewichts," *Zeitschr. f. Elektrochemie*, **10**, No. 22 (1904).

in recent times. When Madsen and I calculated for the first time the action of tetanolysin and antilysin upon each other under the supposition that this action comprehended a reversible process, we knew also very well that the tetanolysin was simultaneously subject to another reaction, which destroyed it slowly. But in this circumstance there was no reason for not employing the known laws for reversible processes. We ascertained for ourselves that the influence of this secondary process may, with the given method of experimentation, be disregarded. Had this not been the case, a correction for the disturbing effect would have been applied. Recently Sachs [1] has shown that another reaction than that investigated by Madsen and myself takes place between tetanolysin and its antitoxin, which may to a certain extent interfere with the chief reaction studied by us. The simple relations found by us seem to indicate that in this case also the perturbations caused by the new factor do not exceed a certain value, of such a magnitude that may be neglected in ordinary experiments on the neutralisation of tetanolysin. We shall later on return to this question.

The incompleteness of the chemical binding process between toxins and antitoxins has aided in retarding the idea that real chemical compounds are formed in the union of these substances. The only chemical reactions which were familiar to the scientists who studied the neutralisation of toxins were the complete reactions. Behring,[2] who was the first in this field, expressed the opinion that

[1] H. Sachs: "Über die Constitution des Tetanolysins," *Berl. klin. Wochenschrift*, No. 16 (1904).

[2] Behring and Kitasato: *Deutsche med. Wochenschrift*, No. 49 (1890).

toxin is destroyed by antitoxin without a diminution of the quantity of the latter substance. Chemically speaking, the antitoxin acted as a catalysator. This idea was incompatible with the measurements of Ehrlich on diphtheria-poison, according to which the double quantity of antitoxin neutralises the double quantity of poison. Ehrlich therefore held the opinion that a real chemical combination takes place. On the other hand, Buchner[1] and Roux[2] supposed that this action of antitoxin on toxin takes place only in the susceptible animal. According to their opinion the antitoxin really reacts upon the animal, stimulating it in the struggle against the poison. This idea was supported by such experiments as those of Calmette, according to which toxin and antitoxin coexist in their mixtures. Against this type of explanation, Ehrlich carried out his experiments on the neutralisation of toxins "in vitro," outside of the living animal, and Martin and Cherry showed that experiments similar to Calmette's might be due to an insufficient time of reaction. The experiments "in vitro" have been multiplied in great number and the influence of the time of reaction has been observed in most cases investigated, so that the idea of the chemical combination between toxins and antitoxins has been generally accepted. But their incomplete knowledge of limited chemical reactions caused Ehrlich and other investigators of these phenomena to suppose that the processes observed are always unlimited; and accordingly they were unable to explain all the phenomena indicating the existence of chemical equilibria between the substances examined. To explain some of these

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[1] Buchner: *Deutsche med. Wochenschrift*, 480 (1903).
[2] Roux and Vaillard: *Ann. de l'Inst. Pasteur*, 8. 724 (1894).

phenomena, Ehrlich and his school invented the artificial hypothesis that these poisons consist really of a mixture of a great number of different poisonous and innocuous substances, which combine with antitoxin. The most thoroughly examined poison, that of diphtheria, contains, according to Ehrlich, not less than eight such different substances. Nearly every new phenomenon led him and his school to invoke the presence of a new substance. Owing to this circumstance the theory of Ehrlich has to a great degree lost its credibility.

The influence of the time of reaction has also not been considered by Ehrlich as much as it should have been. Thus for instance, Madsen and Dreyer[1] had shown that a mixture of diphtheria-poison and its antitoxin, which is innocuous on subcutaneous injection into guinea-pigs, kills rabbits on intravenous injection. This phenomenon was explained by Ehrlich[2] as due to the presence in the diphtheria-poison of a substance which could kill rabbits but not guinea-pigs. The recent investigations of Morgenroth[3] show that the whole difference is due to the different modes of injection. A mixture which is innocuous to guinea-pigs when injected subcutaneously may kill them when injected intracardially, *i.e.* directly into the blood. The reaction between diphtheria-toxin and its antitoxin is not completed in less than a quarter of an hour, as Ehrlich supposed from his subcutaneous injections into guinea-pigs; according to Morgenroth's experiments, this reaction requires several hours at 20° C. to reach the equilibrium. The experi-

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[1] Dreyer and Madsen: *Zeitschr. f. Hygiene,* **37.** 250 (1901).
[2] Ehrlich: *Berl. klin. Wochenschrift,* Nos. 35–37 (1903).
[3] Morgenroth: *Zeitschr. f. Hygiene,* **48.** 177 (1904).

ments that gave different results for rabbits and guinea-pigs were carried out with nearly fresh mixtures of poison and antibody. If such a mixture be injected subcutaneously, it diffuses into the blood very slowly during several hours, and in the meantime the antitoxin binds the toxin. It is not necessary, with Morgenroth, to introduce a new hypothesis; namely, that the tissues of the guinea-pig contain some catalytic agent reacting on the poison. The relatively high temperature ($37°$ C.) of the animal explains the relatively great velocity of the reaction. If, on the other hand, the mixture is injected into the veins, the poison is bound by the tissues of the animal before it has time to react with the antitoxin.

There are some other processes common in sero-therapy which are distinguished by a very high velocity of reaction. Thus, for instance, the agglutinins react with bacteria in less than five minutes even at $0°$ C., according to the experiments of Eisenberg and Volk.[1] (Shorter times of reaction were not examined.) Here again we observe a reversible process, since the agglutinin absorbed by the bacteria (or erythrocytes) may be washed out from them with the aid of a physiological salt-solution, as Landsteiner and Eisenberg and Volk showed. It had been supposed by Bordet that this reaction was of the same nature as the adsorption of a dye by a fibre. But the adsorption phenomenon is one of slow velocity; the dyeing of fibres requires some thirty minutes at $100°$ C. and demands more than two days at common room's temperature ($17°$ C.). Therefore this theory of Bordet is quite improbable. I have come to the conclusion that the process in question is an absorp-

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[1] Eisenberg and Volk: _Ztschr. f. Hygiene_, **40.** 155 (1902).

tion process. If we picture to ourselves bacteria (or other cells) of 5 to 10 μ diameter, shaken with the solution of a substance which enters easily into the cells, we find that even if the diffusion-constant of the substance be so small as 0.001, as for the least diffusible antitoxins, the process of diffusion may reach its equilibrium in as short a time as less than five minutes.

We are led to similar results regarding the velocity of reaction by some experiments of Madsen and Walbum. They added cautiously a little quantity of a solution of tetanolysin to 10 c.c. of a suspension of erythrocytes in a test-tube, so that the solution remained in the uppermost layers of the emulsion, and shook the test-tube after the lapse of thirty seconds. In another experiment they added the suspension precipitously to the poisonous solution, so that the mixing took place immediately. The hæmolysis in the second experiment was nearly double that in the first. This is explained by the fact that the tetanolysin has a very much higher solubility in the erythrocytes than in the surrounding medium. During the short time of thirty seconds the uppermost erythrocytes had absorbed about 30 per cent of the poison, so that only about 70 per cent was left for absorption by the larger fraction of the erythrocytes. In consequence of the great absorption-coefficient of the erythrocytes these retain nearly the quantity of poison which they possessed at the moment of shaking, and the final hæmolysis was about as marked as if only 70 per cent of the quantity of the poison used had been added in the first experiment, supposing that it had been distributed uniformly in the fluid. Other substances which are absorbed by erythrocytes or other cells seem to behave in

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the same manner.   In this category belong the immune-
bodies as well as their compound with alexins, which, as
will be seen later on, in their absorption behave nearly in
the same manner as agglutinins.   Bordet[1] showed that a
given quantity of erythrocytes added to a certain quantity
of blood-hæmolysin gave a greater hæmolysis if added
simultaneously, than if it was added in two portions, the
one after the other.

That even in this case the process is a reversible one,
is shown by an experiment of Morgenroth.[2]   He added
erythrocytes, which had absorbed immune-body, to other
unprepared erythrocytes and mixed the suspension of these
with alexin.   Not only the originally prepared erythrocytes,
but also the others, became hæmolysed, which shows that
a part of the immune-body had left the prepared erythro-
cytes and diffused through the surrounding liquid to the
unprepared ones.   This experiment would not succeed if
a sufficient time (about one hour) was not allotted to the
diffusion process before the alexin was added.   Similar
experiments with analogous results were performed by
Joos[3] with typhoid-bacilli and their agglutinin.

Quite recently Morgenroth[4] has done some experiments
with cobralysin which indicate in a very conclusive man-
ner that this poison is not destroyed but only bound by its
antitoxin.   He added so much antitoxin to the cobra-
poison, that its action was wholly neutralised and a little
over.   After seven days he added to 5 c.c. of the mixture

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[1] Bordet: *Ann de l'Inst. Pasteur*, **14**. No. 5 (1900).

[2] Morgenroth: *Münch med. Wochenschrift*, No. 2 (1903).

[3] Joos: *Zeitschr. f. Hygiene*, **40**. 203 (1902); compare Eisenberg: *Cen-
tralbl. f. Bakteriologie*, **34**. 261, 268 (1903).

[4] Morgenroth: *Berl. klin. Wochenschrift*, No. 50 (1905).

0.25 c.c. of normal hydrochloric acid, which caused the binding between the toxin and antitoxin to be dissolved. This was proved by the acid mixture being heated to 100°C. for thirty minutes, whereby the antitoxin was destroyed, whereas the poison itself was not sensibly weakened. This could be shown by neutralising the solution after it had cooled down to the room's temperature. The toxicity of the solution was nearly the same as that of the original solution without antitoxin. In this case the peculiarity occurs, as Sachs has first shown, that the presence of acid protects the poison from being destroyed by heat.

Morgenroth criticises a theory sketched by Nernst[1] and developed by Biltz, Much, and Siebert,[2] according to which the toxins are "adsorbed" to the "colloidal" anti-toxins and thereafter destroyed. Morgenroth says rightly that this theory, "which as yet is void of any experimental basis," is completely disproved by his experiments.

As will be shown later on, the velocity of reaction of the different toxins changes with temperature according to a law which was deduced from thermodynamical considerations, involving the validity of van't Hoff's law for solutions. The applicability of this law to the velocities of reaction of toxins may therefore be regarded as a new proof that the general laws bearing on the behaviour of common matter are valid even for the processes going on between the substances studied in the phenomenon of immunity. No single proof has been adduced against the

[1] Nernst: *Zeitschr. f. Elektrochemie*, B. 10, No. 22 (1904).
[2] Biltz, Much, and Siebert: *Behrings Beiträge* (1905).

validity of these laws, and it seems to me very unphilo-sophical *a priori* to suppose that other laws should regulate the reaction of toxins and antitoxins, than those which govern the reactions of other substances.

CHAPTER III

VELOCITY OF REACTION. HOMOGENEOUS SYSTEMS

WE have already considered the velocity of the reaction of the most simple kind; namely, the spontaneous destruction of different antibodies in the veins of animals. If, as seems to be the general case in chemistry, every molecule of a substance is decomposed independently of all other molecules, then the number of molecules decomposed during a short interval of time is simply proportional to the number of molecules present at this time. If this number at a certain time, which may be regarded as the beginning-time of the process, is called A, and the number of decomposed molecules at a given time t is called x, then $\dfrac{dx}{dt}$ is the rate of decrease of the active molecules, and we get the well-known equations

$$\frac{dx}{dt} = K(A - x)$$

and

$$\log \frac{A - x_0}{A - x_1} = \frac{K}{2.303}(t_1 - t_0),$$

where t_1 corresponds to x_1 and t_0 to x_0. Many such cases are known in chemistry; for example, the decomposition of hydrogen arsenide $(As\,H_3)$. They are called monomolecular reactions. Monochloracetic acid reacts with water, giving glycolic and hydrochloric acid, a so-called bimolec-

ular reaction. If now the initial concentration of the second group of reacting molecules is termed B, we get

$$\frac{dx}{dt} = K_1(A - x)(B - x),$$

which, if B is very large compared with x and with A, gives the same solution as the differential equation for the mono-molecular reaction, $K_1 = KB$.

In many cases, for instance in the inversion of cane sugar, the one reacting molecule (the hydrogen ion) is reformed by a secondary process so rapidly that its concentration may be regarded as independent of the progress of the reaction. In this case, belonging to the so-called catalytic processes, we may also apply the formula for the monomolecular reaction, putting K proportional to the concentration of the unchanged molecules (here the H-ions).

If $A = B$, the differential equation of the bimolecular reaction gives the solution :

$$\frac{1}{A - x_1} - \frac{1}{A - x_0} = K(t_1 - t_0).$$

As a special case may be regarded that in which two molecules of the same kind react with each other.

If n molecules all at the same concentration react with each other (the so-called n-molecular reaction), we find

$$\frac{dx}{dt} = K(A - x)^n,$$

which gives the solution :

$$\left(\frac{1}{A - x_1}\right)^{n-1} - \left(\frac{1}{A - x_0}\right)^{n-1} = K(t_1 - t_0).$$

This equation was adopted by Madsen for the spontaneous decomposition of antibodies. *n* is in this case no whole number, and the equation may be regarded as a purely empirical rule.

Very regular results corresponding to monomolecular reactions were found at an investigation made by Madsen and Famulener. They studied the attenuation of vibriolysin at 48° C. The hæmolytic power (p) of a solution of vibriolysin, held in a thermostat, was examined at different times (t). The power is inversely proportional to the quantity of the solution which is necessary to produce a certain effect; *e.g.* the hæmolysis of 10 per cent of a 2.5 per cent suspension of erythrocytes within an hour at 37° C. The action evidently follows the law of the monomolecular reactions, according to the equation :

$$-\frac{dp}{dt} = 2.303\ Kp,$$

by the aid of which the calculated values $p_{calc.}$, which are to be compared with the observed ones $p_{obs.}$, are deduced. The temperature was 46.4° C. $K = 0.0079$.

t (min.)	$p_{obs.}$	$p_{calc.}$
0	100.0	100.0
10	78.3	83.2
20	67.6	69.5
30	59.3	57.9
40	49.8	48.3
50	40.8	40.4
60	34.4	33.6
70	25.1	28.1
80	23.0	23.3

The agreement between the observed and calculated values is very satisfactory, so that there is no doubt that the law represented by the differential equation deduced above is really fulfilled.

The influence of temperature on the spontaneous destruction of vibriolysin is very great, as is shown by the following table, taken from a still unpublished work of Madsen and Famulener : —

RATE (K) OF DESTRUCTION OF VIBRIOLYSIN AT DIFFERENT TEMPERATURES

TEMP.	VELOCITY OF REACTION		TEMP.	VELOCITY OBS.	REACTION CALC.
	OBS.	CALC.			
49.975	0.0778	0.0741	46.4	0.0079	0.0081
49.75	0.066	0.066	45.97	0.0063	0.0062
49	0.039	0.041	45.65	0.0057	0.0051
48.15	0.023	0.024	45.145	0.0036	0.0036
47.35	0.0134	0.0128			

The calculated values are found with help of the formula

$$v_1 = v_0 \; e^{\frac{\mu}{2}\left(\frac{T_1 - T_0}{T_0 T_1}\right)},$$

which has been found to agree with the experiments in reaction velocity in other branches of physical chemistry. μ is 128,000, or about five times as great as for the inversion of cane sugar (25,600). The velocity of reaction increases in the proportion of 10 to 1 in an interval of 3.8 degrees.

In a similar manner behaves tetanolysin, as is seen from the following table, borrowed from an investigation of Madsen and Famulener : —

DECOMPOSITION OF TETANOLYSIN

AT 53.5° $K_1 = 0.079$			AT 49.8° $K_0 = 0.0047$		
t (min.)	$p_{obs.}$	$p_{calc.}$	t (min.)	$p_{obs.}$	$p_{calc.}$
0	100.0	100.0	0	100.0	100.0
2	70.9	69.5	20	80.0	80.6
4	43.5	48.6	40	61.1	64.8
6	27.1	33.6	60	52.1	52.3
8	20.4	23.3	80	46.3	42.1
10	14.9	16.2	120	26.8	26.7
12	11.4	11.2	180	14.6	14.3

Here the influence of temperature is still greater than in the decomposition of vibriolysin ; μ is about 162,000. The velocity of reaction corresponds also in this case to that of a monomolecular reaction.

DECOMPOSITION OF VIBRIOLYSIN IN THE PRESENCE OF 0.005 N. SODIUM HYDRATE

AT 48° $K = 0.0313$			AT 46.5° $K = 0.012$		
t (min.)	$q_{obs.}$	$q_{calc.}$	t (min.)	$q_{obs.}$	$q_{calc.}$
0.0	100.0	100.0	0	100.0	100.0
2.5	83.0	82.6	15	51.7	48.6
5.0	69.4	69.8	30	32.2	32.2
7.5	60.1	58.4	45	21.2	21.2
10.0	50.0	48.8	60	14.1	14.1
12.5	36.5	40.3	75	8.6	9.3
15.0	32.2	34.2	90	6.2	6.1
17.5	28.2	28.5	105	4.4	4.1

The decomposition of lysins, and generally speaking of the substances dealt with in sero-therapy, is increased to

a very high degree if acids or bases are added to their solutions. Madsen and Famulener have, for instance, investigated the rate of decomposition of a solution of vibriolysin, which contained so little sodium hydrate that it was 0.005 n. in alkalinity. They found the above figures : —

The constants of the reaction of this monomolecular process are found to be 0.0313 at 48° and 0.012 at 46.5° C. This gives a value of $\mu = 128,000$, or just the same value as that noted for vibriolysin without the addition of alkali. This correspondence may perhaps not be of so great an interest as it seems at first, since the solution of vibriolysin is in itself a little alkaline. But it is not probable that its alkalinity is due to sodium hydrate, but chiefly to weaker organic bases, and therefore the coincidence of μ in the two different cases is still remarkable.

Other substances also exert an influence upon the destruction of vibriolysin and tetanolysin, although to a much less degree than solutions of the alkalies. Of these, ammonia has a less influence than sodium hydrate, but the difference is by far not so accentuated that it would be possible to invoke the action of the OH ions. Thus, for instance, the reaction-constant of vibriolysin, which at 46.2° reaches the value 0.0066 (per min.) is increased to 0.081 if 3.4 c.c. of 1 n. NaOH are present in 100 c.c. of the liquid, and to about 0.075 in the presence of the equivalent quantity of ammonia. An addition of hydrochloric acid gave different results according to its quantity. The first addition diminished the constant of reaction, which reached a minimum 0.00052 in the presence of 4.25 c.c. 1 n. HCl in 100 c.c. of the liquid. With larger amounts the reaction-constant

increased again, and reached the value of 0.022 if 6 c.c., and of 0.109 if 6.75 c.c. of 1 n. HCl was present in 100 c.c. of the liquid. These circumstances seem to indicate that some alkali present in the original culture was neutralised by the first addition of the hydrochloric acid, which there-after exerted its destructive action. The slight difference between the action of sodium hydrate and ammonia leads to the conclusion that the bases act by setting some alkaline substance in the bacterial culture free. The inferiority of the ammonia might be explained by its feebleness, which allows an equilibrium with the base from the culture to be reached before all the base has been set free.

Even the presence of weak acids, e.g. of acetic acid, accelerates the destruction of the vibriolysin. Thus 9 c.c. of 1 n. CH_3COOH in 100 c.c. of culture gives a reaction-constant 0.064, its action corresponding to that of 6.5 c.c. 1 n. HCl. The weak acid exerts much less influence than a strong one, which was to be expected, the neutralised base being of less strength than ammonia, so that a noticeable hydrolytic action must occur.

The reaction-constant is, considering the great errors of observation, practically proportional to the amount of base added (if the alkalinity exceeds 0.01 n.); but for the action of the acids no such regularity is found, even if we calculate from the neutralisation point, which ought to nearly coincide with the point of minimal constant of reaction.

The content of alkali in the natural solution of vibriolysin explains another fact found by Madsen; namely, that the constant of reaction depends on the initial concentration

and is nearly proportional to it. In reality the bouillon in which the vibrions grow is slightly alkaline. Madsen found at 47.8° C. that the original solution of vibriolysin gave $K_1 = 0.0198$. The same solution, diluted to the concentrations 0.5 or 0.25, gave $K_2 = 0.0072$ and $K_4 = 0.0039$ respectively. These three figures are nearly in the proportion $4 : 2 : 1$, or as the corresponding concentrations, which is easily understood if the alkali present exerts a catalytic influence.

Much more complicated is the influence which the addition of acids or of bases exerts upon the attenuation of tetanolysin, which is also in itself alkaline. The velocity of reaction K increases with the amount of alkali added, but decreases at first if acid is added until it reaches a very flat minimum, after which it increases again on further addition of acid. This is evident by an inspection of the following figures (valid at 49.83° C.) : —

98 c.c. of tetanolysin solution +	2 c.c. 1 n. NaOH	$K = 0.0112$
99 c.c. of tetanolysin solution +	1 c.c. 1 n. NaOH	0.0097
99.5 c.c. of tetanolysin solution +	0.5 c.c. 1 n. NaOH	0.0085
100 c.c. of tetanolysin solution		0.0047
99 c.c. of tetanolysin solution +	1 c.c. 1 n. H_2SO_4	0.0071
98 c.c. of tetanolysin solution +	2 c.c. 1 n. H_2SO_4	0.0435

This seems to indicate that the acid first added is bound by some weak base in the bouillon ; and that the first trace of alkali added is also bound, probably in setting some weak alkali free in the bouillon.

The figures for the reaction velocity in the presence of 2 c.c. 1 n. H_2SO_4 in 100 c.c. are as follows, and indicate a monomolecular process : —

Time (min.)	Strength (obs.)	Strength (calc.)
0	100.0	97.5
5	57.3	59.2
10	37.7	36.0
15	20.0	21.7
20	14.0	13.2

With regard to the decomposition of tetanolysin in the presence of acids or bases, some experiments executed by myself gave the following results : —

On addition of equimolecular quantities of hydrochloric, oxalic, citric, and tartaric acid (about 0.003 mol. normal), the velocity of reaction was increased so much that the decomposition of a 1 per cent solution of tetanolysin was three-fourths accomplished in ten minutes. Of sulphuric acid half this quantity was sufficient for the same purpose, and for acetic acid a quantity seventy-five times as great. The said concentrations all contain nearly the same quantity of hydrogen ions, and it seems, therefore, probable that this catalytic action is caused by the hydrogen ions. Sodium hydroxide acts nearly as strongly as a strong acid of the same molecular concentration ; but an ammonia solution of the same concentration had a very little influence. It was necessary to use it in about thirty times as high a concentration as sodium hydrate to obtain the same effect. Both these solutions have also nearly the same concentration of hydroxyl ions.

With the aid of these experiences, it is very easy to interpret a phenomenon observed by Ritchie.[1] He mixed a solution of tetanolysin with a small quantity of hydrochloric

[1] Ritchie: *Journ. of Hygiene*, 1. 130 (1901).

acid at ordinary room temperature. After a certain time he injected this mixture subcutaneously into a guinea-pig. The animal was not killed by the poison; but if before the injection the hydrochloric acid was neutralised by sodium hydrate, the animal was killed. Ritchie inferred that the tetanolysin had been destroyed by the hydro-chloric acid, and was restored by the addition of the base. The simple explanation is the following: The quantity of acid added was not sufficient to cause a rapid destruction of the tetanolysin at the low temperature of the room. But after the subcutaneous injection the temperature of the mixture rose rapidly to about 37° C., and therefore corre-spondingly the destruction of the tetanolysin went on with great speed — according to the figures given above about 200,000 times more rapidly than at 20° C. — before it had time to diffuse into the body of the animal. Therefore the poison was nearly instantaneously destroyed, and had no sensible action on the animal. But if before the injec-tion of the mixture the acid was neutralised (at room temperature) by the addition of an equivalent quantity of sodium hydrate, then the poison was not destroyed after its injection, and could therefore exert its fatal influence. It is not at all necessary to assume such a wonderful pro-cess as a restoration of the destroyed toxin-molecules by the addition of the base.

For antitoxins, very few investigations have been car-ried through regarding their destruction in time. Madsen found for antiricin (serum of an injected goat) that its strength fell at room temperature to 40 per cent in forty-seven days and to 19.6 per cent in eighty-nine days, which corresponds rather closely to a monomolecular process;

that is, the rate of decrease of strength of the solution was proportional to the strength itself.

The greatest increase in the velocity of a reaction with increase in temperature that has ever been found is that of the decomposition of hæmolysin contained in normal goat's serum, which dissolves erythrocytes from the rabbit. Madsen and Famulener give for this reaction the following figures:—

DECOMPOSITION OF A HÆMOLYSIN AT 53° AND 51°C. (MADSEN AND FAMU-LENER)

t (min.)	$q_{obs.}$	$q_{calc.}$	t (min.)	$q_{obs.}$	$q_{calc.}$
0.0	100.0	100.0	0	100.0	100.0
2.5	58.3	58.2	5	74.3	73.4
5.0	44.3	33.4	10	58.3	62.5
7.5	17.9	19.3	15	48.8	53.3
			20	44.9	45.4
			25	40.0	38.7
			30	33.7	33.0
			35	28.4	28.1
			40	25.2	24.0

μ was for this monomolecular reaction found to be 198,500; with the aid of this figure the following table is calculated:—

INFLUENCE OF THE TEMPERATURE ON THE DECOMPOSITION OF A HÆMOLYSIN

TEMP.	VELOCITY OF REACTION		TEMP.	VELOCITY OF REACTION	
	OBS.	CALC.		OBS.	CALC.
53.0	0.095	0.095	51.5	0.025	0.024
52.5	0.060	0.060	51.0	0.0139	0.0145
52.0	0.038	0.038			

Here we evidently observe two concomitant processes. Probably the process which corresponds to the observed

figures, is that of the decomposition of hæmolysin into immune-body and alexin, and the alexin is destroyed still more rapidly.　But the reverse may also be true.　The figures indicate that at 60° C. a heating for 0.1 minute will practically decompose the hæmolysin totally and give a solution of immune-body free from alexin.

The first reaction of enzymes — namely, the influence of emulsin on salicin, studied by Tammann according to methods used in physical chemistry — seems to be of a complicated nature.　The process seems to be monomolecular at low temperatures, as is shown by the following figures, indicating the strength (concentration) of a solution containing in the beginning 3.007 g. salicin and 0.08 g. emulsin in 100 c.c.

DESTRUCTION OF SALICIN BY EMULSIN AT 25° C.

TIME (hours)	STRENGTH (obs.)	STRENGTH (calc.)
0	100	100
1	87	88
3	68	67
5	42	52
8	35	35
12	24	21
26	9	3

$K = 0.057.$

The last figures seem to indicate that the process is retarded at its end.　This is still more conspicuous at higher temperatures.　Therefore we should not overestimate the accuracy of μ calculated from Tammann's figures and giving $\mu = 3330$,[1] corresponding to an increase in the proportion of only 1.2 : 1 in an interval of 10° C.

[1] Tammann, *Zeitschr. f. ph. Ch.* **18.** 436 (1895), gives $\mu = 2A = 5870$, which is evidently due to some misprint or other error.

Tammann investigated also the slow destruction of emul-
sin in 0.5 per cent solution and in the form of dry powder.
Even in this case the reaction was monomolecular. The
content of emulsin in the solution or in the powder at
different times was determined by estimating its decom-
posing influence on salicin. He found for the solution
(between 60° and 75°) $\mu = 45,000$, corresponding to an in-

DESTRUCTION OF A 0.5 PER CENT EMULSIN

TEMP.	$K_{obs.}$	$K_{calc.}$
40	0.04	0.011
50	0.18	0.099
60	0.80	0.800
65	2.86	2.180
70	5.90	5.740
75	14.70	14.700

$$\mu = 45,000$$

crease in the proportion 7 : 1 in an interval of 10 degrees.
For the solid powder μ was only 26,300; that is, 60 per
cent of that for the solution (the determination is rather
uncertain). Furthermore, the decomposition of the powder
at 80.5° C. proceeds about 500 times more slowly than
that of the 0.5 per cent solution. As will be seen in the
following, rennet behaves in a similar manner, and it
seems to be a very common observation that the sub-
stances with which we are dealing are much more resist-
ing to heat in dried form than in solution. This reminds

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us in a certain sense of the great resistance of dried spores to high temperatures as compared with the pronounced destructive influence of higher temperatures on developed bacilli in fluid media.

Dr. Euler has collected the experimental data[1] bearing upon the catalytic action of ferments. In most cases we find that the influence of the time of reaction may be represented as a monomolecular process. Thus, for instance, he finds for the decomposition of hydrogen peroxide (H_2O_2) by solutions containing 3.4 or 5 c.c. of the juice of the *Boletus scaber* to the 200 c.c. the following values at 15° C.[2] q is the quantity of H_2O_2, determined by titration with 0.01 n. $KMnO_4$; n is the concentration of the "catalase," t time in minutes.

$n = 3$			$n = 4$			$n = 5$		
t	q	K	t	q	K	t	q	K
0	8.0	—	0	8.0	—	0	8.2	—
6	6.9	0.0107	8	6.2	0.0138	2	7.5	0.0193
12	5.8	0.0116	10	5.6	0.0154	7	6.0	0.0193
19	5.0	0.0107	13	5.1	0.0150	16	4.0	0.0195
55	2.5	0.0100	19	4.2	0.0147	22	3.15	0.0190
		0.0107			0.0147			0.0193

The constant of reaction K, calculated for a monomolecular reaction, increases with the concentration slightly more than proportionally. In the same manner, according to the investigations of Bredig and Müller von Berneck,

[1] Euler: "Katalyse durch Fermente," *Zeitschr. f. ph. Ch.* **45.** 420 (1905).
[2] Euler: "Zur Kenntnis der Katalasen," *Beiträge zur chemischen Physiologie,* **7.** *h.* 1–3 (1905).

behaves the catalytic action of a colloidal solution of platinum on hydrogen peroxide.

In an analogous manner, according to Euler's measurements, the catalase extracted from lard saponifies a concentrated solution of ethyl-butyrate (at 35° C). q is the quantity of butyric acid set free after t minutes, as determined by titration with barium hydrate.

t	q	$q_\infty - q$	K
0	0.0	2.70	—
2	0.30	2.40	0.0256
6	0.75	1.95	0.0235
9	1.05	1.65	0.0237
16	1.65	1.05	0.0250
∞	2.70	—	—

The reaction is clearly monomolecular, as is indicated by the constancy of K. A more complicated formula was found empirically by Henri[1] to be valid for the inversion of cane sugar by means of invertin extracted from yeast cells.

Henri's formula is the following : —

$$K_1 = \frac{1}{t} \log \frac{a+x}{a-x}.$$

It differs from the formula for monomolecular reactions by the expression $(a+x)$ instead of simply a in the nominator after log. As example I quote the reaction of 4 c.c. solution of diastase with 0.5 n. sugar at 25° C. $\frac{x}{a}$ is the inverted sugar proportional to the initial quantity of cane sugar.

[1] V. Henri: *Zeitschr. f. ph. Ch.* **39**. 194 (1901).

t (min.)	$\dfrac{x}{a}$	$K=\dfrac{1}{t}\log\dfrac{a}{a-x}$	$K_1=\dfrac{1}{t}\log\dfrac{a+x}{a-x}$
75	0.037	0.000218	0.00022
186	0.103	254	24
499	0.228	281	25
505	0.292	297	26
557	0.322	303	26
1120	0.589	345	26
1172	0.611	350	26

Whereas K increases regularly with time in the proportion $1 : 1.6$, K_1 is nearly constant. The quantity transformed in a certain time is proportional to the quantity of diastase present, but not to the concentration of the cane sugar in the solution. In a rather large interval (0.15–0.6 n.), the transformed quantity is independent of the concentration of cane sugar, as Duclaux maintained, but at low and high concentrations we get lower values than at mean concentrations. At very low concentrations the transformed quantity is proportional to the concentration of cane sugar.[1]

In some other cases also the formula of Henri has been verified. Still it seems probable that in dilute solutions of sugar the reaction is of the pure monomolecular type. Only at higher concentrations do deviations occur. Barendrecht[2] has suggested a very singular theory, according to which the deviations might be due to an absorption by the molecules of the sugar of a kind of radiation emanating from the enzyme, which emanation is said to produce

[1] Adrian Brown: "Enzyme Action," *Journ. Chem. Soc.* **81**. 387 (1902).
[2] Barendrecht: *Zeitscr. f. ph. Ch.* **49**. 456 (1904).

the inversion. This is not the place to enter upon a discussion of this peculiar view.

Regarding the influence of temperature on the action of invertase on cane sugar we possess some old observations of Kjeldahl.[1] They give an optimum at about 52.5° and $\mu = 9080$. They are reproduced in the table:—

Temp.	0	18	30	40	45	48	50	52.5	55	60	65	70°C.
Vel. obs.	17	60	113	179	228	250	260	267	260	179	21	0
Vel. calc.	21	(60)	112	181	(228)	261	285	318	353	436	534	651

In a recent memoir[2] Henri analyses the hydrolysis of maltose by means of maltase. In this case the reaction-product is glucose, and the constant of reaction increases with the progress of the reaction. Henri's formula $K = \frac{1}{t} \log \frac{a+x}{a-x}$ gives a very nearly constant value for K. This constant decreases as the quantity of maltose increases. For the first three hours it is, for instance, for solutions of the following concentration, 2 per cent sol. $K = 368 \cdot 10^{-5}$, 4 per cent sol. $K = 164 \cdot 10^{-5}$, 6 per cent sol. $K = 106 \cdot 10^{-5}$. It is nearly inversely proportional to the quantity of maltose present. It would therefore be reasonable to put—

$$K = \frac{K_2}{(a-x)},$$

where K_2 is a new constant, and $(a-x)$ the concentration of the maltose. But as the maltose as well as the glucose has a retarding influence on the process, it would be more rational to put—

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[1] Kjeldahl: cited from Duclaux, *Traité de microbiologie*, **2.** 177 (1899).
[2] Victor Henri: "Recherches physico-chimiques sur les diastases," *Archivo di Fisiologia*, **2.** 1 (Nov. 1904).

$$K = \frac{K_2}{(a-x)+nx} \quad \text{and} \quad -\frac{dx}{dt} = \frac{K_2(a-x)}{(a-x)+nx},$$

which, integrated, gives the formula of Bodenstein:—

$$K_2 t = a\left\{(1-n)\frac{x}{a} + n\log_{\text{nat}}\frac{a}{a-x}\right\}.$$

In this case we find a sufficient agreement with the experimental results, if we suppose $n = \frac{1}{3}$, as Henri has shown. For invertase acting on saccharose $n = 0.5$, and for emulsin acting on salicin $n = 2$. Of course the introduction of a new constant n is favourable to the agreement between the calculation and the observation. Still Henri finds that the agreement for the maltose is not very satisfying (K_2 varies between 0.028 and 0.046).

Henri therefore proposes to introduce still a new empirical constant in the equation for K_2. But it is to be feared that the calculation will not repay the work laid out upon it as long as the experiments are so contradictory as now. For instance, I have calculated some observations of Mr. Terroine cited by Henri (*l.c.* p. 6) according to his formula and found K very nearly constant $= 564 \cdot 10^{-5}$, as will be seen from the following figures for a 2 per cent solution of maltose:—

t (min.)	$x\%$	$K = \frac{1}{t}l\frac{100+x}{100-x}$	$K_1 = \frac{1}{t}l\frac{100}{100-x}$
62	0.373	549.10^{-5}	327.10^{-5}
90	0.515	550	349
120	0.656	569	389
150	0.772	593	428
176	0.832	589	440
210	0.860	535	407
240	0.914	561	444
362	0.965	(483)	402

According to the figures of Terroine K does not change more than 10 per cent (the last value, as there are only 3.5 per cent of the matose left, is too unreliable to be used for the calculation). But in the same memoir, on p. 4, Henri gives values for a 2 per cent solution of maltose for which the values of K change between $319 \cdot 10^{-5}$ and $489 \cdot 10^{-5}$ *i.e.* 53 per cent. The errors of observation must therefore be very great, and the figures should not be applied to delicate calculations. Still greater is the disagreement in the figures of Armstrong,[1] who finds that the con-

MALTOSE 5% AT 30° (E. F. ARMSTRONG)

t (hours)	x %	$K_1 = \frac{1}{t} l \frac{100}{100 - x}$
1	7.3	0.0329
2	13.9	0.0325
4	24.4	0.0304
7.25	31.7	0.0229
23	35.2	0.0082

stant K_1, calculated from the formula for monomolecular reactions, sinks with the progress of the reaction; whereas Henri finds that K, calculated from his formula, is nearly constant; *i.e.* that K_1 as is seen in the figures given above, increases very rapidly with the progress of the reaction, and this is valid not only for 2 per cent, but in a still higher degree for 4 per cent and 6 per cent solutions.[2]

Armstrong has communicated additional measurements

[1] E. F. Armstrong: *Proc. Roy. Soc.* **73**. 508 (1904).

[2] Brown and Glendinning (*Journ. Chem. Soc.* **81**. 388, 1902) find that the formula of Henri's is valid even for the hydrolysis of soluble starch by means of diastase.

on the inversion of milk sugar by means of lactase or emulsin, which follow a wholly different law from that used in Henri's formula. The regularity is evident only if the enzyme is present in great quantity. We reproduce only the first two tables (I and VII) illustrating the hydrolytic action of 100 c.c. of lactase-extract or 0.2 g. of emulsin on 2 g. of milk sugar at 36° C.

ACTION OF LACTASE ON MILK SUGAR			ACTION OF EMULSIN ON MILK SUGAR		
t (hours)	$x\%$	$\dfrac{x}{\sqrt{t}}$	t (hours)	$x\%$	$\dfrac{x}{\sqrt{t}}$
1	22.1	22.1	0.5	3.2	4.5
2	31.2	22.0	1	4.8	4.8
3	38.9	22.5	2	6.4	4.5
4	45.8	22.9	3	7.6	4.4
5	51.5	23.0	4.5	9.0	4.2
6	56.6	23.1	6	10.0	4.1
10	69.0	21.8	23	19.7	4.1
17	84.2	20.4	29	22.0	4.1
23	92.4	19.3	48	29.0	4.2
29	95.3	17.7	53	30.7	4.2
38	98.0	15.9	144	62.2	5.2
			264	77.5	2.9

Here the quantity transformed is very nearly proportional to the square root of the time of action. This corresponds to the differential equation

$$\frac{dx}{dt} = \frac{K}{x} = \frac{K(a-x)}{x\,(a-x)};$$

or, in other words, the reaction-constant calculated with the common formula for a monomolecular reaction should be inversely proportional to the product of the rest of the milk sugar and of the reaction-products. This will happen,

provided that the reaction-products are present in sufficient quantity, and that one molecule of the milk sugar and one molecule of the reaction-products unite with one molecule of the enzyme to form a partially dissociable compound.

It has been long known that the enzymes differ from chemical catalysors in one very important point. This experiment was done in 1883 by Duclaux,[1] with solutions containing 10, 20, and 40 resp. g. of sugar. In 100 c.c. dissolve 20 mg. of invertase and elevate the temperature to 37° C.; then after four hours we shall find that nearly the same quantity of sugar (about 5 g.) will be hydrolysed in the three cases; whereas if we had added 20 mg. of an acid, the inverted quantity would have been 2.5 and 5 g. in the two first cases, if it was 10 g. in the last. Armstrong[2] gives some good instances of this regularity, which holds only if the quantity of enzyme is small. The tabulated quantity is the number of grammes of hydrolysed milk sugar in 100 c.c.

Solution Containing % Milk Sugar	Grammes Milk Sugar, Hydrolysed by a Small Quantity of Lactase in			Grammes Milk Sugar, Hydrolysed by a Small Quantity of Emulsin in	
	24 h.	46 h.	144 h.	23 h.	48 h.
10	1.42	2.22	3.34	1.97	2.98
20	1.41	2.18	3.38	2.12	3.06
40	1.44	2.21	3.30	2.10	3.06

On the other hand, if there is present a great quantity of enzyme and a relatively small quantity of sugar, the action is proportional to this latter quantity, just as if an acid acted.

[1] Duclaux: *Traité de microbiologie*, **2.** 136 (1899).
[2] Armstrong: *l. c.*, pp. 508–510.

ACTION OF INVERTASE ON 100 C.C. OF SUGAR SOLUTION (BROWN)[1]

PER CENT SACCHAROSE	GRAMMES INVERTED IN 1 H.
0.25	0.060
0.5	0.129
1.0	0.249

ACTION OF LACTASE ON 100 C.C. OF MILK SUGAR SOLUTION (ARMSTRONG)

PER CENT MILK SUGAR	AMOUNT CHANGED IN 3 H.
0.2	0.042
0.5	0.098
1.0	0.185

If the enzyme is present in small proportion, the transformed quantity is proportional to its quantity, as is seen from the following experiments of O'Sullivan and Tompson[2] and Armstrong.

HYDROLYSIS OF CANE SUGAR TO 78 PER CENT BY MEANS OF INVERTASE AT 15.5° (O'SULLIVAN AND TOMPSON)

0.15 g. inv. time 283 min. (calc. 307)
0.45 g. inv. time 94.8 min. (calc. 92.1)
1.5 g. inv. time 30.7 min. (calc. 30.7)

HYDROLYSIS OF A 5 PER CENT SOLUTION OF MILK SUGAR (100 C.C.)

SOLUTION CONTAINING	AFTER 24 HOURS	
	OBS.	CALC.
0.66 c.c. of lactase	2.3 p. c.	2.1
1 c.c. of lactase	3.2 p. c.	3.1
2 c.c. of lactase	6.3 p. c.	6.2
5 c.c. of lactase	15.4 p. c.	15.5

[1] Brown: *Journ. Chem. Soc.* **81**. 387 (1902).
[2] O'Sullivan and Tompson: *Journ. Chem. Soc.* **57**. 865 (1890).

A very good instance is given by Henri [1] of the inversion of cane sugar by means of invertase at constant concentration. The following table gives the number (n) of milligrammes inverted during the first minute of solutions of cane sugar of the concentration, c. normal (1 normal = 342 g. per liter).

$c =$	0.01	0.025	0.05	0.1	0.25	0.5	1	1.5	2
$n =$	0.58	1.41	2.40	2.96	4.65	5.04	4.45	2.82	1.15

As will be seen from these figures n is at first nearly proportional to c, then it reaches a maximum at c=0.5, only later on to sink again at very high concentrations, at which the solvent may be regarded as changed.

In good agreement with this experience Terroine found that the quantity of maltose transformed by means of a given quantity of maltase in a 100 c.c. of solution is nearly proportional to the concentration of the maltose until it reaches about 2 per cent and is thereafter independent of the concentration. This regularity may be detected in some experiments executed by Tammann as early as in 1889, on the decomposition of different quantities of amygdalin by means of a constant quantity of emulsin.[2]   All these

DECOMPOSITION OF AMYGDALIN (IN GRAMMES) BY MEANS OF EMULSIN

Quantity of amygdalin		2.555	5.11	10.229
Time (min.) =	14	0.61	0.61	0.50
	19	0.77	0.85	0.73
	23	0.79	0.98	0.86

experiments lead us to the assumption that the substance which really is decomposed into the reaction-products is in

[1] Henri: "Lois générals de l'action les disastases: Thèse," Paris (1899).
[2] Tammann: *Zeitschr. f. ph. Ch.* **3.** 33 (1889); *Hoppe Seylers Zeitschr.* **16.** 315 (1892).

these cases a compound of the enzyme and the substrate upon which it acts. If the enzyme is in excess (in the said cases), nearly the whole quantity of the substrate acted upon enters into the compound, the quantity of which is therefore proportional to the quantity of this substrate. If, on the other hand, the substrate is in excess, the quantity of compound is nearly proportional to the quantity of enzyme. We may therefore from these experiments draw conclusions as to the quantities of enzyme that are equivalent to a certain quantity of, *e.g.*, sugar or milk sugar. These conclusions indicate that the equivalent weights of the enzymes are not so high as is often assumed. The circumstance that enzymes are not in general pure substances hinders the estimation of their true equivalent weights.

The formula of Bodenstein and Henri may be deduced in the following manner. The quantity[1] of ferment from the beginning may be called F, that of the decomposing substance A. Of this a part, x, may be decomposed so that only $A - x$ remains. The ferment, substance A, and reaction-products may enter into compounds consisting of I molecule of ferment with p molecules of A and q molecules of different reaction-products. Of these compounds z, z_1, etc., molecules may be present. Then for every one of these kinds of molecules a formula of the following form is valid : —

$$z = k_1 \, F_1 \, (A - x - \Sigma pz)^p \, (x - \Sigma qz)^q, \text{ where } F = F_1 + \Sigma z.$$

If we suppose Σz to be small as compared with A and x, we get — $\qquad z = kF_1 \, (A - x)^p x^q.$

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[1] Cf. Henri: *Compt. rend.* 135. 916.

Further we have : —

$$\frac{dx}{dt} = k_1 \ F_1 \ (A - x - \Sigma pz)^n = k_1 \frac{F (A - x)^n}{1 + \Sigma k \ (A - x)^p x^q}.$$

In many cases the first term, 1, in the denominator is little as compared with the terms under the sign of summation and may therefore be neglected. In this case we get the formula of Bodenstein if we put $n = 1$, and suppose the terms under Σ to be two, one in which $p = 1$ and $q = 0$, another in which $p = 0$ and $q = 1$. In nearly all cases hitherto investigated the velocity of reaction is nearly proportional to the first power of the concentration of enzyme present; therefore the deduction is given only for this case. The more general formula, in which this concentration enters to another power than 1, is very complicated. A very common case is that in which only one term with $p = 0$ and $q = 1$ enters under the sign Σ ; this is the case in the digestion of egg-white by pepsin and trypsin and for the hydrolysis of fat by means of lipases. Here also the term 1 may be dismissed. This holds good even for the experiments of Armstrong, in which the product of reaction is proportional to the square root of the time of reaction. Here $p = 1$ and $q = 1$, i.e. the chief influence is due to the formation of a compound between the enzyme, the reaction-product, and the original substance. The formula is evidently not valid, if the quantity of substance acted upon by the enzyme or the reaction-product is to a great part bound by the enzyme. Therefore the formula gives no good values for the experiments of Terroine with small quantities of maltose. It will be necessary to carry out a large number of experiments in order to verify this

view, which seems to be the best expression of our present knowledge.

A very prominent place in this connection is held by the rule of Schütz, according to which some processes, especially the peptic digestion, proceeds through a certain time nearly proportionally to the square root of the time and thereafter more slowly.

To understand the meaning of this rule, we at first examine a very well-known process, the saponification of ethyl-acetate by means of ammonia, on which I have carried out some experiments. A mixture was made of 18 c.c. distilled water and 1 c.c. (=0.91 grammes) of ethyl-acetate, in a vessel used for determining conductivities, held at a constant temperature (16° C.). At a certain moment 1 c.c. of a 0.02 normal solution of ammonia was added and the vessel shaken. Afterwards the resistance was determined. It decreased at first rapidly, then slowly, indicating that the well-conducting acetate of ammonia was formed from the less-conducting ammonia, according to the equation : —

$$NH_3 + CH_3COO\,C_2H_5 + H_2O = NH_4CH_3COO + C_2H_5OH.$$
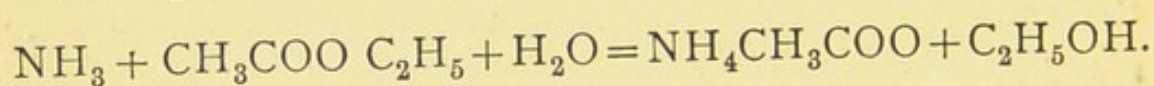
By means of the conductivity the progress of the chemical process was determined. If we take the quantity of ammonia at the beginning, like 1000, then the following quantities of ammonium-acetate, $x_{obs.}$, were observed at the given times (t in minutes).

The table contains two calculated values of x, viz. $x_{calc.\,1}$, found by means of a formula given below, and $x_{calc.\,2}$, deduced by means of Schütz's rule. At $t = 14$ the latter quantity exceeds 1000, which is evidently impossible.

PROGRESS OF SAPONIFICATION OF ETHYLACETATE BY MEANS OF AMMONIA
AT 16° C.

t	$x_{obs.}$	$x_{calc.}1$	$x_{calc.}2$
1	320	352	303
1.5	394	412	371
2	453	469	428
3	539	548	525
4	607	608	606
5	657	656	677
6	701	685	742
7	733	728	801
8	764	757	856
10	810	803	958
14	878	864	——
20	936	923	——
32	980	973	——

The agreement with Schütz's rule is rather imperfect, especially for high values of x. This is a general feature of the said rule, and it is quite clear that the rule would give values of x exceeding 1000 for high values of t, which is evidently impossible.

In order to deduce a rational formula for this process, we must remember that the saponification is a catalytic process, with a specific velocity of reaction proportional to the concentration of the hydroxyl-ions and of the ethyl-acetate. This latter is here present in such an excess, that only about 0.2 per cent of it is transformed during the process, consequently its concentration may be taken as a constant (P). The concentration y of the hydroxyl-ions is subject to the following formula:—

$$y = \frac{K(A-x)}{x + ky},$$

where A is the quantity of ammonia present at the time $t = 0$, x the quantity of ammonium-acetate formed, and consequently $A - x$ the quantity of ammonia at a given time, t. The velocity of reaction $\dfrac{dx}{dt}$ is proportional to y and to the quantity P of ethyl-acetate, so that the equation

$$\frac{dx}{dt} = \frac{K(A-x)}{x+ky} \cdot P$$

is also valid. If x is not too small, ky may be neglected, and we find the integral : —

$$A \log_{\mathrm{nat}}\left(\frac{A}{A-x}\right) - x = KPt.$$

From this formula the values $x_{\mathrm{calc.1}}$ are calculated. A is set as 1000. Here KP is 82. The agreement may be regarded as very satisfying. At low values of t the observed value x is less than the calculated one, due to the neglection of ky, as compared with x, which is not allowed if x is small.

If x is very small compared with A, *i.e.* in the beginning of the experiment, we may as a first approximation put $A - x = A$ and

$$\frac{dx}{dt} = \frac{KAP}{x}$$

which gives — $\qquad x^2 = KAP,$

x is proportional to the square root of the time and also to the square root of the concentration of the ammonia at the beginning of the experiment, and to the square root of the concentration of the ethyl-acetate. This is the rule of Schütz.

As is seen from these equations, x is only a function of

APt, *i.e.* of the product of the initial concentration of the catalytic agent — here ammonia — of the substrate — here ethyl-acetate — and of time. Evidently here the product *KP* may be regarded as the constant of the reaction.

From the analysis given above we find that the validity of the rule of Schütz indicates that the active part of the catalysor, ammonia or pepsin, etc., is inversely proportional to the products formed. This occurs in the case of ammonia because the product, NH_4-ions, gives a compound with the active part, the hydroxyl-ions, which is dissociated to a very low degree. Probably the case is similar in all the processes in homogeneous media studied below and belonging to this group (peptic or tryptic digestion). In heterogeneous media other circumstances, such as change of solubility, may play a rôle; perhaps this occurs in the saponification by means of lipases.

One of the most interesting investigations in this line was done as early as 1895 by Sjöqvist.[1] He dissolved 2.23 g. of egg-albumen, which by dialysis had been nearly purified from salts, in 100 c.c. of four solutions, which furthermore contained 0.005 gramme-molecules (=0.1875 g.) HCl and 2.5, 5, 10, or 20 c.c. of pepsin. In this homogeneous system the albumen slowly digested at 37° C. At the same time the conductivity gradually diminished, and this diminution was regarded as a measure of the velocity of digestion. At given intervals samples were taken from the solution and rapidily cooled to 18° C., at which temperature the velocity of digestion might be practically neglected. The determination of the conductivity was made at this temperature in the ordinary manner.

[1] Sjöqvist : *Skandinav. Archiv f. Physiologie,* 5 (1895).

He found the following figures : μ is the conductivity, Δ its decrease, $\Delta_{calc.}$ the corresponding calculated quantity, and $f = \dfrac{2.236\,\Delta}{\sqrt{P}}$, where P is the concentration of pepsin.

DIGESTION OF EGG-ALBUMEN AT 37° C. BY MEANS OF HCl AND PEPSIN

TIME (hours)	$P=0.025\%$				$P=0.05\%$			
	μ	Δ	$\Delta_{calc.}$	$f.$	μ	Δ	$\Delta_{calc.}$	$f.$
0	188.4	—	—	—	188.4	—	—	—
0.5	—	—	5.3	—	—	—	7.8	—
1	—	—	7.8	—	178.2	10.2	10.5	120
2	177.3	11.1	10.5	157	172.8	15.6	15.6	156
4	171.1	17.3	15.6	245	164.7	23.7	21.6	237
6	167.4	21.0	(19.6)	297	159.5	28.9	(26.9)	289
8	164.5	(23.9)	21.6	338	155.5	(32.9)	29.7	329
9	163.1	25.3	(23.0)	358	153.5	34.9	(33.7)	349
12	159.9	28.5	(26.9)	403	149.4	39.0	(36.6)	390
16	156.4	(32.0)	29.7	452	145.2	(43.2)	41.6	432
20	152.9	35.5	(34.7)	502	141.0	47.4	(46.8)	474
32	146.2	42.2	(41.6)	597	133.1	55.3	(53.1)	553
48	139.8	(48.6)	(48.8)	673	126.2	(62.2)	(61.2)	622
64	135.0	(53.4)	53.1	755	121.4	(67.0)	68.2	670
96	127.9	60.5	(61.2)	856	114.4	74.0	(77.0)	740

TIME (hours)	$P=0.1\%$				$P=0.2\%$			
	μ	Δ	$\Delta_{calc.}$	$f.$	μ	Δ	$\Delta_{calc.}$	$f.$
0	188.4	—	—	—	188.4	—	—	—
0.5	179.2	9.2	10.5	65	176.0	12.4	15.6	62
1	174.2	14.2	15.6	100	167.8	20.6	21.6	103
2	165.9	22.5	21.6	160	157.9	30.3	29.7	153
4	154.8	33.6	29.7	237	144.5	43.9	41.6	220
6	148.0	40.4	(36.6)	286	137.2	51.2	49.2	256
8	143.2	(45.2)	41.6	320	133.0	(55.4)	53.1	277
9	140.8	47.6	(44.5)	337	130.1	58.3	55.7	292
12	136.1	52.3	49.2	370	125.8	62.6	61.1	313
16	130.9	(57.5)	53.1	407	121.6	(66.8)	68.2	334
20	125.7	62.7	(58.6)	443	117.3	71.1	73.3	356
32	119.4	(69.0)	68.2	488	109.8	78.6	83.7	393
48	113.1	75.3	(77.0)	533	102.3	86.1	91.8	431
64	109.1	(79.3)	83.7	561	97.4	(91.0)	96.5	455
96	101.8	86.6	91.8	612	91.2	97.2	101.4	486

The figures in *brackets* are interpolated. $KP = 125$, if P is expressed in per cent; and $A = 1000$.

Sjöqvist found that f was nearly constant for constant and small values of t: in other words, the quantity of transformed egg-albumen is proportional to the square root of the acting pepsin.   But there is also another relation which holds good even for long times of reaction: the digested quantity of albumen is a function of Pt only, *i.e.* the quantity n of pepsin digests the same quantity in the time $\dfrac{t}{n}$, as the unit quantity of pepsin in the time t. This is seen from the following table : —

$Pt=0.05$	0.1	0.2	0.4	0.8	1.6	3.2	4.8	6.4	9.6
$P=0.025$	11.1	17.3	23.9	32.0	42.2	53.4	—	—	—	—
0.05	10.2	15.6	23.7	32.9	43.2	55.3	67.0	74.0	—	—
0.1	9.2	14.2	22.5	33.6	45.2	57.5	69.0	75.3	79.3	86.6
0.2	—	12.4	20.6	30.3	43.7	55.4	66.8	73.6	78.6	86.1
Mean	10.2	14.9	22.7	32.2	43.8	55.4	67.6	74.3	79.0	86.4
V	11	15.6	22	31.1	44					

From this it follows, as is seen in the mean values, that if Pt does not exceed a certain value (1.0) the quantity of digested albumen is nearly proportional to the square root of Pt, as is also seen from the calculated values (V) written below the means.

This was already observed by Emil Schütz [1] and has been confirmed by Jul. Schütz,[2] who dissolved the quantity, p, of pepsin, 10 c.c. of egg-albumen (containing 1–1.2 g. of coagulable substance), and 29 c.c. 1 per cent HCl in 100 c.c. In a digestion of 15 hours at 38° he found the following digested quantities, d, of egg-albumen, *i.e.* pepton, which is not coagulable : —

[1] Emil Schütz : *Zeitschr. f. ph. Ch.* **9.** 577 (1885).
[2] Jul. Schütz : *Zeitschr. f. ph. Ch.* **30.** 1 (1900).

$g.$	$d._{obs.}$	$d._{calc.}$
1	0.0212	0.0213
4	0.0471	0.0426
9	0.0652	0.0639
16	0.0799	0.0852
25	0.0935	0.1065
36	0.1031	0.1278

Here we have a very nice example of a monomolecular reaction, where the rate of transformation is proportional as well to the quantity of pepsin present as to that of the albumen present, but where, because of the perturbing influence of one of the reaction-products, the simple law of the monomolecular processes is altered. It reminds one in this regard strongly of the monomolecular process of transformation of acetamid studied by Ostwald,[1] or of the bimolecular process of saponification of ethyl acetate by ammonia, examined by myself.[2]

In such cases the experimental fact that x, the transformed quantity, is only a function of qt, the product of the quantity of the reacting substance and the time, gives an answer to the question, whether the action is, *ceteris paribus*, proportional to the quantity, q, of the reacting substance. In an analogous manner if x is only dependent upon q^2t or generally upon $f(q)t$, this circumstance indicates that the action of the substance is proportional to its square or in general q to the function $f(q)$.

By the aid of the integral formula the calculated values are found which are tabulated above beside those found by Sjöqvist. The agreement is very close and

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[1] Ostwald: *Journ. f. prakt. Ch.* **27.** 1 (1883).
[2] Arrhenius: *Zeitschr. f. ph. Ch.* **2.** 289 (1888).

indicates that the method of Sjöqvist was very useful and even that the theoretical views are rather concordant with the facts. It would have been desirable to have varied the quantity of hydrochloric acid in these experiments. Some experiments of Sjöqvist on the digestion in the presence of other acids than HCl, namely, sulphuric, nitric, and phosphoric acid, seem to indicate that the action of these acids is (about 16, 24, and 37 per cent respectively) less than that of hydrochloric acid; but more empirical material is desirable before definite conclusions may be induced.

E. Schütz and Huppert[1] have made a large number of measurements of the digestion of egg-white by means of hydrochloric acid and pepsin. The egg-white was freed of globulins. The influence of the concentration of acid was such that if 1 g. of egg-white in 100 c.c. was digested, the digested quantity in a given time increased with the quantity of acid, but more slowly than proportionally to this, until its concentration was 0.2 per cent; thereafter it was nearly constant, or in some cases, even decreased a little. This agrees with the opinion that the really reacting substance is the albuminat-ion, but further investigations are necessary before this may be stated with certainty.

In another series of experiments the temperature was varied. The digested quantity of 0.922 g. egg-white in 100 c.c. of 0.2 per cent HCl was found to be: —

At 30° C.	0.544 g. = 59.0 per cent	Calc. 59.0 per cent
" 35 "	0.660 " 71.6 "	" 71.4 "
" 27.5 "	0.713 " 77.3 "	" 77.4 "
" 40 "	0.775 " 83.0 "	" 83.0 "

[1] Schütz and Huppert: Pflüger's Archiv. 80. 470 (1900).

By means of the formula used for the calculation of peptic digestion I have determined μ, which was found to be 15,570, and from this value I have deduced the calculated values given above. The agreement is very satisfactory, but additional investigations will be necessary to give a definite value for μ. According to Schütz and Huppert the process has an optimum at about 50° C.

On analogous topics many interesting experiments have been carried out in the Danish Serum Institute. Different quantities (q c.c.) of pepsin were added to a solution of 2 c.c. of 7 per cent thymolgelatin, 1 c.c. 0.4 per cent solution of HCl, and $1 - q$ c.c. of 1 per cent solution of NaCl. The liquid was thereafter well mixed, and the whole was put in a test-tube which was placed in a thermostat at 36.6° C, and held at this temperature during a certain time, t. Then the test-tube was placed on ice until the next day. In this manner different test-tubes with varying q were prepared. The contents of tubes with high q were liquefied. It was noted which value of q was just high enough to produce liquefaction. In this way the following table was found:—

DIGESTING INFLUENCE OF PEPSIN ON THYMOLGELATIN

t (hours)	q	$q \cdot t$	t (hours)	q	$q \cdot t$
1.33	0.6	0.8	8	0.13	1.04
2	0.47	0.94	10	0.095	0.95
3	0.3	0.9	12	0.08	0.96
4	0.26	1.04	14	0.07	0.98
6	0.18	1.08	20	0.045	0.90
			24	0.038	0.91

The mean value is $q \cdot t = 0.96$, and the observed values

do not vary from it more than may be attributed to the experimental errors.

In quite the same manner experiments were done at different temperatures, and the observed values of $K = \dfrac{1}{qt}$ compared with the values calculated from the formula above ($\mu = 10,750$). The results are tabulated below :—

INFLUENCE OF TEMPERATURE ON THE DIGESTION OF THYMOLGELATIN

TEMP.	$K_{obs.}$	$K_{calc.}$
20	0.36	0.36
25	0.45	0.48
29.8	0.62	0.65
36.8	1.00	0.96
40.7	1.20	1.21

Segelcke and Storch [1] had already stated that a solution of rennet coagulates milk in times which are inversely proportional to the concentration of the solution. Soxhlet confirmed these results by more accurate measurements. On the basis of the proportionality of the digestion and the coagulating power of peptic solutions of different preparation Pawlow concluded that rennet is probably identical with pepsin. Hammarsten objected to Pawlow's opinion, because the pepsin was said to follow the rule of Schütz, which was not the case with the rennet, according to Soxhlet's experiments. This contradiction is evidently not proved and Sawjalow therefore infers that all experiments are in favour of Pawlow's opinion. Recent investigations of Bang, Hemmeter, and Schmidt-Nielsen (cf. Chapter IX) seem to indicate that pepsin in acid solution

[1] Segelcke and Storch : *Ugeskrift for Landmaend* (1870).

coagulates casein, just as rennet in neutral, or even in acid solution. The two coagulating enzymes are therefore not identical.

The influence of rennet on the coagulation of milk has been investigated in a manner analogous to that used in the study of pepsin. Thus, for instance, Madsen adds different quantities, q, of rennet to a given quantity of milk. Then he places the mixtures in test-tubes and places these during a time, t, in a thermostat at given temperature. After this the tubes are rapidly cooled, and it is determined which is the least quantity, q, sufficing for coagulation. The results are quite concordant with those for pepsin, as indicated by the following table:—

COAGULATING POWER OF DIFFERENT CONCENTRATIONS OF RENNET IN MILK AT 36.55° C.

t (min.)	q	qt	t	q	qt
4	0.08	0.32	35	0.007	0.25
6	0.05	0.30	50	0.005	0.25
9	0.033	0.30	70	0.004	0.28
11	0.024	0.26	80	0.0032	0.26
12	0.019	0.23	100	0.0028	0.28
14	0.0175	0.25	120	0.0025	0.30
20	0.013	0.26	180	0.00185	0.33
25	0.01	0.25	240	0.0017	0.41
30	0.007	0.21			

The mean value of qt is 0.28 (or if the two last observations are excluded 0.267). The last values display a notable increase of qt, that is, a decrease in the velocity of reaction with increasing time. This agrees well with the other series of observations from Copenhagen, as well

as with those of other observers, and may perhaps be ascribed to an attenuation of the rennet with time.

The influence of temperature on the coagulating power of rennet was investigated by Fuld.[1] He found the following values of the time of coagulation, t, for the same quantity of rennet : —

TEMPERATURE	t (sec.)	$K_{obs.}$	$K_{calc.}$
25.05	54	185	185
30	32	312	327
35	17	588	574
140	10.2	980	980
44	9	1111	1491
50	14.7	680	2742

$$\mu = 20,650$$

The values of $K_{obs.}$ are 10,000 divided by t. They agree quite well with the calculated values up to a temperature of 40° C.; above this the agreement fails. This evidently depends on the fact that above 40° C. the destruction of the rennet proceeds at such a speed that the observations are disturbed thereby. This explains also the occurrence of an optimum at about 44° C., which optimum therefore must not be regarded as real. The observed effect depends upon μ being many times greater (in this case 4.4 times) for the spontaneous destruction than for the process of fermentation. The same observation may be applied to the other cases in which such optima occur (cf. p. 53).

Reichel and Spiro[2] have contributed some interesting

[1] Fuld: *Hofmeisters Beiträge*, **2**. 169 (1902).
[2] Reichel and Spiro: *Hofmeisters Beiträge*, **7**. 478 (1905).

figures regarding the coagulation of milk at different dilutions or with the addition of different quantities of calcium chloride. The most interpretable results were yielded by the additions of calcium chloride. The milk itself contained 0.6 per cent if diluted in the proportion 10 : 8, which was employed in these experiments. Eight c.c. of milk were mixed with 1, 0.5, or 0.25 c.c. of a rennet solution, R, and with different amounts of a solution of calcium chloride and water until the total volume was 10 c.c. The authors give the following values : —

CaCl$_2$ IN %	TIME OF COAGULATION			CONSTANT $= (p + 0.6)t$		
p	1 c.c. R	0.5 c.c. R	0.25 c.c. R	1 c.c. R	0.5 c.c. R	0.25 c.c. R
0	95	48	24	57	28.8	14.4
0.05	88.6	45.6	23	57.6	29.6	15.0
0.1	79	41.6	22	55.3	29.1	15.4
0.2	66.4	36	19	53.1	28.8	15.2
0.5	48	26.4	14	52.8	29.0	15.4
1.0	30	18.2	10.6	48.0	29.1	17.0
2.0	17	11	6.8	44.2	28.6	17.7
5.0	10	7.4	5.4	56.0	41.4	30.2
10.0	13	9.2	6.2	137.8	97.5	65.7
20.0	22	15	8.6	453.2	309	177.2

As will be seen from these figures, the product $(p + 0.6)t$ is nearly constant, if p does not exceed 2 %. This relation is quite like that representing the connection between the quantity of rennet and time of coagulation. If the quantity of rennet is called R, the complete equation for the time of action is $R(p + 0.6)t = $ const. The value 0.6 which must be added to p to obtain a constant value may be regarded as the quantity of calcium ions

present if no calcium chloride is added.[1]   Chlorides of barium and magnesium exert a similar influence to that of calcium ; the less ionised magnesium sulphate has an analogous but weaker action.

In another series of experiments the concentration of the casein was changed.   The solvent by means of which the attenuation was accomplished was milk freed of casein, *i.e.* whey.   The experimental values are given below ; the quantity of rennet present was always the same, 1 c.c. of a given solution.

Milk	Whey	Time of Coagulation	
		obs.	calc.
c.c.	c.c.	sec.	sec.
0.2	8.8	110	110
0.4	8.6	70	64
0.6	8.4	50	49
0.8	8.2	42	42
1.0	8.0	39	37.4
1.25	7.75	33	33.8
1.5	7.5	31.2	31.5
1.75	7.25	29.6	29.9
2	7	28.6	28.6
2.5	6.5	27	26.8
3	6	26	25.7
4	5	24	23.2
5.5	3.5	23	23.0
8	1	22	21.8

The calculated values for the time of coagulation (t) are found by means of the empirical formula : —

$$t - 21.6 = 1.75 \cdot \frac{10 - M}{M}$$

[1] As the authors assert, this supposition assumes that only about 15 per cent of the calcium salts present in milk are in ionic state.

where M denotes the quantity of milk (in cubic centimeters) present in the solution.

Very complicated results were obtained on dilution with 0.9 per cent solution of sodium chloride, as is seen from the following table, in which M and R respectively designate the number of cubic centimeters of milk and rennet respectively present in 10 c.c. of the solution. (The rennet contained calcium salts.) The rest ($10 - M - R$) is the quantity of salt solution added. The tabulated figures give the time of coagulation in seconds.

$R =$		2	1.5	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3
$M =$1.25	c.c.	3	4	9	12	14.5	19	33	50	107	810
3	c.c.	4	6	10	11.5	14	18	24	32	45	78
8	c.c.	8	9	11	12	14	16	19	23	29	42

If $R = 0.8$ or 0.9 c.c., the time of coagulation is nearly independent of the dilution of the milk; at lower concentrations of R the time increases with dilution, at higher concentrations of R the opposite is true.

According to the views of Sawjalow the coagulation of milk is only a special case of digestion in which one of the products coagulates. This agrees very well with experiments at low temperatures first executed by Morgenroth,[1] and then repeated by Fuld. They prepared mixtures of rennet and milk, which were held at low temperatures. Then no coagulation occurred, but the digestion process did occur. After these mixtures had been held for some time at the low temperature and were then heated to 20° or more, they coagulated instantaneously.

[1] Morgenroth: *Archives internationales de pharmarcodynamie*, 7. 265 (1900).

As we have seen above, the albumose-salts produced in the digestion exert an enormous retarding influence on the velocity of digestion. In the same manner according to Sawjalow the addition of peptone to a mixture of milk and gastric juice increased the time of coagulation to a notable degree. This time, for instance, increased from 29.8 seconds to 791 seconds on the addition of 4 c.c. of peptone solution (1 c.c. was equivalent to 5.5 mg. HCl) instead of 4 c.c. of water to a mixture of 1 c.c. stomachal fluid with 10 c.c. of milk. The action was therefore diminished to about 4 per cent. A like influence is exerted by peptone solution on the velocity of reaction on milk of pancreatic juice or of solution of papayotin.

As peptone is very nearly related to albumose, this seems to indicate that the digestive action of pepsin is identical with the coagulating influence of rennet or pancreatic liquid or papayotin, as Pawlow and Sawjalow have contended. A substance that acts in a similar manner to pepsin on thymolgelatin is trypsin. On the action of different quantities (from 0.0022 to 0.3 c.c.) at 47.3° C. the following investigations were carried out by Madsen and Walbum. In this case no acid was added to the liquid in the test-tubes, but only 2 c.c. of thymolgelatin, q c.c. of trypsin solution, and $(2 - q)$ c.c. of 1 per cent salt solution. These investigations indicate that in this case, as well as in those treated above, the product of the time of reaction and reacting quantity is a constant, if the magnitude of reaction is the same. In the following table the signs t and q denote time and quantity: —

LIQUEFACTION OF THYMOLGELATIN BY MEANS OF TRYPSIN AT 47.3° C.

q	t (hours)	qt	q	t (hours)	qt
0.3	0.16	0.048	0.0072	8	0.058
0.105	0.5	0.052	0.006	10	0.060
0.05	1	0.050	0.0037	16	0.059
0.027	2	0.054	0.0032	18	0.058
0.02	3	0.06	0.0027	20	0.054
0.015	4	0.06	0.0025	22	0.055
0.011	5	0.055	0.0022	24	0.053
0.009	6	0.054		Mean	0.056

With another preparation of trypsin the effect of temperature was studied. The inverse value of qt may be regarded as the constant of reaction; it was found to have the following values : —

$$K = \frac{1}{qt}, \quad \mu = 10{,}570.$$

INFLUENCE OF TEMPERATURE ON THE DIGESTION OF GELATIN BY MEANS OF TRYPSIN

TEMP.	$K_{obs.}$	$K_{calc.}$
22.6	4.18	4.04
24.75	4.44	4.60
29.2	5.72	5.98
36.5	9.01	9.06
39.8	10.89	10.85
44.96	14.52	14.30
50.2	18.35	18.74

In most of the series of Madsen and Walbum an increase of qt with time is observed. This may be explained by the binding of a little of the trypsin or by the weakening of it with time. μ is nearly the same as for pepsin.

Bayliss [1] has investigated the progress of the action of trypsin on casein by means of the method of Sjöqvist. A solution of 8 per cent of the sodium salt of casein was prepared and to 6 c.c. of this solution 2 c.c. of 0.5 normal ammonia and 2 c.c. of a 2 per cent solution of trypsin were added. The conductivity was measured at different times and its increase (at 39° C.) plotted in a curve. This curve tends to an upper limit. By measuring the ordinates (Y) in millimeters of this curve I have found the following values : —

PROGRESS OF DIGESTION OF CASEIN WITH TRYPSIN AT 39° C.			PROGRESS OF DIGESTION OF EGG-WHITE BY TRYPSIN AT 39° C.		
TIME (hours)	$Y_{obs.}$	$Y_{calc.}$	TIME (hours)	$Y_{obs.}$	$Y_{calc.}$
0.3	25.0	29.0	1	10.2	10.2
0.5	35.5	35.7	2	15.0	13.8
0.7	41.0	40.8	4	18.8	18.5
1.0	48.0	46.4	6	22.0	22.0
1.5	55.5	53.7	8	24.0	24.1
2.0	59.2	58.4	10	25.5	26.5
2.5	62.2	61.4	15	29.2	30.3
3.0	64.6	64.2	20	31.8	33.2
3.5	66.0	66.4	25	33.6	35.3
4	67.5	68.1	30	35.4	36.9
5	70.0	70.8	40	38	39.4
6	71.4	72.6	50	41	41
7	72.7	73.3	∞	45	—
8	74.2	74.8			
∞	(77.0)	—			

The calculated values are found with the aid of the formula $KP = 320$, adopted for the pepsin action. They

[1] Bayliss: *Archives des sciences biologiques*, **11**. Suppl. p. 261, St. Petersburg, 1904.

agree very closely with the observed ones. The same is the case for the digestion of the solution of egg-white, which had been previously heated to 100 ° C. in order to destroy its content of antitrypsin. The solution contained 10 c.c. of a mixture of 10 per cent of egg-white and 90 per cent of distilled water, and further 1 c.c. of a 1 per cent solution of trypsin. Here $KP = 30$. The reaction with casein increases (the determination is not very reliable) in the proportion $1 : 5.3$ between 20.7° and 30.7° C. ($\mu = 29,500$) and $1 : 2.6$ between 30.7° and 38.7° and has probably an optimum, due to the instability of trypsin at higher temperature.

In this case the increase of conductivity depends probably on the formation of ammonium salts of the reaction-products. These also react with the trypsin, so that the quantity of free trypsin is nearly inversely proportional to the quantity of reaction-products, whereby the reaction progresses proportionally to the square root of the time in its first period. Bayliss showed that the addition of digestion-products, as well as asparagin, glycin (Merck), leucin, and amphopepton (Grübler), retards the action of trypsin to a high degree.

Henri and Larguier de Bancels[1] have used the method of Sjöqvist for the study of digestion by pancreatic juice. They found that this process of tryptic digestion follows the laws for monomolecular reactions and give as an illustration the following figures for the changes in the conductivity with time (at 44°, 4 per cent solution of gelatine).

[1] Victor Henri and Larguier de Bancels : *C. R. de la Soc. de Biol.* **55.** 787, 789, and 866 (1903).

Time (min.)	Change of Conductivity			Mean	$7.37\sqrt{t}$
10	27	28	27	27.3	29.3
20	46	44	42	44	41.5
30	53	55	51	53	50.0
40	58	60	58	58.7	58.7
55	66	66	65	65.7	68.8

As the end-value was not measured, we may only conclude that the change of the conductivity is very nearly proportional to the square root of the time of digestion, as is seen in the last column. It might therefore be regarded as probable that these experiments are not in opposition with those made by other observers, who have found that the rule of Schütz[1] holds good during the first time of digestion. The same authors have also digested casein dissolved in a 2 per cent solution of sodium carbonate in the same manner. They found the following changes of the conductivity (at 44° C.) : —

Time	Change	$5.59\sqrt{t}$
10	24	24
20	36	34
30	41	42
40	42	48
50	44	54

During the first time (30 min.) the change is nearly proportional to the square root of the time.

Another series of experiments were concerned with the problem of the digestion of different quantities of gelatine or casein, or of both simultaneously, by means of a con-

[1] Schütz and Huppert : *Pflüger's Archiv.* **80.** 470 (1900).

stant quantity of trypsins. The results are given below together with some calculated figures : —

TIME	G. 3.5 %		G. 1.75 %		C. 2.5 %		C. 1.25 %	
	obs.	calc.	obs.	calc.	obs.	calc.	obs.	calc.
10	17	18	11	13	23	26	20	18
20	30	25	18	18	39	37	26	26
30	37	29	20	22	46	45	28	32

G. 3.5 % + C. 2.5 %		G. 3.5 % + C. 1.25 %		G. 1.75 % + C. 2.5 %		G. 1.75 % + C. 1.25 %	
obs.	calc.	obs.	calc.	obs.	calc.	obs.	calc.
25	32	28	24	25	29	28	22
50	45	45	34	47	41	42	32
63	55	55	41	58	50	47	39

The calculated values are obtained on the basis of the assumption that the digested quantities for the gelatin are proportional to $3.05\sqrt{c \cdot t}$, and for the casein to $5.2\sqrt{c \cdot t}$, where c is the concentration of per cent and t the time in minutes. (In the table above G represents gelatin and C represents casein.) The agreement seems to be within the errors of observation, so that we may well conclude that the mass digested is, for short times, proportional to the square root of the concentration as well as of the time. The calculated values for the mixtures are made under the assumption that 1 per cent of casein is, so to speak, equivalent to 2.9 per cent of gelatin $\left(2.9 = \left(\frac{5.2}{3.05}\right)^{2}\right)$. Therefore 3.5 per cent gelatin + 2.5 per cent casein are equivalent to $3.5 + 7.3 = 10.8$ per cent gelatin. The observed effect is in most cases greater than the calculated one for the mixtures; only for a brief period do they correspond closely. It therefore seems

probable that the rule for this calculation agrees well with the facts only for short times; a closer examination of this circumstance can be done only when we know the limit values of the change of conductivity, and even then new observations ought to be carried out and multiplied.

The above calculation is carried through on the assumption that the products of digestion from casein exert the same binding influence on the reacting bodies (probably the trypsin) as do the corresponding derivatives from gelatin, since they cause the same change of the conductivity. This, of course, need not necessarily be the case; it is only the simplest hypothesis we may introduce in the present undeveloped state of research on this point. Hence it is not necessary at all to suppose that the trypsin is bound by the gelatin and the casein to explain that the observed effect on digesting gelatin and casein simultaneously is less than the sum of the effects of the digestion of the two substances separately, as Henri and Larguier suppose. Their hypothesis might be well founded, if we could observe the digestion in such an early period where the quantity digested was proportional to time, or, in other words, when the products of digestion would be much inferior in quantity (according to equivalents) to the quantity of trypsin. But this condition is not fulfilled in these or in any other experiments on tryptic digestion.

With the use of a different substratum, however, the action of trypsin exhibits a different behaviour. As determined by Taylor,[1] when protamin is digested by trypsin, the acceleration produced by the ferment is directly pro-

[1] Taylor, University of California Publications, Pathology, I, 21, 1904.

portional to its mass. This has been confirmed by Euler,[1] who digested glycyl-glycin by means of trypsin.

Henri and Lalou[2] have also carried out some measurements on the decomposition of amygdalin and salicin by means of emulsin at 26° C. The readings were determined by means of a polarimeter. An example may be given : —

Time (min.)	Salicin 2%	Amygdalyn 2.5%	Salicin 2% + Amygdalin 2.5%	Salicin 4%	Amygdalin 1.25%
46	0.67	0.97	1.05	1.08	0.90
130	1.58	2.38	3.63	2.25	1.57
268	2.32	3.15	4.22	3.45	1.56
∞	3.15	3.17	6.32	6.30	1.59

The decomposition of the mixture is much slower than the sum of the decompositions of the constituents. For comparison the rates of decomposition of 4 per cent salicin and of 1.25 per cent amygdalin under similar conditions are given in parallel. The explanation of the difference is evidently the same as that of the non-proportionality of reaction and concentration, but it offers no more stringent conclusions, regarding the combination of enzyme and substrate, than does this latter circumstance.

Weis[3] has carried out a great number of experiments on the digestion of the protein from wheat by means of trypsin or pepsin (extract of malt). A great interest is attached to those experiments in which the quantity of protein was varied. In the experiments on digestion cited above, the

[1] Euler: *Arkiv für Kemi*, **2**. No. 31, p. 8, Stockholm, 1907.
[2] Henri and Lalou : *C. R. de la Soc. de Biol.* **55**. 868 (1903).
[3] Fr. Weis : *Meddelelser fra Carlsberg-Laboratoriet*, **5**. 127 (1903).

protein was always present in constant amount. In the following table the quantity of protein is given in per cent n of the total liquid examined. After this, is tabulated the quantity of protein, in per cent of the whole quantity, which had been digested in 2 or in 5 hours. If, for instance, the digested quantity is 10 per cent of the original quantity of protein, the total quantity of digestion-products is double as great if $n = 2$, as if $n = 1$. Now the velocity of reaction is inversely proportional to the quantity of digestion-products, therefore the point where 10 per cent are digested is reached later if $n = 2$, than if $n = 1$. The digested quantity is therefore nearly inversely proportional to the square root of n, as is seen from the following figures, which are compared with figures calculated from the general formula on p. 64 : —

n (per cent)	DIGESTED QUANTITY AFTER				$K = \dfrac{13 \cdot 5t}{n}$
	2 HOURS		5 HOURS		
	obs.	calc.	obs.	calc.	
1	22.0	21.4	36.2	32.4	
2	17.0	15.6	25.9	23.8	
3	13.1	12.9	20.3	19.8	
4	9.1	11.2	16.0	17.3	
5	7.9	10.0	13.2	15.6	

The agreement seems to be satisfying considering the rather complex composition of the substrate. It indicates that the quantity of digestible matter enters in the formula in the same manner as the quantity of enzyme. In all cases of digestion or lipolysis which are subject to the formulæ given above, p. 64, that is to Schütz's rule, if the process has not gone too far, the concentration of the sub-

strate plays the same rôle as that of the enzyme or as the time, which is also probable on the theoretical grounds.

The products from a culture of *Bacillus pyocyaneus* exert a liquefying action on thymolgelatin, just as do pepsin or trypsin. This action was investigated by Madsen and Walbum. It was found, as for pepsin and trypsin, that the time necessary for liquefying the gelatin to the stated degree is inversely proportional to the quantity of culture employed. The bouillon in which the *B. pyocyaneus* had been cultivated for two weeks was filtered through a Chamberland filter and a certain quantity (q c.c.) added to 2 c.c. of 7 per cent thymolgelatin and $(2-q)$ c.c. of 1 per cent NaCl solution. The experiments were done at 34.5° C.

DIGESTION OF THYMOLGELATIN BY MEANS OF PYOCYANEUS CULTURE

q	TIME t (hours)	qt	q	TIME t (hours)	qt
1.6	0.5	0.8	0.11	8	0.88
0.8	1	0.8	0.09	10	0.90
0.46	2	0.92	0.08	12	0.96
0.3	3	0.9	0.06	16.5	0.99
0.22	4	0.88	0.044	18	0.79
0.2	4.5	0.9	0.042	20	0.84
0.17	6	1.02	0.035	25	0.88
					Mean 0.89

Madsen and Walbum have investigated the destruction of coli-agglutinin by means of trypsin at 35.6°C. The strength of the agglutinin was determined by means of its agglutinating power. The observed quantity, q, is that quantity of agglutinin which must be added to a suspension of *Bacillus coli* to obtain a given agglutination in a given time. These values are tabulated below. The strength S is the inverse value of q. The reaction is

calculated according to the formula for bimolecular reactions. $K = 68 \cdot 10^{-5}$.

DESTRUCTION OF 1 C.C. OF COLI-AGGLUTININ SOLUTION BY MEANS OF 5 C.C. OF 1% SOLUTION OF TRYPSIN AT 37.5°

TIME (hours)	$S_{obs.}$	$S_{calc.}$	TIME (hours)	$S_{obs.}$	$S_{calc.}$
0	1000	1000	6	189	197
0.5	775	763	8	149	155
1	610	600	10	140	128
2.25	389	395	12	108	109
3	280	329	14	88	95
4.17	259	261	23	61	57
5	233	227	25	59	56

The agreement is very satisfactory at the beginning, if we consider that the errors of observation are rather large. This method gives a direct determination of the destruction, and not an indirect one, as do the measurements of the electrical conductivity.

The destruction of rennet increases very rapidly with increasing temperature, as is shown by the following table:

WEAKENING OF RENNET IN 2% SOLUTION AT 47.55° C.			WEAKENING OF RENNET (2% SOL.) AT DIFF. TEMPERATURES		
t (min.)	$S_{obs.}$	$S_{calc.}$	TEMP.	$K_{obs.}$	$K_{calc.}$
0	20	17.9	44.51	0.0127	0.011
2.5	14.3	14.3	46.04	0.0231	0.022
5	10.5	11.4	47.55	0.039	0.0414
7.5	8.3	9.1	48.57	0.0646	0.0647
10	7.1	7.1	49.12	0.072	0.0836
12.5	5.9	5.9	49.6	0.101	0.102
15	5.0	4.8			
17.5	4.0	3.7			
20	3.0	3.0			
22.5	2.2	2.2			
25	1.8	1.9			

$K = 0.0386.$

$\mu = 89,130.$

The reaction is monomolecular.   The influence of temperature is characterised by a rather high value of μ. A second series gave $\mu=91,000$, so that in the mean $\mu=90,000$.   This attenuation is much more marked in weaker than in stronger concentrations, as is indicated by the following figures, valid for the temperature $46.15°C$.:

Concentration	Velocity of Destruction	Concentration	Velocity of Destruction
7	0.00372	1	0.028
6	0.003	0.5	0.032
5	0.0049	0.25	0.039
4	0.0077	0.125	0.060
3	0.0154	0.0625	0.073
2	0.0212		

Dried powder of rennet is very resisting to the influence of temperature; $K=0.0414$ at $158°$ C.   The reaction was monomolecular.

The destruction of the rennet is to a high degree accelerated by the presence of an alkali.   To 200 c.c. of a 10 per cent rennet solution Madsen added from 0.5 c.c. to 3 c.c. of 1 n. NaOH.   He found that the velocity of destruction proceeds more slowly than that of a monomolecular reaction.   This circumstance is probably due to a retarding influence of the reaction-products.   This influence may in the following example, in which 3 c.c. of 1 n. NaOH acted upon 200 c.c. of a solution of rennet at $46°$ C., be put proportional to the third root of the quantity of rennet. $q_{obs.}$ indicates the quantity of rennet which is necessary to cause coagulation in ten minutes. $q_{calc.}$ is the corresponding calculated quantity.

DESTRUCTION OF RENNET IN PRESENCE OF SODIUM HYDRATE AT 46° C.

t (min.)	$q$obs.	$q$calc.	t (min.)	$q$obs.	$q$calc.
0	0.040	0.040	10	0.50	0.48
2	0.073	0.079	12	0.70	0.66
4	0.130	0.139	14	1.00	0.88
6	0.20	0.22	16	1.15	1.15
8	0.35	0.33			

The calculated values are deduced from the formula :—

$$q_1^{\frac{1}{3}} - q_0^{\frac{1}{3}} = 0{,}044\,(t_1 - t_0).$$

Madsen has also investigated the destruction of trypsin by means of alkalies and found similar regularities as for the destruction of pepsin.

Regarding the spontaneous attenuation of pepsin, Madsen and Walbum found that the reaction is monomolecular, which is shown in the following table. The increase of this reaction with temperature gives $\mu = 75{,}600$, that is, $\frac{5}{6}$ of the value for rennet (2 per cent solution). As the preparations are not identical and even used in different concentrations, this difference does not afford any data bearing on the possible identity of rennet and pepsin. The strength of the pepsin was determined by means of its digesting influence on thymolgelatin.

WEAKENING OF PEPSIN (5% SOL.) AT 66.8°			WEAKENING OF PEPSIN AT DIFFERENT TEMPERATURES		
t (min.)	STRENGTH obs.	STRENGTH calc.	TEMP.	K obs.	K calc.
0	17.5	17.6	57	0.00112	0.00112
5	11.1	12.8	60	0.0047	0.0036
10	8.33	8.71	63.3	0.0109	0.0098
20	4.35	4.34	64.8	0.0141	0.0160
30	2.22	2.25	66.8	0.0305	0.0305
40	1.11	1.06			

$K = 0.0305.$

$\mu = 75{,}600.$

Trypsin behaves in a manner very similar to that of rennet and pepsin. The reaction of the spontaneous attenuation is monomolecular and the velocity constant of reaction increases very rapidly with temperature ($\mu=62,000$). The values are given below:—

DESTRUCTION OF TRYPSIN AT 64.03° C.			DESTRUCTION OF TRYPSIN AT DIFFERENT TEMPERATURES		
t (min.)	STRENGTH$_{obs.}$	STRENGTH$_{calc.}$	TEMP.	$K_{obs.}$	$K_{calc.}$
0	12.5	12.1	60.72	0.00127	0.00127
5	11.8	11.6	61.95	0.0018	0.0018
11	11.1	11.1	63	0.0024	0.0024
15	10.5	10.8	64.03	0.0032	0.0032
20	10.0	10.4	67.15	0.0073	0.0074
30	9.1	9.6	72.15	0.0274	0.0274
40	8.7	9.0	74.35	0.049	0.049
50	8.3	8.3			
60	7.7	7.7			
80	6.7	6.7			
100	5.9	5.9			
120	5.6	5.0			

$K = 0.00317.$ $\mu = 62,034.$

The concentration of the trypsin solution exerts no sensible influence on the rate of destruction in this case. Solutions containing 2, 4, 6, 8, or 10 per cent of trypsin all gave satisfying results when calculated with the value $K=0.0073$ (at 67.15° C.). The strength of the trypsin was measured by means of its property of liquefying thymolgelatin.

Trypsin exerts, as we saw, a destructive influence on coli-agglutinin, and this destruction proceeds faster at higher temperature than at lower, as is seen from the following measurements of Madsen and Walbum. At 44.2° C. the calculated values differ for some time rather

widely from the observed ones, probably because of the spontaneous destruction of the trypsin.

DESTRUCTION OF COLI-AGGLUTININ (1 C.C.) BY TRYPSIN (5 C.C. 1 % SOL.) AT DIFFERENT TEMPERATURES

TIME (hours)	AT 33.5° C.			AT 37.2° C.			AT 44.2° C.		
	$q_{obs.}$	Strength obs.	calc.	$q_{obs.}$	Strength obs.	calc.	$q_{obs.}$	Strength obs.	calc.
0	0.001	1000	1000	0.001	1000	1000	0.001	1000	1000
1.25	0.0017	589	602	0.002	500	515	0.0025	400	407
3	0.0023	435	386	0.0027	370	308	0.005	200	222
5	0.0038	263	274	0.0047	213	211	0.008	125	146
8	0.005	200	191	0.008	125	141	0.0095	105	96
12	0.008	125	136	0.01	100	100	0.0115	87	66
	$10^5 K = 53$			$10^5 K = 75$ (calc. 70)			$10^5 K = 117$		
				$u = 14200$					

The method used for measuring the potency, *i.e.* the inverse value of the quantity $q_{obs.}$ necessary for the production of a certain degree of agglutination, is that described by Madsen and Jörgensen.[1] From the observations the values K are calculated, by means of which the calculated values for the potency are deduced according to the formula for bimolecular reactions. The agreement with the observed values is satisfactory. From the two values at 33.5° and 44.2° C. the value $\mu = 14,200$ is calculated by means of the general formula. With this value of μ we find $10^5 K = 70$ at 37.2° C. in satisfactory agreement with the observed value 75.

A peculiar regularity is found for the coagulating power of fibrin ferment on plasma. Here the product of time of reaction and concentration is not nearly constant, as in the coagulation of milk by rennet, but the product of

[1] *Festskrift ved Indvielsen af Statens Serum Institut*, Copenhagen, 1902, No. 5.

the concentration to the power $\frac{2}{3}$ and time of reaction gives a nearly constant value, as indicated by the figures below, found by Madsen and Walbum, as well as by Fuld:[1]

PLASMA AND MUSCLE EXTRACT FROM HORSE (MADSEN AND WALBUM [2])						BLOOD PLASMA OF GOOSE (2 C.C.) WITH 6 C.C. OF EXTRACT OF GOOSE MUSCLE (FULD)		
t (min.)	C	$t(10\,C)^{\frac{2}{3}}$	$t=$TIME (min.)	CONC. (C)	$t\,C^{\frac{2}{3}}$	C	$t=$TIME (seconds)	$t\times(10\,C)^{\frac{2}{3}}$
105	0.6	330	80	2	127	0.2	80	127
155	0.3	322	120	1	120	0.1	120	120
230	0.15	301	180	0.5	113	0.05	180	113
328	0.10	328	290	0.25	115	0.025	290	115
595	0.05	375						

The reaction is therefore not proceeding in proportion to the concentration of the fibrinogen, but to the power $\frac{2}{3}$ of it. Closer investigations seem desirable.

Even the process of precipitation is subject to the same influence of temperature as the other reactions studied.[2] The precipitating substances were 10 n. sulphuric acid or albumen precipitin, produced by subcutaneous injection of egg-albumen into a rabbit. The following values were observed with a solution of 2 c.c. of egg-albumen in 98 c.c. of physiological salt-solution.

10 N. H_2SO_4 (90 MIN.)			PRECIPITIN (73 MIN.)		
TEMP.	$q_{obs.}$	$q_{calc.}$	TEMP.	$q_{obs.}$	$q_{calc.}$
35.8	0.10	0.11	36.1	0.25	0.25
29.7	0.16	0.16	30.1	0.30	0.31
25.4	0.22	0.21	20.0	0.45	0.44
19.9	0.30	0.29	13.9	0.55	0.55
14.5	0.40	0.41			

$$\mu = 11,000.$$ $$\mu = 6,300.$$

[1] Fuld: *Hofmeisters Beiträge*, **2**. 514 (1902).
[2] Madsen and Walbum: *Oversigt over det kgl. danske Vid-selsks Forh.* (1904).

The two processes are undoubtedly of a very different nature. Here it is supposed that the precipitins, like rennet, obey the law that the product of time and reacting mass was constant. Then if, for instance, 0.1 c.c. of 10 n. H_2SO_4 acting upon 8 c.c. of egg-albumen solution coagulates it in 90 minutes at 35.8°, we expect that 0.3 c.c. of this acid will give coagulation in 30 minutes. Now this quantity cogulates the egg-white solution in 90 minutes at 19.9°. Therefore we say that the velocity of reaction is three times greater at 35.8° C. than at 19.9° C. In this manner the variation of the velocity of reaction with temperature may be calculated and from that the value of μ. In an analogous manner we observe with the precipitin the quantity necessary to give a unit of precipitate in a given time, and from this we calculate the different times in which the same quantity of precipitin will give the same degree of precipitation at the different temperatures. Probably a closer investigation will show that the premises of our calculation are fulfilled.

An interesting instance of a bimolecular reaction has been found by Madsen and Walbum[1] in the interaction between tetanolysin and pepton. A solution of 2 g. of Witte's pepton in 100 c.c. water and another of 2 per cent tetanolysin in physiological salt-solution were prepared. Mixtures of 4 c.c. of the solution of lysin heated to 36.1°C. with 0.15, 0.20, and 0.25 respectively of the solution of pepton (of 36.1°) and so much physiological salt-solution of 36.1°, that the whole equalled 8 c.c. were placed in a water-bath at this temperature and the hæmolytic power determined

[1] Madsen and Walbum: *Centralbl. f. Bakteriologie*, **40**. 409 (1906).

at different times. For this purpose a part of the mixture was rapidly cooled in a test-tube surrounded by ice, whereby the destruction of the lysin was practically brought to an end. The quantity of lysin remaining was determined in the ordinary manner by measuring its hæmolytic action upon a suspension of erythrocytes.

In this manner the following values were obtained:—

Time In hours (t)	Toxicity (obs.)	$q_{calc.}$	$\frac{1}{q_{obs.}}$	$\frac{1}{q_{calc.}}$
		0.15 c.c. Pepton Added		
0	100	(55.8)	0.01	(0.0179)
0.5	47.7	47.7	0.021	0.0210
1	39.7	41.4	0.0252	0.241
2	30.3	33.0	0.0330	0.0303
4	22.3	23.4	0.0448	0.0427
6	18.1	18.1	0.0552	0.0551
8	17	14.8	0.0588	0.0675
		0.20 c.c. Pepton Added		
0	100	(60.0)	0.01	(0.0167)
0.5	41.6	41.6	0.024	0.024
1	30.3	31.5	0.033	0.0313
2	20	21.5	0.050	0.0460
4	12.2	13.5	0.082	0.0754
6	9.6	9.7	0.105	0.1048
8	8	7.5	0.125	0.1342
		0.25 c.c. Pepton Added		
0	100	(70)	0.01	0.0142
0.5	35.2	35.2	0.0284	0.0284
1	22	23.4	0.0455	0.0426
2	13	14.0	0.0769	0.0711
4	6.0	7.8	0.1666	0.128
6	5.3	5.4	0.1886	0.185
8	3.8	4.1	0.2631	0.242

Evidently the reaction follows very nearly the law for a bimolecular reaction, in regard to the quantity of free toxin (q), so that: —

$$-\frac{dq}{dt} = K \cdot q^2;$$

and:

$$\frac{1}{q_2} - \frac{1}{q_1} = K(t_2 - t_1).$$

The observed and calculated values agree within the errors of observation. An exception is evidently displayed by the first value ($t = 0$). Just as in similar chemical processes it is difficult to determine the time zero, since the reaction does not end instantaneously when the mixture is removed from the water-bath. We therefore employ the same method that has been applied to similar cases studied before, namely, to reduce the time to that of the first experimental determination of the toxicity, which was made after the mixture had been held at 36.1° C. for half an hour.

The constant (K) increases very rapidly with the concentration of the pepton, as the following table shows: —

Concentration of pepton, C	0.15	0.20	0.25
Constant, K, obs.	0.0062	0.0147	0.0285
Constant, K, calc. = 1.833 C^3	0.0062	0.0147	0.0286

The velocity of reaction is proportional to q^2C^3, which may be explained by supposing that at first there is formed a compound of two molecules of tetanoysin with three molecules of peptone, which is decomposed very rapidly with the destruction of the tetanolysin.

Madsen and Walbum have also investigated the influence of the temperature on this process, which in this case is peculiarly low, μ being found to be 10,500 only. As a comparison we may cite the saponification of ethyl-acetate, where μ has a value of the same order of magnitude, namely 11,160. They investigated the reaction velocity of a solution containing 4 c.c. of the solution of tetanolysin, 0.08 c.c. of a 2 per cent solution of Witte's pepton, and 3.9 c.c. of physiological salt-solution at 37.1, 31.2, 27.5, and 17.8° C. respectively.

They found the following figures :—

INFLUENCE OF THE TEMPERATURE ON THE DESTRUCTION OF TETANOLYSIN
BY PEPTON

	AT 37.1°			AT 31.2°	
t (hours)	$q_{obs.}$	$q_{calc.}$	t (hours)	$q_{obs.}$	$q_{calc.}$
0	100	100	0	100	100
0.33	41.5	41.7	0.33	46.7	48.8
1	18.8	18.9	1	26.3	24.2
2	12.1	10.4	2	13.2	13.8
	AT 27.5°			AT 17.8°	
0	100	100	0	100	100
0.33	48.9	54.9	0.33	69.8	69
1	28.3	28.2	1	46.1	40.8
2	16.7	16.4	2	21.5	25.7
4	9.5	8.9	4	13	14.8

The agreement between the observed and the calculated values is very satisfying; even at 17.8° C., where the deviations are the greatest, they still fall within the possible errors of observation.

The calculated constants (K) of reaction, according to the bimolecular formula, are given in the following table ($\mu = 10,240$):—

Temp.	$K_{obs.}$	$K_{calc.}$
37.1	0.0431	0.0431
31.2	0.0313	0.0313
27.5	0.0255	0.0255
17.8	0.0144	0.0144

Here the coincidence is nearly exact. In the cases investigated above, where the interval of temperature reached only four degrees, a simple exponential formula would have given just as good results as the more complicated one adopted from physical chemistry; but in this case the interval of temperature is so great that the exponential formula would have given a markedly inferior agreement with the observations, though still the differences would not have exceeded the errors of observation.

It may be remarked that not all peptons exert this weakening influence on tetanolysin; for instance, Chapeautaud's pepton is without influence in this respect.

As a general result of these investigations we find that the spontaneous destruction of the substances studied increases in solution very rapidly with temperature. In most cases the destruction of these substances by catalytic agencies increases much more slowly, about at the same rate as the catalytic processes studied in general chemistry.

The observed values of μ are compared in the following table : —

Spontaneous destruction of dry emulsin $\qquad \mu = 26,300$

(Tammann)

Spontaneous destruction of lipase from castor beans in sol. $\mu = 26,000$

(Nicloux, cf. next chapter)

Spontaneous destruction of emulsin in 0.5 per cent sol. $\quad \mu = 45,000$

(Tammann)

H

Spontaneous destruction of trypsin in 2 per cent sol. $\mu = 62,034$
Spontaneous destruction of pepsin in 2 per cent sol. $\mu = 75,600$
Spontaneous destruction of rennet in 2 per cent sol. $\mu = 90,000$
Spontaneous destruction of vibriolysin in sol. $\mu = 128,000$
Spontaneous destruction of tetanolysin in sol. $\mu = 162,000$
Spontaneous destruction of comp. hæmolysin in sol. $\mu = 198,500$
Spontaneous destruction of dibromsuccinic acid $\mu = 22,220$
(van't Hoff)

Destruction of salicin by emulsin $\mu = 3,300$
(? Tammann)

Destruction of cane sugar by invertase $\mu = 9,080$
(Kjeldahl)

Destruction of tetanolysin by pepton $\mu = 10,240$
Destruction of coli-agglutinin by trypsin $\mu = 16,500$
Coagulation of milk by rennet $\mu = 20,650$
Coagulation of digestion of casein-salt by trypsin $\mu = 29,500$
Precipitation of egg-white by sulphuric acid $\mu = 11,000$
Precipitation of egg-white by precipitin $\mu = 6,300$
Digestion of gelatin by means of pepsin $\mu = 10,750$
Digestion of gelatin by means of trypsin $\mu = 10,570$
Digestion of egg-white by means of pepsin $\mu = 15,570$
Hydrolysis of sugar by acids $\mu = 25,600$
(Spohr)

Saponification of cotton oil by lipase from castor beans $\mu = 7,540$
(Nicloux, cf. next chapter)

Saponification of triacetin by lipase from castor beans $\mu = 16,700$
(A. E. Taylor, cf. next chapter)

Saponification of ethyl-acetate by NaOH $\mu = 11,150$
(Warder)

The spontaneous destruction of the different substances investigated by Madsen increases from three to nine times more rapidly with temperature than the corresponding phenomenon for dibromsuccinic acid ($C_4H_4O_4Br_2 = C_4H_3O_4Br + HBr$). The values of μ found by Madsen even exceed those found for emulsin by Tammann. On the other hand, the μ for the catalytic actions is of the same order of magnitude as those known heretofore (especially that of saponification). The similarity of the values

of μ in the two cases of digestion of gelatin by means of pepsin or of trypsin is very startling. In any case it is not possible to retain the opinion that μ is of the same order of magnitude for all velocities of reaction, corresponding closely to an increase in the proportion $1:2$ for an increase in temperature of ten degrees ($\mu = 11{,}850$ at $20°$, $\mu = 14{,}400$ at $50°$, and $\mu = 17{,}200$ at $80°$ C.). As most of Madsen's determinations were carried out in the proximity of $50°$, we may say that μ is between four and fourteen times greater, that is, the increase for $10°$ C. may be from $2^3 = 8$ to $2^{13} = 8200$ times the usual rate.

CHAPTER IV

VELOCITY OF REACTION. HETEROGENEOUS SYSTEMS

MOST of the reactions concerned in sero-therapy occur in heterogeneous systems, and may therefore be regarded as analogous to, *e.g.*, the solution of a metal or a carbonate in an acid. To this category belong, for instance, the hæmolytic reactions, which possess such great importance for theoretical researches. Madsen and I[1] have made some experiments on the velocity of hæmolysis by sodium hydrate, ammonia, and tetanolysin. The reagents, solutions of the hæmolytic substances and suspensions of blood-emulsions, were heated to 37°C., mixed and allowed to act upon each other for a certain time, then the mixture cooled down to 0°C. in order to practically check the reaction, and rapidly centrifugalised. The colour of the solution indicates the degree of hæmolysis.

The velocity of the reaction was calculated under the assumption that the transformed quantity in the unit time is proportional to the number of erythrocytes present. The hæmolytic agent was present in such an excess that its quantity was many times greater than that necessary for complete hæmolysis. This quantity, being in excess, was therefore regarded as approximately constant during the short time of reaction. Hence the velocity of reaction should be that of monomolecular order in a homogeneous

[1] *Festskrift*, Copenhagen (1902), No. 3.

system.   A series of experiments with 0.5 c.c. 0.1 n. NH_3 mixed with 10 c.c. 2.5 per cent suspensions of blood gave :

Time (minutes)	0	6	14	23	31
Intact blood (per cent)	100	97	82	60	35
Constant of reaction	—	0.00022	0.0062	0.0096	0.0147

The "constant" increases very rapidly as the process advances.   This phenomenon depends evidently upon something similar to the "time of induction," observed in reactions in heterogeneous and sometimes also in homogeneous systems (action of light upon a mixture of chlorine and hydrogen).   In this case it is easy to understand that a certain quantity of ammonia must diffuse into the erythrocytes and act there for some time before the hæmoglobin leaves the cells.   According to different circumstances, such as varying resisting power of the cells, distance from the molecules of ammonia in the moment of mixing, the different cells are attacked more or less slowly.

Still it was possible to prove that the time necessary for hæmolysing a given number of erythrocytes is inversely proportional to the strength of the hæmolytic agent.   Thus, for instance, in experiments with solutions the dilutions (inverse concentrations) of which were proportional to 1 : 0.44 : 0.23 : 0.133 the following times in minutes were necessary for the hæmolysis of 3, 10, 20, 30 and 40 per cent respectively of the erythrocytes.   In parentheses are printed the calculated values obtained according to the rule mentioned.

Hæmolysis		3	10	20	30	40 per cent.
Dilution	1	13 (13)	26 (26)	35 (35)	44 (44)	53 (53) min.
Dilution	0.44	6 (5.7)	10 (11.5)	15 (15.4)	18 (19.4)	23 (23.3) min.
Dilution	0.23	——	5.5 (6.0)	9 (8.0)	12 (10.1)	14 (12.2) min.
Dilution	0.133	——	1.8 (3.5)	4 (4.7)	6.2 (5.9)	8 (7.1) min.

The agreement is very satisfactory.

Similar experiments were done with sodium hydrate and tetanolysin. They led to similar results. If different quantities of ammonia are allowed to act upon a unit amount of blood corpuscles during a given time, it follows from the considerations given above that the hæmolysed quantity increases more rapidly than in proportion to the amount of ammonia. If we consider the quantities a and $2a$, and let the first act during the time $\frac{t}{2}$, the second during the time t, the action of the two will be equal. If thereafter the quantity a be allowed to act during the time $\frac{t}{2}$, the velocity of reaction will be much greater during this interval than in the first period. Therefore the quantity hæmolysed in the time t by the quantity $2a$ is more than double that hæmolysed in the same time by the quantity a (provided that the total hæmolysis is not near completeness, and that we observe in the first stages of the process). It is often found that the quantity hæmolysed is roughly proportional to the square of the concentration of the poison, if this does not act very rapidly as is the case with ammonia and tetanolysis. This rule, which may be of use for many calculations, is illustrated by the following examples (a is the concentration of the poison, b the degree of hæmolysis in per cent, $c = \sqrt{b} : a$).

In other cases, generally of rapidly acting hæmolytic agents, the rule does not hold, but the value of c sinks rapidly when the degree of hæmolysis falls below 10 per cent. Such are the strongly dissociated bases, caustic potash, soda, and lithium, and even solanin.

INFLUENCE OF THE CONCENTRATION OF A POISON ON THE HÆMOLYSIS

TETANOLYSIN			AMMONIA		
a	b	$c=\dfrac{\sqrt{b}}{a}$	a	b	$c=\dfrac{\sqrt{b}}{a}$
0.91	45	7.4	0.84	65	9.6
0.74	25	6.8	0.67	55	11.1
0.57	14	6.6	0.50	37	12.3
0.48	7	5.5	0.40	27	12.0
0.43	6	5.7	0.36	16	11.1
0.38	3.5	5.9	0.31	12	11.1
0.29	2.5	5.5	0.27	6	9.2
0.24	2.5	6.6	0.22	5	10.2
0.20	1.7	6.5			

The blood often binds a certain quantity of the poison added, so that under a certain concentration no hæmolysis occurs; this was, *e.g.*, the case with 10 c.c. of a suspension containing 10 per cent of erythrocytes and less than 0.015 milligramme equivalent of caustic soda or ammonia. The quantity bound is very strictly proportional to the quantity of erythrocytes.

An analogous case is seen in telanolysin, according to the experiments of Madsen and Henderson-Smith. They added different quantities, q, of telanolysin to 10 c.c. of 2 per cent suspension of erythrocytes from the horse and determined the times which were necessary at 37° C. to produce a certain degree of hæmolysis. The results are given below on p. 104.

The time ∞ indicates that the quantity 0.25 telanolysis does not give an appreciable hæmolysis. Evidently the product of the reacting quantity $(q-0.25)$ and the time of action is constant.

HÆMOLYSIS BY MEANS OF DIFFERENT QUANTITIES OF TETANOLYSIN AT 37° C.

q	t (min.)	$(q-0.25)\,t$	q	t (min.)	$(q-0.25)\,t$
1.0	2	1.50	0.4	11.8	1.77
0.8	2.8	1.54	0.35	13.5	1.35
0.6	4.5	1.48	0.3	28.5	1.42
0.5	6.1	1.52	0.25	∞	
0.45	7.1	1.42			

The agglutinins display in their general behaviour a very great similarity to the hæmolysins. This was determined by experiments in which different quantities, q, of an agglutinin (against Bacillus coli) were allowed to act upon similar suspensions of this bacillus at 37° C. Then the time necessary to produce a given degree of agglutination was found to be inversely proportional to q as will be seen from the following table.

Action of different quantities of agglutinin on Bacillus coli at 37° C.

q	t (min.)	qt	q	t (min.)	qt
0.08	20	(1.6)	0.008	140	(1.12)
0.035	30	1.05	0.006	150	0.90
0.025	45	1.11	0.0055	165	0.91
0.017	60	1.02	0.005	180	0.90
0.012	90	1.08	0.004	210	0.84
0.008	120	0.96	0.0035	240	0.84
0.005	180	0.90	0.003	300	0.90
0.004	240	0.96	0.0022	420	0.92
0.003	300	0.90	0.002	480	0.96
0.0027	360	0.97			
		Mean 0.99			Mean 0.90

The first figures indicate an activity known to be too low (too long time). This may be due to errors in the measuring of the time. It is supposed that the liquids are immediately brought to the temperature of the thermostat in which they are placed. Evidently this is not quite true, and therefore in a more exact calculation a certain time should be subtracted from the observed one.

Henri has carried out some interesting experiments on the hæmolysis of chicken erythrocytes by means of normal dog-serum. He added different quantities of the serum to 30 c.c. of a 10 per cent suspension of the erythrocytes and so much of 0.9 per cent NaCl solution as to bring the whole volume to 40 c.c. Then he observed that the hæmolysis proceeded at first rapidly, later on more slowly, until it reached a limit value. This value was found to be:—

Quantity of serum in *c.c.* (q)	0.3	0.4	0.5	0.75	1	1.5
Limit of hæmolysis in per cent	15	19.5	30	56	93	100
$93\,q^{\frac{3}{2}}$		15.3	23.5	33	60	93	(100)

The limit value increases more rapidly than the first power of the quantity of serum, but not as much as the square of it; the last figures indicate that the limit is nearly proportional to the power $\frac{3}{2}$ of q.

To illustrate the progress of hæmolysis with time Henri gives the following figures:—

Quantity of serum in c.c.	0.15	0.2	0.3	0.4	0.5	0.75	1	1.5	2
Hæmolysis in per cent after 12 min.	—	—	—	—	—	—	8.5	19.1	30
							(8.5)	(19.1)	(34)
Hæmolysis in per cent after 36 min.	—	—	5.0	6.9	10.0	28.2	66.6	95.6	—
			(4.5)	(8.0)	(12.5)	(28.1)	(50)	(100)	

Hæmolysis in per
cent after 76 min. —   4.1   8.4   13.0   19.5   47.0   78.5   98.3   100
                        (3.1) (7.0) (12.5) (19.5) (43.9) (78)   (100) (100)

Hæmolysis in per
cent after 107 min. 3.3 5.5   11.7   15.7   23.6   50.0   85.0   100   100
                   (2.0) (3.6) (8.0) (14.3) (22.3) (50.0) (89.0) (100) (100)

Hæmolysis in per
cent after 200 min. 4.8 7.9   14.4   18.3   29.0   55.0   90.0   100   100
                   (2.4) (4.3) (9.6) (17.1) (26.7) (60)   (100) (100) (100)

In the brackets are written the figures which are proportional to the squares of the quantities of hæmolysing serum. As will be seen from these, the agreement with the observed figures is fairly good (within the errors of observation), if the time lies below 76 minutes (in most cases the time of reaction was about one hour in similar experiments). For longer times of reaction the action of small quantities is somewhat greater than the rule of the square root demands.

Henri[1] found that the progress with time follows very closely the law of monomolecular reactions. He mixed 30 c.c. of a 10 per cent suspension of chicken erythrocytes with 9.5 c.c. of 0.8 per cent sodium chloride solution and the following quantities of dog-serum. The hæmolysis, x, is given with its limit value as unit. The reaction-constants are calculated from the formula

$$K = \frac{1}{t} \log \frac{1}{1-x}.$$

Quantity of serum in *c.c.*	0.3		0.4		0.5		0.75	
	x	K	x	K	x	K	x	K
Time of reaction, 24 min.	0.33	0.0072	0.35	0.0077	0.33	0.0072	0.50	0.0125
Time of reaction, 63 min.	0.56	0.0057	0.67	0.0076	0.65	0.0072	0.83	0.0122
Time of reaction, 94 min.	0.78	0.0070	0.80	0.0094	0.79	0.0072	0.90	0.0106
Time of reaction, 190 min.	0.96	0.0071	0.94	0.0065	0.96	0.0071	0.98	0.0090

[1] Victor Henri: *C. r. de la Société Biologique*, **58**. 36–39 (Jan. 4, 1605).

Another series of experiments seems to indicate that the value of K is independent of the quantity of erythrocytes, but increases more rapidly than in proportion to the quantity of serum. (It is rather peculiar that this behaviour is not indicated in the figures given above.)

Madsen, aided by Walbum and Nugochi, has carried out a large number of experiments on the velocity of reactions of different substances at different temperatures. Their method consisted in determining the qualities of the same agent, *e.g.* hæmolysin, which were necessary to produce a certain effect in a given time, *e.g.* 10 minutes. The lower the temperature, the greater the quantity of the agent necessary for the effect, generally speaking. Taking ammonia as an illustration, we know that, if the quantity exceeds greatly the amount necessary for complete hæmolysis in a very long time, the time necessary to secure a certain effect is inversely proportional to the quantity of ammonia used. If therefore, as the experiments indicate, the addition of 0.085 c.c. of a solution of ammonia to 8 c.c. of a 1 per cent suspension of erythrocrytes from horse blood at 34.8° C. will give in 10 minutes the same effect as 0.17 c.c. of the same solution at 29.7° C., the other conditions being the same, we conclude that 0.085 c.c. of the ammonia would require 20 minutes at 29.7° C. to attain the same effect as in 10 minutes at 34.8°; *i.e.* the velocity of reaction at 34.8° C. is double that at 29.7° C. Generally speaking, the velocity of reaction is in these experiments inversely proportional to the quantity used. Evidently this conclusion is valid only for those cases in which, as for ammonia, the rate of the reaction is proportional to the quantity of reacting substance; but this seems

to be generally the case if a correction be introduced for the first fraction of the poison, which is neutralized in the erythrocytes. This correction, in most cases, seems to be of minor importance.

We may again use the formula—

$$\frac{v_1}{v_0} = e^{\frac{\mu}{2} \frac{(T_1 - T_0)}{T_0 - T_1}},$$

where v_1 and v_0 indicate the velocities at the absolute temperatures T_1 and T_0 and μ is a characteristic constant. Here we have only to replace the velocity, v, by the inverse value of the concentration necessary to produce the desired effect. Experimentally it was found that the results agreed very well with the formula, as shown by the following figures,

AMMONIA, 0.5 NORMAL SOLUTION

$z = $ 10 MIN.			$z = $ 20 MIN.			$z = $ 30 MIN.		
t	$q_{obs.}$	$q_{calc.}$	t	$q_{obs.}$	$q_{calc.}$	t	$q_{obs.}$	$q_{calc.}$
39.5	0.04	0.043	39.2	0.03	0.029	39.5	0.027	0.023
34.8	0.085	0.083	34.8	0.05	0.05	34.8	0.035	0.039
29.7	0.17	0.17	30.2	0.085	0.081	29.7	0.07	0.072
25.9	0.3	0.3	25.7	0.19	0.17	25.9	0.13	0.12
21.0	0.60	0.64	21.0	0.3	0.32	21.0	0.25	0.23
			15.7	0.55	0.69	15.2	0.5	0.5
$\mu = $ 26,760			$\mu = $ 24,360			$\mu = $ 23,020		

$z = $ 60 MIN.			$z = $ 100 MIN.			$z = $ 180 MIN.		
t	$q_{obs.}$	$q_{calc.}$	t	$q_{obs.}$	$q_{calc.}$	t	$q_{obs.}$	$q_{calc.}$
39.2	0.019	0.014	39.1	0.02	0.018	39.0	0.015	0.015
34.8	0.024	0.022	34.6	0.025	0.025	34.7	0.185	0.185
30.2	0.035	0.035	30.7	0.025	0.034	30.9	0.023	0.023
25.7	0.050	0.056	25.9	0.045	0.051	25.7	0.030	0.029
15.7	0.17	0.17	21.3	0.060	0.075	21.5	0.040	0.037
12.2	0.26	0.26	16.1	0.115	0.12	16.1	0.050	0.050
			12.2	0.17	0.17			
$\mu = $ 19,150			$\mu = $ 14,920			$\mu = $ 9,441		

giving the observed concentrations compared with calculated values; z gives the time of reaction, t the temperature, q the quantity added, *i.e.* the inverse value of the velocity v.

As will be seen from these figures the formula may be well used in this case. We observe also that the magnitude μ increases with decreasing time of reaction. With a prolonged reaction-time, the quantities of ammonia used do not exceed very much the quantity necessary for total hæmolysis and then our premises are not fulfilled. Hence the real value of μ, corresponding to the theoretical premises, is that to which the values of μ converge with decreasing time of reaction. It does not seem to differ very much from that valid for $z = 10$ min. The applicability of the known equation from physical chemistry for even longer times renders it highly probable that it would hold for the limit-value, which would be reached in an exceedingly short time of observation, if it were possible to observe it directly. In heterogeneous systems the direct observation of the velocity of reaction meets in most cases with great difficulties, as has been indicated above, so that the indirect observations such as those executed by Madsen, Noguchi, and Walbum are necessary to obtain a knowledge of these phenomena. I therefore reproduce the values of μ found by them for different bases and acids. The interval of temperature was always between about 17 and 39° C. The experiments were arranged in the same manner as those dealing with ammonia.

These figures give occasion to remarks similar to those made regarding the behaviour of ammonia. If we could carry out experiments extended through a very long time,

0.2 n. Sodium Hydrate		0 2 n. Potassium Hydrate		0.1 n. Formic Acid		1 n. Acetic Acid		1 n. Propienic Acid		1 n. Butyric Acid	
Time min.	μ	Time min.	μ	Time min.	μ	Time min.	μ	Time min.	μ	Time min.	μ
10	15,200	10	11,700	10	8,800	10	23,600	10	24,900	10	21,600
20	9,500	20	9,200	20	7,600	15	22,200	20	18,100	20	19,900
30	9,400	30	8,300	30	4,300	20	18,100	30	15,900	30	15,200
40	9,200	40	8,000	40	2,600	40	15,500	40	15,100	40	14,000
50	7,400	60	6,100	50	2,900	50	14,200	60	13,700	50	13,200
60	7,200	120	5,200	180	900	60	13,200	90	7,700		
80	6,900	180	4,100			90	8,800	120	5,100		
100	6,300					120	7,500	180	4,800		
180	5,700					210	5,100				

1 n. Maleinic Acid		1 n. Citraconic Acid		1 n. Itaconic Acid		0.1 n. Oleinic Acid	
Time min.	μ	Time min.	μ	Time min.	μ	Time min.	μ
10	12,700	10	13,500	15	17,000	10	25,800
20	9,100	20	11,700	20	15,600	22	23,400
30	8,300	50	4,400				

the temperature would probably exert a very minute influence. To attain a certain effect, for instance total hæmolysis or the first appreciable trace of hæmolysis, a certain amount of acid or base would be necessary, equivalent to the quantity of erythrocytes present. Madsen and I found the following values (q) of 0.05 n. NaOH or 0.037 n. NH_3 to be the greatest quantities which could

	NaOH		NH_3	
n	$q_{obs.}$	$q_{calc.}$	$q_{obs.}$	$q_{calc.}$
20	0.60	0.60	0.82	0.82
10	0.27	0.30	0.42	0.41
5	0.14	0.15	0.19	0.20
2.5	0.09	0.075	0.11	0.10
1.25	0.047	0.038	0.055	0.05
0.62	0.018	0.019	0.027	0.025
0.31	0.011	0.010	0.014	0.013

be added without producing a sensible effect on 10 c.c. of an n per cent suspension of horse-blood.

The calculated values are obtained under the assumption that 0.6 c.c. of the 0.05 n. NaOH solution, which corresponds to 0.082 c.c. of the 0.037 n. NH_3 solution, are equivalent to 10 c.c. of a 20 per cent suspension in producing the first trace of hæmolysis. This idea of the equivalency is evidently true, though the time of reaction was here only one hour at 37° C. To this corresponds indeed the fact found by Madsen and Walbum that equivalent quantities of the four bases investigated (namely, NH_3, NaOH, KOH, and $Ba(OH)_2$) must be added to the same quantity of blood to produce the first trace of hæmolysis. For 19 different acids the corresponding quantity was in all cases equivalent, but it was double as great as for the bases. The time of reaction was 24 hours at 16.2° C. Here we have evidently not to do with a velocity of reaction, but with a strong chemical binding of the acids or the bases to some substance in the erythrocytes.

The same is true according to the experiments of Madsen, Walbum, and Noguchi[1] even for the other reaction which they observed, which was not far from complete hæmolysis, if we regard the figures for prolonged times and high temperature (37–39°), where the reaction has nearly come to an end. The three bases yield for this end-value: KOH, 0.008; NaOH, 0.008; and NH_3, 0.0075 c.c. respectively of 1 n. solutions; within the errors of observation these quantities are equivalent. For the acids examined we find the following figures (in c.c. of 1 n. solutions): formic, 0.012; acetic, 0.015; propionic, 0.017; butyric, 0.017;

[1] Madsen, Noguchi, and Walbum: *Oversigt*, 1904, No. 6, pp. 425 and 447.

maleinic, 0.013; and citric acid, 0.012. These figures do not differ very much from each other; they are really a little higher for the weaker than for the stronger acids, perhaps indicating a slightly marked hydrolytic effect. But on the whole they indicate a combination, although of not quite so strong a nature as with the bases. For very long times of reaction we therefore observe no real velocity of reaction, but a binding, and hence we might expect that for these long times of reaction the values of μ converge to zero. This convergence takes place much earlier for the strong acids and bases, which react more rapidly, than for the weak ones. Thus, for instance, a dosage of 0.1 c.c. normal NH_3 needs 210 minutes to yield the same hæmolytic effect that the equivalent quantity of the three strong bases examined give in 15 minutes (at 16.2°); and an addition of 0.05 c.c. of dichloracetic acid produces the same hæmolytic effect in 5 minutes as the equivalent quantity of acetic acid in 60 minutes. Therefore even the shortest time (10 minutes) used in these investigations of strong acids or bases seems to be much too long to give a value, μ_0, for μ, which approaches that for an infinitely short time, corresponding to the real value for the velocity of reaction. On the other hand, the values for the shortest times for weak acids do not differ much from each other, and probably also not from the theoretical limit-value, which seems to be about $\mu = 27,000$. A little higher, perhaps, lies the true limit-value for ammonia, $\mu = 29,000$. These limit-values are probably valid even for the other acids and bases. The great difference in the speed of reaction between the strong and the weak bases and acids seems to indicate that we have even here to do with an ionic reaction, although the

differences would be much greater than those observed if the phenomenon were not disturbed by the relatively long time of observation.

Oleic acid differs rather widely from the other acids, exhibiting the same effect as about five times larger quantities of other weak acids. The hæmolytic agent in this acid is therefore probably not only the hydrogen ion, as in other acids, but the undissociated molecules exert also a hæmolytic action.

The lysins of bacterial origin reach the end-value of their hæmolytic effect much more slowly even than ammonia. Hence it is probable that the deduced value of μ for these substances does not differ materially from the theoretical value. I reproduce some figures from Madsen and Walbum's series : —

INFLUENCE OF TEMPERATURE ON THE ACTION OF LYSINS OF BACTERIAL ORIGIN

STREPTOLYSIN (20 MIN.)			VIBRIOLYSIN (20 MIN.)			TETANOLYSIN (60 MIN.)		
Temp.	$q_{obs.}$	$q_{calc.}$	Temp.	$q_{obs.}$	$q_{calc.}$	Temp.	$q_{obs.}$	$q_{calc.}$
36.1	0.08	0.08	35.3	0.17	0.10	30.4	0.40	0.37
31.1	0.20	0.21	29.8	0.25	0.25	25.6	0.50	0.53
25.8	0.40	0.54	26.3	0.4	0.4	21.9	0.80	0.66
22.9	1.30	1.15	20.6	1.0	1.0	15.4	1.00	1.00
						12.1	1.30	1.27
$\mu = 31,900$			$\mu = 27,300$			$\mu = 10,900$		

The values of μ for streptolysin and vibriolysin probably do not differ very much from the limit-value, μ_0. A comparison of the observed with the calculated values seem to indicate rather great errors of observation, which make the value of μ relatively uncertain. For vibriolysin, there

I

are given four values corresponding to the times, 20, 40, 100, and 180 minutes respectively; they are 27,300, 24,400, 19,300, and 15,800 respectively. They seem to indicate that the limit-value lies a little above 27,300, but the calculation would give a better agreement if, in the first instance, μ were fixed at 25,000, which value therefore perhaps comes near to the end-value. The only statement that can be made is that these values are of nearly the same magnitude as those for the hæmolytic action of (weak) acids and bases, and that a closer investigation might bring them into agreement; this would have a physical meaning, namely, that it is probably a change in the condition of the erythrocytes that causes the increase of the reactivity with temperature.

Two other hæmolytic substances have been investigated by the same authors, namely, the oleate of sodium and triolein. They gave, with the same suspension of erythrocytes as was used in the other experiments, namely 1 per cent of red cells from the horse, the following results : —

HÆMOLYSIS BY MEANS OF

0.01 n. SODIUM OLEATE (10 MIN.)			0.01 n. TRIOLEIN (15 MIN.)		
Temp.	$q$obs.	$q$calc.	Temp.	$q$obs.	$q$calc.
36.3	0.125	0.125	38.9	0.17	0.17
31.4	0.14	0.14	35.7	0.20	0.20
24.1	0.18	0.16	30.9	0.32	0.26
15.9	0.19	0.19	24.0	0.40	0.40
12	0.20	0.21			
4	0.25	0.25			
$\mu = 3800$			$\mu = 13,800$		

With regard to the magnitude of μ, the oleate and the

triolein seem to differ from the other lysins. The formula gives the observed results within the limits of the errors of observation.

The change of the agglutinating power with temperature was investigated in an analogous manner. The values for mercuric chloride, ricin, coli- and typhoid-agglutinin are given below for the shortest times used : —

INFLUENCE OF TEMPERATURE ON AGGLUTINATION BY MEANS OF

0.1 n. HgCl₂ (45 MIN.)			RICIN (30 MIN.)		
Temp.	$q$obs.	$q$calc.	Temp.	$q$obs.	$q$calc.
35.8	0.085	0.085	36.2	0.05	0.05
30.9	0.15	0.15	27.9	0.12	0.11
25.9	0.25	0.24	15.6	0.33	0.37
21.9	0.55	0.36	10.1	0.70	0.66
16.1	0.60	0.65	0.9	1.30	1.80
12.3	1.00	0.98			
	$\mu = 17,900$			$\mu = 17,200$	
COLI-AGGLUTININ (10 MIN.)			TYPHOID-AGGLUTININ (10 MIN.)		
Temp.	$q$obs.	$q$calc.	Temp.	$q$obs.	$q$calc.
38.6	0.005	0.0043	38.2	0.002	0.002
34.9	0.0055	0.0072	31.1	0.007	0.0083
30.9	0.015	0.0135	27.4	0.02	0.018
24.9	0.045	0.042	19.2	0.13	0.09
21.2	0.065	0.068	15.9	0.20	0.20
12.9	0.30	0.30	11.5	0.55	0.55
	$\mu = 30,100$			$\mu = 37,200$	

The two agglutinins acting upon equine erythrocytes give remarkably similar values of μ, although the time of reaction is rather long.

The values of μ for longer times seems to indicate that the limit-value of μ for the two bacterio-agglutinins

probably differs little from that given above. They are for the typhoid-agglutinin, $\mu_{40} = 33,000$, $\mu_{120} = 18,700$, and $\mu_{180} = 8500$ (the indices represent the time in minutes). For the coli-agglutinin there are two different series. The one proceeds regularly, so that μ sinks with increasing time. In the other series an irregularity occurs; the μ-value at first sinks, then passes through a minimum and thereafter through a maximum, and then later on falls toward zero with increasing time. The two series were carried out with the same preparations, so that the differences may be regarded as purely accidental. But the limit-value μ_0 seems to be nearly the same in the two series.

The phenomenon of agglutination bears a great similarity to that of hæmolysis in its dependence on time and temperature. We know from Eisenberg and Volk's experiments that the absorption of the agglutinins reaches the state of equilibrium in a few minutes (less than 5). But still the agglutination continues through hours, especially at low temperatures. The phenomenon proceeds with time just as does hæmolysis, although the hæmolytic substance is absorbed before the time of the first observation. The following figures, giving the quantity of coli-agglutinin necessary to reach the observed degree of agglutination at 36.6 and 12.3° C., may illustrate this peculiarity : —

Time (min.)	20	30	50	65	80	120
Q obs. at 36.6	4	1	0.5	0.2	0.13	0.1
Q obs. at 12.3	85	26	10	2	1.6	0.32

The figures indicate that the limit-value for infinite time is nearly reached in 120 minutes at the higher temperature, but not for the lower one; and that this limit-value is of

the same order of magnitude (probably equal) in the two instances. The progress of agglutination with time indicates that after the agglutinin has been absorbed (in the first five minutes) some change (probably coagulation) takes place slowly within the bacteria, which thereby obtain their agglutinating properties. In the same manner, after a hæmolysin has been very rapidly taken up by erythrocytes, these are subject to a slow chemical change, which causes them to give up their colouring matter. This circumstance seems rather incompatible with the commonly adopted idea that the agglutination might depend upon some electric charge of the bacteria, due to the absorption of the agglutinin, since the electric charge very likely follows immediately upon the absorption of agglutinin.

These far-reaching investigations of Madsen and his pupils have also made us familiar with some substances, the action of which increases with sinking temperature, or has a maximum or minimum at a certain temperature.

HÆMOLYTIC ACTION AT DIFFERENT TEMPERATURES

POISON OF WATER-MOCCASIN				COBRA POISON		STAPHYLOLYSIN			
Time, 5 min.		Time, 15 min.		Time, 15 min.		Time, 15 min.		Time, 45 min.	
Temp.	q_{obs}.	Temp.	q_{obs}.	Temp.	q_{obs}.	Temp.	q_{obs}.	Temp.	q_{obs}.
39.3	0.38	39.3	0.28	37.3	0.225	36.5	0.35	36.4	0.35
35.2	0.36	35.2	0.28	30.8	0.25	29.1	0.17	28.6	0.08
30.7	0.35	30.7	0.3	24.1	0.30	24.3	0.23	24.4	0.07
28.2	0.35	28.2	0.33	17.7	0.30	19.5	0.33	19.7	0.075
19.2	0.3	19.2	0.3	14.2	0.25	13.6	1.8	11.3	0.6
14.8	0.3	14.8	0.25	10.8	0.25				
10.6	0.25	10.6	0.23						

As illustrations may be given the hæmolytic action of the snake-venoms of the water-moccasin (*Ancistrodon piscivorus*), and cobra (*Naja tripudians*) and of staphylolysin, produced by staphylococcus.

The snake-poisons were tested on horse-blood, the staphylolysin on rabbit-blood. Probably the observed phenomenon is of complex nature. A maximum effect might result if the hæmolytic agent were decomposed with increasing temperature. At low temperatures, then, where the decomposition is insignificant, the poisons behave normally, the velocity of reaction increases with temperature. If then at higher temperatures the decomposition or dissociation of the poisonous substance increases more rapidly with temperature than the velocity of the real reaction, then the quantity of poison necessary for a given hæmolytic effect (in a given time) must increase with temperature. A closer investigation of these complicated phenomena will be necessary to show whether an explanation analogous to that sketched above may be assumed.

The phenomena studied above in this chapter are, generally speaking, due as well to velocities of reaction as to real chemical equilibria. These prevail the more, the longer the time of reaction and the higher the temperature. The observations have great importance because they correspond to the method of working actually used, which is determined by the nature of the material employed. Hence their discussion is useful for the comprehension of the results of the ordinary method of working, and for the arrangement of similar experiments. Therefore I have discussed them in a rather detailed manner, although their theoretical meaning is not very simple.

Rather similar to these hæmolytic and agglutinating processes in which the chemical attack is directed against cells suspended in the acting medium, are some other processes in heterogeneous systems, namely, the decomposition of coagulated protein, or of small emulsified fat drops by so-called lipases. These processes are not very different from those in homogeneous systems. The coagulated proteins are introduced in the state of a fine powder and the suspension held in uniform concentration in all its parts by shaking. In this manner the digestion of coagulated egg-white by means of pepsin (Sjöqvist) or for the digestion of casein by means of trypsin (Madsen and Walbum) have been examined.

Perhaps the most important of the processes is the digestion of coagulated albumen by pepsin (in the presence of acids). Regarding this process Schütz[1] had found the rule, that the digested quantity of albumen hydrolysed in a given time is proportional to the square root of the time and of the concentration of pepsin. Against the figures of Schütz, which, according to a criticism of Sawjalow,[2] give no very accurate results, he quotes the experiment of Sjöqvist[3] as indicating that the reaction is proportional to the time, *i.e.* follows the laws for a monomolecular reaction. In Sjöqvist's experiment the protein was coagulated egg-albumen in the state of a powder, which was brought in 100 c.c. of a solution at 37° C. containing 50 c.c. of 0.1 n. hydrochloric acid, 2.5, 5, 10, or 20 c.c. of a 0.067 per cent solution of pepsin and water to con-

[1] Schütz: *Zeitschr. f. ph. ch. v. Hoppe-Seyler*, **9.** 557 (1885).
[2] Sawjalow: *Zeitschr. f. ph. ch. v. Hoppe-Seyler*, **46.** 307 (1905).
[3] Sjöqvist: *Skand. Archiv. f. Physiologie*, **5.** (1895).

stant volumes. After a certain time (1 to 20 hours) a sample of the solution was cooled to 0° C., the albumen was centrifugalised, and the content of nitrogen in the liquid determined according to the method of Kjeldahl.

In this manner the digestion was followed. Sjöqvist found that the same quantity (Q) is digested if the time (t) of digestion is inversely proportional to the concentration (c) of the pepsin, as is shown by the following figures:

c	t (hours)	Q
0.5	16	6.90
1	8	6.70
2	4	6.77
4	2	7.00

During the first time there are some deviations. The progress with time is indicated by the following figures ($KP = 52$):—

t (hours)	$Q_{obs.}$	$Q_{calc.1}$	$Q_{calc.2}$
0.5	2.25	3.14	2.18
1	3.16	3.47	3.00
2	4.08	4.08	4.04
3	4.72	4.64	4.78
4	5.24	5.15	5.35
6	6.10	6.04	6.21
8	6.84	6.77	6.84
10	7.38	7.39	7.35
12	7.84	7.90	7.76
16	8.54	8.67	8.39
20	8.94	9.21	8.83
30	9.39	9.93	9.43
40	9.85	10.21	9.90
∞	10.40	(10.40)	(10.40)

The calculated figures $Q_{calc.1}$ are found from the common formula for the monomolecular reaction, those signed $Q_{calc.2}$ from the formula given above for the digestion of

dissolved egg-albumen. As will be seen, the latter formula agrees very closely with the experiments, and much better than the common formula for monomolecular reactions. This indicates that the digestion follows the same laws in both cases.

In experiments on digestion there is often used a method of including the coagulated albumen in short capillary tubes, so-called tubes of Mett. By the aid of this method Borissow found the rule of Schütz to be valid, if a solution of acid and pepsin diffused into the tubes; whereas Sawjalow, who mixed the pepsin with the albumen, found then that the height of the digested albumen-pillar was proportional to the quantity of pepsin, and not to the square root of it, as Borissow had found. As is seen above, the results of Sjöqvist agree in the most satisfactory manner with the values $Q_{calc.2}$, which for short times coincide with values calculated from Schütz's rule. It should be emphasised that experiments with Mett's tubes ought not to be used in investigating these questions. For in them the rate of diffusion interferes with the chemical reaction, and if it is the slower of these processes, it causes the digestion to proceed proportionately to the square root of the time and concentration. An analogous experiment may be made with diffusion of alkali into a Mett's tube filled with an acidulated jelly solution plus phenolphthalein. At a given time (t) the concentration of alkali in the Mett's tube surrounded by a solution of the concentration 2 is at a given point double as great as at the corresponding point in a Mett's tube surrounded by an alkaline solution of the concentration 1. Now the height to which the alkali in a given concentration has reached

after a certain time (t) is proportional to its square root. Therefore at the time $\dfrac{t}{\sqrt{2}}$ the distribution of alkali in the first tube is nearly the same as the distribution of alkali in the second tube after the time t. This may be seen in the height of the pink-coloured column of jelly which is the same in the two cases. Experiments with the diffusion of pepsin are of quite the same type. The indicator is in this case not the reddening phenolphthalein, but the liquefying solution of albumen. — One measures the length of the liquefied albumen-pillar at a given time. — Therefore Pawlow's and Borissow's experiments, which indicate that the liquefaction of coagulated albumen or gelatin goes on proportionally to the square root of time or of reacting enzyme (pepsin, trypsin, etc.), are not conclusive in favour of Schütz's rule.

A process which is very analogous to the digestion of egg-white is that of the saponification of fats by means of gastric juice or of extracts of gastric tissues (steapsin). Volhard[1] used glycerine extracts of the pig's stomach and observed that the quotient, $q : \sqrt{f} = k$, of the digested quantity q (in per cent), and the square root of the concentration of steapsin, f, is nearly constant. This ob-

GLYCERINE EXTRACT (24 HOURS)			GASTRIC JUICE (1 HOUR)		
f	q	k	f	q	k
1	4.7	4.7	1	4.1	4.1
4	8.5	4.3	4	11.2	5.6
9	15.0	5.0	9	17.3	5.8
16	19.5	4.9	16	21.3	5.3
25	24.5	4.9			

[1] Volhard: *Zeitschr. f. klin. Medicin*, **42**. 414, and **43**. 397 (1901).

servation was confirmed by Stade,[1] as may be seen from the above figures (temp. 40°).

If the digestion proceeds further than 25 per cent the rule of Schütz is no longer applicable. We then may use the general formula (p. 64) as is seen by the next figures valid for digestion by gastric juice during 16 hours at 40° C. $(KP = 27)$: —

f	$q$obs.	$q$calc.
1	21.6	21.6
4	40.7	39.6
9	50.9	54.6
16	67.4	66.7

Stade has further done a series of measurements of the progress of saponification of an emulsion of the fat from the yolk of egg by neutralised gastric juice. These measurements follow nearly the same formula as those of Sjöqvist, as may be seen from the calculated values.

PROGRESS OF SAPONIFICATION OF YOLK OF EGG BY MEANS OF GASTRIC JUICE

TIME (hours)	$q$obs.	$q$calc.	TIME (hours)	$q$obs.	$q$calc.
2	20.4	18.6	12	24.1	24.5
4	25.6	25.7	16	25.4	27.9
6	29.8	30.8	21*	28.5	31.4
8	35.3	34.8	37*	39.5	40.0
10	37.6	38.3	39*	39.8	40.9
25*	49.5	55.2	43*	41.7	42.6
29*	51.5	58.2	46	46.2	43.8
31*	55.4	59.6	65	53.6	50.2
35*	60.9	62.0			
75	77.5	78.4			

[1] Stade : *Hofmeisters Beiträge*, **3**. 291 (1902). The dates with an asterisk are the means of two measurements.

The constants, KP, are respectively 10 and 3. For short times the digested quantity is proportional to the square root of the time, so that if as well time, t, as quantity, f, of steapsin change, the digested quantity is proportional to the square-root of the product of $f \cdot t$. Even this rule has been verified by Stade. Stade's work has been repeated by Engel[1] for the glycerine extract of " pancreatin Rhenania " as well as for gastric juice, acting on an emulsion of egg-yolk. The following figures reproduce two series of observations, the one at 30°, the other at 40° with 1.24 times as much extract of pancreatin. The time of digestion was 6 and 18 hours respectively. From these observations we may calculate the variation of K with temperature. We so find $\mu = 15,600$.

$t = 30°$ C.			$t = 40°$ C.		
$f = 1$	$p_{obs.} = 4.7$	$p_{calc.} = 5.0$	$f = 1.24$	$p_{obs.} = 12.8$	$p_{calc.} = 14.1$
4	10	9.8	4.96	24.7	26.8
9	15	14.6	11.16	39.5	38.2
16	19.5	19.0	19.84	51.6	48.2
25	22.3	23.4	31.0	59.5	57.1
36	24.4	27.6	44.64	63.1	64.8
	$K = 1.3 : 6$			$K = 8.9 : 18$	

Stade has furnished some measurements on the influence of the concentration (c) of gastric juice at 40° C. on this process, which we reproduce below. The tabulated figures concern the hydrolysed quantity in per cent (p).

[1] Engel: *Hofmeisters Beiträge*, 7. 77 (1905).

	TIME OF ACTION 1 HOUR		TIME OF ACTION 4 HOURS		TIME OF ACTION 9 HOURS		TIME OF ACTION 16 HOURS		TIME OF ACTION 25 HOURS	
c	$p_{obs.}$	$p_{calc.}$	$p_{obs.}$	$p_{calc.}$	$p_{obs.}$	$p_{calc.}$	$p_{obs.}$	$p_{calc.}$	$p_{obs.}$	$p_{calc.}$
1	4.1	6.2	13.7	12.1	19.2	17.8	21.6	23.2	31.5	28.4
4	11.2	12.1	28.0	23.2	39.8	33.3	40.7	42.5	46.0	50.7
9	17.3	17.8	38.9	33.3	49.4	46.7	50.9	58.0	65.6	67.6
16	21.3	23.2	44.0	42.5	52.9	58.0	57.4	70.3	73.6	79.6
25	23.6	28.4	45.4	50.7	63.3	67.6	68.4	79.6	74.1	88.1

If we calculate the constants for the five series, we find : —

$$1.8 \quad 10.7 \quad 21 \quad\quad 27 \quad\quad \text{and} \quad 41 \quad \text{or}$$
$$1.1,8 \quad 4.2,7 \quad 9.2,3 \quad 16.1,7 \quad \text{and} \quad 25.1,64$$

or in mean the constant is $2\,t$. With this constant the calculated figures are obtained. The observed values agree in general as well as might be expected with the calculated ones, and probably the deviation is not greater than might be referred to the experimental errors. (Perhaps real or so-called false equilibria disturb the observations for high values of p.)

The seeds of *Ricinus communis* (castor bean) contain an enzyme which decomposes the oil from these seeds into fatty acid and glycerine. This process seems also to follow the same laws as the decomposition of fats by gastric juice. I reproduce some figures from the article of Connstein, Hoyer, and Wartenberg.[1] They prepared an emulsion by grinding 5 g. of Ricinus seeds and 6.5 g. of castor oil, and thereafter adding 4 g. of an acid. The

[1] Connstein, Hoyer, and Wartenberg: *Berichte d. d. chem. Ges.*, **35**. 3988 (1903).

emulsion was held at constant temperature and at given times samples were removed and titrated for their free acid. (The acid initially added was subtracted.) The first figures concern an emulsion in 0.1 n. sulphuric acid, the later figures are found for emulsions in 0.1 and 0.4 n. acetic acid.

SULPHURIC ACID 0.1 N.			ACETIC ACID 0.1 N.			ACETIC ACID 0.4 N.		
Time (min.)	$p_{obs.}$	$p_{calc.}$	Time (hr.)	$p_{obs.}$	$p_{calc.}$	Time (hr.)	$p_{obs.}$	$p_{calc.}$
15	12	20	1	50	48.6	1	65	63.9
30	20	27	2	65	62.8	2	86	78.8
45	30	32	3	70	71.5	3	84	86.5
60	33	36	4	72	77.6	4	84	91.2
90	41	43	24	80 (?)	99.5	24	91 (?)	99.9
150	54	53						
210	59	59						
330	68	69						
1620	81 (?)	97						

The constants are 1.47 for the first, 180 and 380 respectively for the later experiments.

The deviation of the figures for the later times seems to indicate that we have here to do with either real equilibria in the presence of lipases acting on ethylic butyrate, monoglycerid of butyric acid, and triacetin, and known from Kastle and Loewenhart's, Hanriot's, and A. E. Taylor's investigations,[1] or some perhaps false equilibrium. These are very common with enzymes, as Tammann (*l.c.*) found. Even the figures for less than 45 minutes indicate a deviation from the premises of the calculation. There are also

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[1] Kastle and Loewenhart: *Amer. Chem. Journ.*, **24.** 491 (1900); Hanriot, *C. R.*, **132.** 212 (1901); A. E. Taylor: *Journ. Biol. Chemistry*, **2.** 87 (1906).

some observations regarding the influence of the quantity of enzyme. With 0.5 g. of Ricinus seed were emulsified 5, 10, 15, 20, 25, or 50 g. of Ricinus oil and the same quantities of 2 per cent acetic acid. The results are indicated in the table below. The calculated figures are found under the assumption that the acting mass of the enzyme is proportional to its quantity. The agreement between calculated and observed values is very satisfactory, except for the greatest quantities. The constant for two days is, within the errors of observation, double that for one day. The concentration in the presence of 50 g. oil and 50 g. acid is taken as unit.

ACTION OF 0.5 G. RICINUS SEEDS ON DIFFERENT QUANTITIES OF RICINUS OIL

Oil	Acid	AFTER 1 DAY		AFTER 2 DAYS	
		$p$obs.	$p$calc.	$p$obs.	$p$calc.
50	50	49	49	49	59
25	25	60	63	74	74
20	20	71	69	80	78
15	15	77	75	87	84
10	10	81	83	86	91
5	5	89	94	92	98
		KP = 186		KP = 300	

In this case the presence of a certain quantity of free acid, about 4 c.c. of a 0.2 normal solution, the same quantity for all acids, weak or strong, is necessary. This circumstance resembles very closely the action of acids in peptic digestion. If no acid is added, the acid in the seeds acts, and new acid is procured by the process.

A lipolytic process, which probably should be regarded as occurring in a homogeneous system, is that studied by

Zeller [1] on the lipolytic agent in the mushroom (*Amanita muscaria*). An aqueous extract of the powder of this mushroom had no lipolytic action. But if the powder were mixed with olive oil or tallow, they were slowly decomposed. I reproduce some figures for olive oil. In this case the reaction is decidedly monomolecular (the constant used for the calculation is 0.00045). The acid from the olive oil evidently has no sensible influence on the enzyme.

Time (hours)	48	118	160	304	485	631
Decomposition (per cent)	4.8	11.5	14.2	28.5	38.9	46.3
Decomposition (calc.)	4.8	10.9	15.3	27.0	39.5	48.0

Regarding the lipolytic action of the cytoplasma of the seeds of *Ricinus communis*, a large number of experiments have been carried out by Nicloux.[2] He found that the catalytic agent is not soluble in water, which seems to arrest its action. The conditions of the reaction seem, therefore, to have been nearly the same as in the experiments of Zeller. The reaction proceeds (at low temperatures) very closely according to the law valid for monomolecular reactions. The cytoplasma was suspended in the oil examined, in most cases cotton oil, and thereafter water containing a small quantity of acetic acid was added. The following figures, valid for 18° C., indicate that the process is monomolecular : —

Time (min.)	30	45	60	90	127	150	210	450
Saponification, per cent (x)	23.6	33.1	40.4	54.8	67.0	73.2	85.5	94.4
$K = \dfrac{100}{t} \log \dfrac{100}{100-x}$	0.388	0.387	0.375	0.382	0.392	0.381	0.399	0.278

[1] Zeller: *Sitz. ber. d. Ak. d. Wiss. zu Wien* (chemical memoirs = *Monatshefte für Chemie*), **36**. 727 (1905).

[2] M. Nicloux: *C. R. de la Soc de. Biol.* **56**. I. 701, 702, 839, 840, and 868 (1904).

At higher temperatures, above 25° C., the constant K decreases during the period of reaction, as will be seen from the following figures, which give the mean values of $K.10^{-4}$ during the time intervals 30–90 and 90–180 minutes. From these an extrapolated value for the time 0, the beginning of the reaction, is calculated : —

Temp.	5	10	15	20	25	30	35	40	45
0 min.	7.7	12.5	14.6	20.6	26.6	30.3	40.1	34.4	27.2
30–90 min.	9.0	12.6	13.7	19.3	24.2	26.6	29.7	19.2	10.5
90–180 min.	6.3	12.4	15.4	21.9	21.5	22.6	20.4	9.1	3.2
$60\dfrac{d \log K}{dt}$	—	—	—	—	0.041	0.057	0.130	0.259	0.413

K has a maximum at about 37° C. This evidently depends upon the rapid destruction of the catalytic agent at higher temperatures. Nicloux has found that at 55° C. its action is totally nullified in ten minutes. This destruction is measured by the decrease of $\log K$ during the progress of the saponification, if the process is monomolecular, as indicated by the values found at lower temperatures. At temperatures below 25° C. this decrease is insensible; at higher temperatures the destruction of the enzyme proceeds at about double the speed with increase of the temperature of 5° C. This corresponds to a value of $\mu = 26,000$.

The extrapolated value of K itself at the time 0 shows a remarkably small increase with temperature. An increase of about 14° C. is necessary to increase it in the proportion 2 to 1. The value of μ calculated from the figures for 10° and 30° C. is only 7540.

This investigation affords a good insight into the real meaning of optima. If we regard the time 0, the optimum

K

seems to occur at about 37° C.   In the interval, 30–90 min., the optimum seems to lie at about 33° C., and in the interval 90–180 min. at about 29° C.   The position of the optimum evidently depends on how rapidly the experimental manipulations are done.   If it were possible to examine K immediately after mixing the reagents, the optimum would probably be found to lie at a much higher temperature; or there would perhaps be no optimum at all.

It is worthy of mention that the dry cytoplasma suspended in pure oil resists a temperature of 100° C. for twenty hours; at 120° C. its activity decreases one-third in a quarter of an hour.

Quite recently A. E. Taylor[1] has carried out an investigation on the action of the lipase from the castor bean on the triglycerid of acetic acid, commonly termed triacetin. This compound is rather soluble in water, so that homogeneous solutions were prepared containing 0.5, 1, or 2 per cent (up to 3 per cent) of triacetin together with 1 g. of the powder of castor beans, from which the fat had been extracted, in 100 c.c. of water.   The following values were obtained, indicating a monomolecular process.   The velocity-constant is $K = \dfrac{1}{t} \log \dfrac{A}{A - x} \cdot 10^4.$

The agreement between the three values of K indicates that the decomposed quantity is very closely proportional to the quantity of the substrate.   The regularity of this process is evidently higher than that of any other fermentative action hitherto studied.   The velocity of reaction increased in the proportion of 1 to 2.6 if the temperature

[1] A. E. Taylor: *Journ. Biol. Chemistry*, **2**. 87 (1906).

was elevated from 18° C. to 28° C. This corresponds to a value for μ of 16,700.

SAPONIFICATION OF TRIACETIN BY MEANS OF LIPASE FROM THE CASTOR BEAN AT 18° C. (A. E. TAYLOR)

t (hours)	0.5 PER CENT		1 PER CENT		2 PER CENT	
	$A-x$	K	$A-x$	K	$A-x$	K
0	100	—	100	—	100	—
4	90.4	109	91.7	94	90.2	112
8	83.8	96	82.6	104	82.6	104
16	71.3	92	66.2	112	67.7	106
24	58.2	98	58.2	98	56.9	102
28	51.1	104	51.2	104	49.8	108
32	52.3	88	45.8	106	51.5	90
40	37.7	106	39.1	102	40.5	98
48	34.8	97	34.5	96	36.4	91
Mean		99		102		101

Taylor thereafter investigated a suspension of triolein (2 per cent) in water containing 1 g. of castor bean powder in 100 c.c. The liquid was shaken vigorously. He found the following values of $A - x$ for the days, termed t.

SAPONIFICATION OF A 2 PER CENT EMULSION OF TRIOLEIN AT 18° C. BY LIPASE FROM THE CASTOR BEAN (TAYLOR)

t (days)	0	1	2	3	4	5	7	9	11	15	18
$A-x$	100	97	95	92.5	91	89	84	78.4	72.8	66.9	61.9
$x:t$	—	3	2.5	2.5	2.3	2.2	2.3	2.4	2.5	2.4	2.1

Here evidently the transformed quantity (x) is, as the last line indicates, proportional to the time. This is easily explained. Through the strong shaking the water is a concentrated solution of triolein, its content of triolein is constant, independent of time. Therefore, as the concen-

tration of the ferment is also unaltered in time, the quantity of triolein decomposed in the unit of time is constant, and the total quantity proportional to the time of reaction. We might perhaps at first expect, with Taylor, that the velocity of reaction might increase about in the proportion 1 : 2.6 for an increase of 10° in temperature; instead of this Taylor found the proportion 1 : 1.2. This will be easily understood if the solubility of the triolein diminishes in about the proportion 1 : 2.2 for an elevation of 10° C. The velocity of reaction will then increase in the proportion $1 : \dfrac{2.6}{2.2} = 1 : 1.2$, as found by Taylor. Taylor's conclusion from the low value 1.2 that we do not observe a velocity of reaction but of some other process, such as diffusion, seems not very convincing.

Kastle and Loewenhart[1] had already (1900) found that the velocity of reaction of monobutrin with animal lipase is proportional to the concentration of the ferment.

Here we observe evidently a very simple monomolecular process. New investigations on the different results of Connstein, Hoyer, and Wartenberg seem therefore very desirable.

Taylor found that the process is reversible, the ester may be formed from glycerine and fatty acid under the influence of the lipase. The equilibrium is reached very slowly and does not differ sensibly from that attained under the influence of an acid. The following end-values were obtained by means of normal sulphuric acid and lipase (after several months) :—

[1] Kastle and Loewenhart : *Amer. Chem. Journ.* 24. 491 (1900), as cited by Taylor.

EQUILIBRIUM IN SOLUTION OF TRIACETIN AT 18°C. (A. E. TAYLOR)

CONCENTRATION per cent	HYDROLYSIS BY MEANS OF		
	H_2SO_4	Lipase	Calc.
0.5	88	86	88
1.0	82	79	80.1
2.0	78	70	69.8

The calculated values are found according to the law of Guldberg and Waage, under the assumption that the first figure (88) is correct. As will be seen, the figures found for the action of lipase agree better with the theory than those for the catalytic action of the sulphuric acid. As probably the same end-value is reached, be the catalytic agent sulphuric acid or lipase, we conclude that the spontaneous hydrolysis of triacetin would reach the same end-value, and that the catalysor only accelerates the process, as is generally assumed and especially set forth by Ostwald.

This circumstance, that equilibria are reached under the action of ferments, so that the reaction may be carried in the one or in the other direction, is a direct illustration of the fact that we may apply the laws of physical chemistry to biological science. The first discovery that ferments may synthetise as well as decompose was made by Hill[1] in 1898 and excited a very great interest. He found that it is possible to synthetise a maltose (according to Emmerling,[2] it is an isomaltose) from glucose by means of yeast, according to the formula : —

$$2\,C_6H_{12}O_6 \rightleftarrows C_{12}H_{22}O_{11} + H_2O.$$
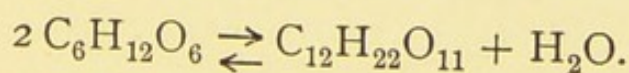
[1] Hill: *Journ. Chem. Soc.*, **73**. 643 (1898).
[2] Taylor: University of California Publications, *Pathology*, Vol. I., 33 and 65 (1904). For references on literature see this last memoir.

The sign $\rightleftharpoons$ indicates that the decomposition of the disaccharide by means of yeast is also possible, and this is the normal process. In a similar manner it is possible to reverse this reaction under the influence of sulphuric acid as the catalysor, as was shown by Musculus, Wohl, and Fischer.

Fischer and Armstrong synthetised isolactose by means of kephir yeast (lactase) from d-glucose and d-galactose.

Kastle and Loewenhart, as well as Hanriot, accomplished the synthesis of ethyl and monoglyceryl butyrate by means of animal lipase; and Taylor synthetised olein-triglycerid by means of lipase from the castor bean, but he did not succeed in reversing tryptic digestion.[1] Emmerling added a maltase from yeast to a mixture of glucose and nitril mandel glucoside and recovered amygdalin after the lapse of three months. As is seen from these illustrations, the reversibility of processes accelerated by ferments is a normal phenomenon.

All the experiments cited which give a constant value of qt seem to indicate that even here the reagents diffuse into the small particles of the substance examined with such a speed that the time of diffusion may be regarded as very low compared with the time of reaction. For the erythrocytes and bacteria this may be easily understood, because their linear dimensions are very insignificant (cf. p. 25). This may even be the case for the

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[1] Quite recently experiments on the synthetic action of trypsin or pepsin seem to have been followed by success. Taylor (Univ. of Calif. Publ., *Pathol.* I, No. 9, 1907, p. 343) synthetised protamin from its products of decomposition by means of trypsin, and T. Brailsford Robertson (Univ. of Calif. Publ., *Pathol.* III, No. 9, 1907, p. 59) synthetised paranuclein from its products of decomposition by subjecting them to the action of pepsin.

finely powdered coagula of egg-white or the emulsified fat drops in Volhard's, Stade's, and Engel's experiments. But with the pillars of coagula this cannot be said to occur. In this case we must suppose that the liquefied part of the column is carried away into the liquid about, so that this is able to attack new portions of the pillar. Here, therefore, the reaction is going on at the surface of the coagulated substrate, and immediately below it, and the constancy of the product qt shows that under constant conditions the number of molecules digested in one second is proportional to the concentration of the ferment in the surrounding liquid (and to the surface, which remains nearly constant).

Madsen and Walbum have investigated the progress of dissolution of casein by means of trypsin. Ten g. of casein in the form of the powder were suspended in 100 c.c. of a 1 per cent solution of trypsin and held at constant temperature. In order to prevent the casein from subsiding, the flask containing the mixture was steadily shaken. After different time intervals samples were removed, and the undissolved quantity of casein determined by means of its content of nitrogen — determined according to the method of Kjeldahl. The progress of dissolution is shown by the following table (temperature, 34.1°) : —

Time (hours)	Nitrogen (obs.)	Nitrogen (calc.)	Time (hours)	Nitrogen (obs.)	Nitrogen (calc.)
0	0.11	0.11	48	0.060	0.058
0.5	0.108	0.109	72	0.0486	0.0464
2.5	0.102	0.105	101	0.0374	0.0376
6	0.100	0.099	125	0.0329	0.0325
11	0.096	0.091	168	0.0274	0.0269
24	0.076	0.076	192	0.0236	0.0236
33	0.070	0.068			

The nitrogen (calc.) is calculated according to the equa-
tion for a bimolecular process, and, as will be seen, the
agreement is very satisfying.   The relation which, after
Bayliss's experiment, is very unexpected, may be regarded
as a purely empirical one.   The reaction-constant is 0.173.
It increases with temperature, as might be expected.   At
37° C. it reaches the value 0.194, corresponding to an in-
crease in the proportion 1.485 : 1 in an interval of 10° C.
($\mu = 7400$).

Many of the most important processes of normal life
occur in heterogeneous systems.   As is seen from the fol-
lowing examples, they behave as regards the velocity of
reaction quite like enzymic processes.   All of them pos-
sess an optimum at about 40°–50° C., and in the vicinity of
0° C. the velocity of reaction sinks rapidly with tempera-
ture.

The most important of these processes are the phenom-
ena of assimilation and of respiration of carbonic acid by
plants.   As van't Hoff[1] remarks, the observations of
Clausen[2] seem to indicate that in the interval of tempera-
ture 0°–25° C. the quantity of carbonic gas given off by
seedlings of wheat, lupins, and flowers of syringa increases
with temperature in the proportion 1 : 2.5 for an increase
of 10 degrees ($\mu = 14,800$).   The three processes show an
optimum at 41, 38 and 42° C. respectively.

Godlewski[3] showed that the assimilation of a leaf of
four different plants — the most regular results were those

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[1] Van't Hoff: *Vorlesungen über theoretische und physikalische Chemie*, 2d
ed., 1. 224 (1901); E. Cohen: *Vorträge f. Arzte*, p. 43 (1901).

[2] Claussen: *Landwirtsch. Jahrb.*, 19. 892 (1890).

[3] Godlewski: *Arbeiten des botanischen Instituts in Würzburg*, 1. 243
(1872).

of *Typha latifolia* — increases with the content of carbonic acid in the surrounding air, and nearly proportionally with it, up to about 2 per cent. An optimum occurs at about 6 per cent. At further increase the assimilation decreases, but very slowly. This process was recently studied by Miss Gabrielle Matthaei,[1] who found that the assimilation of carbonic acid by a leaf of *Prunus Laurocerasus* increases with temperature in the following manner (for a leaf of 50 cm.^{2} and in one hour) : —

TEMP.	ASSIMILATION MILLIGRAMMES OF CO_2	
	obs.	calc.
0	1.75	1.75
10	4.2	3.79
20	8.9	7.81
30	15.7	15.3
37	23.8	23.8

The process is in all points analogous to the action of ferments. The chlorophyll may here be regarded as the acting ferment. The value of μ is 11,940. In these figures we observe already a greater increase at lower temperature than the formula indicates. If the temperature falls below 0°, the assimilation sinks very rapidly, so that at − 6° C. the observed figure is only 0.2 mg., corresponding to a value of μ between 0 and −6° C., about ten times greater than between 0 and 37. Above this latter temperature again the assimilation sinks with rising temperature until the leaf dies. This evidently depends on a destruction of the functions of the chlorophyll at very low or at higher temperatures.

[1] Gabrielle Matthaei: *Phil. Trans. Roy. Soc.*, Ser. B, **197**. 47 (1904). Cf. Kanitz: *Zeitschr. f. Elektroch.*, No. 42 (1905).

Similar observations may be made regarding the growth of eggs of animals after their fertilisation, as observed by Hertwig and Karl Peter.[1] The development of these eggs was followed by means of observations of the cell-division which went on much more rapidly at higher than at lower temperatures. Even chemical influences, especially a small addition of hydroxyl-ions to the sea water, are often very remarkable. The eggs of Arbacia develop a little more rapidly in sea water containing 2 c.c. of 0.1 n. alkali in 100 c.c. of water than if no alkali has been added. An equivalent amount of HCl retards the development.[2] The increase (I) for increase of the temperature 10° C. and the corresponding value of μ are given below. They are valid for a mean temperature of about 16° C. : —

Eggs of *Echinus microtuberculatus* —	First stage	$I=2.29$	$\mu=13,700$
	Later stages	2.03	11,700
Eggs of *Sphærechinus granularis*	First stage	2.30	13,800
	Later stages	2.08	12,100
Eggs of *Rana fusca* (Hertwig)	First stage	2.23	13,300
	Later stages	3.34	20,000

As we see, μ is of quite the same order of magnitude as that for the production or assimilation of carbonic acid by green plants. Further, at low temperatures (3–5°C.) the μ for the eggs of the frog has a very high value — about ten times those given above; and at the temperatures of over 37° C. the life process is hindered by increase of temperature. In a similar manner behaves the heart-beat of

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[1] Hertwig: *Archiv f. mikrosk. Anatomie*, **51**. 319 (1898); Karl Peter: *Archiv f. Entwicklungsmechanik*, **20**. 130 (1905).

[2] J. Loeb: *Archiv f. Entwicklungsmechanik*, **7**. 631 (1898). Cf. above, the influence of alkalies and acids on the velocity of destruction of lysins.

the Pacific terrapin (*Clemmys mamorata*), according to observations of Charles Snyder.[1] His observations give the following values for the number of heart-beats in one minute : —

Temp.	0	2.5	5	10	15	20	25	30	35	37.5	40
Number	0.75	3.2	4.9	7.8	9.7	20.7	27.3	46	48.6	48.2	43.3
Do. calc.	2.4	3.2	4.2	7.0	11.4	18.5	29.3	46	71	87.7	106

μ is found to be 16,060. As is seen from the figures, the action of the heart has an optimum. This circumstance probably depends upon changes in the protoplasms near $0°$ and $50°$; in the latter case coagulation occurs.[2] Therefore the calculated values coincide with the observed ones only within a certain interval, 2.5–30 degrees. The rapid increase in the neighbourhood of the freezing point is very similar to that found for the two other phenomena of life studied above.

Another process of great practical importance caused by living cells is the production of alcohol and carbonic acid from glucose by yeast plants. This process has been investigated very thoroughly by Aberson.[3] He found that the formula of Henri is valid for this reaction, as will be seen from the following figures, in which the quantity of glucose $(A - x)$ was determined by means of a polarimeter. A is the beginning concentration, $A - x$ the corresponding quantity after t minutes. The temperature was for the first series $17.5°$, for the second $27°$ C.

[1] Charles D. Snyder: University of California Publications, *Physiology*, 2. 125 (1905).

[2] Loeb : *Vorlesungen über die Dynamik der Lebenserscheinungen*, p. 155 (1906).

[3] Aberson: *Rec. d, trav, chim. l, Pays-Bas et de la Belgique*, 22. 78 (1903).

PRODUCTION OF ALCOHOL BY MEANS OF YEAST

t	$A-x$	$K=\dfrac{10^5}{t}\log\dfrac{A+x}{A-x}$	t	$A-x$	$K=\dfrac{10^5}{t}\log\dfrac{A+x}{A-x}$
0	30.7	—	0	30.5	—
43	29.9	26.3	41	28.3	76.5
107	28.7	26.5	86	25.8	78.5
196	27.1	26.1	149	22.6	77.3
269	25.7	26.5	240	18.2	77.2
393	23.5	26.4	300	15.8	76.1
432	22.8	26.5	357	13.5	76.5
512	21.4	26.5	493	11.7	77.5
590	20.5	26.3	514	9.6	76.5
613	19.7	26.5	529	8.1	78.1
790	17.1	26.2	752	4.1	76.0

The mean values are 26.85 and 77. From these we calculate the value of $\mu = 15,607$. By means of this value Aberson calculated his other experiments, and found an excellent agreement with the observations. As illustration the following figures may be cited : —

TEMP.	$K_{obs.}$	$K_{calc.}$
15.4	31.2	31.2
21.0	55.6	52.4
27.0	90.0	89.2
32.0	139.8	136.8

In many cases the quantity of yeast was augmented by cellular multiplication during the experiment; in such cases a correction for this circumstance was applied. The quantity of glucose, as well as that of the reaction-products, had an influence upon the constant, whereby the deviation of the velocity of reaction from that valid for monomolecular reactions may be explained.

The production of alcohol and carbonic acid from glucose is, as Buchner showed, not a peculiarity for the living cell, as Pasteur had supposed, but may be performed by dead yeast-cells or an extract from them called zymase. With dead yeast-cells (Herzog)[1] and with zymase (Euler)[2] experiments have been carried out,—in the latter case the reaction occurs in a homogeneous medium. Euler followed the progress of the reaction by measuring the quantity of carbonic acid developed, or with the polarimeter the destruction of the sugar. The quantity of glucose was 20 c.c. of 1 n. solution. The quantity of zymase used was 3.6 g. in the first, 1.2 g. in the second series.

PRODUCTION OF ALCOHOL FROM GLUCOSE BY MEANS OF ZYMASE

OBSERVATIONS OF THE PRODUCED QUANTITY OF CO_2			OBSERVATIONS WITH THE POLARIMETER		
Time, t (min.)	$A - x$	$K = \frac{10^4}{t} \log \frac{A}{A-x}$	Time, t (min.)	$A - x$	$K = \frac{10^4}{t} \log \frac{A}{A-x}$
0	790	—	0	1075	—
198	709	2.37	81	1018	2.95
237	700	2.21	124	990	2.90
323	672	2.17	224	930	2.81
413	644	2.15	324	877	2.73
			355	860	2.73
			381	851	2.67
			406	841	2.63
			730	700	2.55

Here we find a slow decrease in the values of K and not an increase, in which case the formula of Henri might be applicable. It is therefore probable that the agreement of Henri's formula with the measurements of Aberson is

[1] Herzog: *Hoppe-Seylers Zeitschr.* **37.** 149 (1903).
[2] Euler: *Hoppe-Seylers Zeitschr.* **44.** 53 (1905).

due to some perturbation introduced by the life-processes of the yeast-cells. Euler, however, also observed some complications which require additional experimentation before they can be explained. The velocity of reaction increases more rapidly than the concentration of the enzyme, and not proportionally to a simple power of it. The quantity decomposed does not increase with the concentration of the sugar, but shows on the contrary a relative decrease, if the concentration of sugar is raised.

The study of the velocities of reactions in heterogeneous systems indicates that they behave very nearly in the same manner as in homogeneous systems. This observation has often been made concerning the velocity of reactions in heterogeneous systems.[1] It depends upon the circumstance that by means of the experimental arrangements the diffusion goes on so rapidly that it does not perturb the chemical processes. If capillary tubes are employed, this cannot be said to be the case, and therefore Mett's tubes should not be used for quantitative measurements.

Even the influence of temperature on these reactions is of the same order of magnitude as for those processes in which different substances react with one another in homogenenous systems. Remarkable is the low value for the hæmolysis by means of sodium oleate ($\mu = 3800$). The agglutination of erythrocytes by means of mercuric chloride and ricin give nearly the same values for μ (17,900 and 17,200) and the values for μ for the two different bacterio-agglutinins are not of very different order ($\mu = 30,100$ for coli-agglutinin, $\mu = 37,200$ for typhoid-agglutin). Near this value also stands that for hæmolysins of bacterial

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[1] Cf. Goldschmidt : *Zeitschr. f. physikal. Chemie*, **31**, 235 (1899).

origin (streptolysin, $\mu = 31,900$; vibriolysin, $\mu = 27,300$; for tetanolysin the time of reaction has been too long to yield correct value for μ). Close to these values are the highest ones of those for the hæmolytic action of acids and bases (ammonia, $\mu = 27,000$; propionic acid, $\mu = 25,000$). The values of μ for these substances have already been discussed at length above.

For comparison I tabulate the other values of μ given in this chapter : —

Digestion of powdered casein by means of trypsin . . . . $\mu = 7,400$
Saponification of emulsion of yolk by means of pancreatic juice $\mu = 13,600$
Respiration of different plants (mean value) . . . . . . $\mu = 14,800$
Production of alcohol by means of yeast-cell . . . . . . $\mu = 15,600$
Assimilation process in plants . . . . $\mu = 12,000$
Cell-division in different eggs (mean value) . . . . . . $\mu = 14,100$
Heart-beats of the Pacific terrapin . . . . $\mu = 16,060$

The μ values are in general of the same order of magnitude as in the case of homogeneous reactions. The great similarity of the values of μ for the different life-processes would scarcely seem to be accidental.

CHAPTER V

EQUILIBRIA IN ABSORPTION PROCESSES

THE most simple of all processes belonging to this domain seems to be the absorption of agglutinin by the corresponding bacteria. If we add an agglutinin to a suspension of bacteria (whether living or dead makes little difference), the bacteria clump together and fall to the bottom of the container. If after the sedimentation of the bacteria, we decant the supernatant fluid and again determine its agglutinating power, we find that it has decreased in large measure. We conclude, therefore, that a large fraction of the agglutinin has been absorbed by the bacteria. Eisenberg and Volk have studied this phenomenon on a rather large scale. The two following tables give their results with the serum of a horse that had been injected with typhoid bacilli, and that of another horse that had been injected with cholera vibrions. Different concentrations of these agglutinin-holding sera — prepared by dilution with physiological sodium chloride solution — were brought in contact with the same quantities of suspensions of typhoid bacilli or of cholera vibrios. The quantity of agglutinin added is given in the column headed T, the quantity of agglutinin absorbed by the bacteria is found under C; the quantity remaining in the liquid is called $B_{obs.}$ Evidently $B_{obs.} + C = T$. In the next column is tabulated the figures for $B_{calc.}$, calculated in a manner to be indicated below.

ABSORPTION OF TYPHUS AGGLUTININ BY TYPHOID BACILLI

T	C	$B_{obs.}$	$B_{calc.}$	K	
2	2	0	0.02	—	
20	20	0	0.7	—	
40	40	0	2.1	—	
200	180	20	19.7	24.4	
400	340	60	52.9	22.6	Mean
2,000	1,500	500	478	23.7	24.7
10,000	6,500	3,500	3,890	28.2	
20,000	11,000	9,000	9,160	25.4	

ABSORPTION OF CHOLERA AGGLUTININ BY CHOLERA VIBRIOS

T	C	$B_{obs.}$	$B_{calc.}$	K	
2	2	0	0.03	—	
20	20	0	1.0	—	
40	38	2	2.8	24	
67	60	7	6	16.4	
200	120	80?	27	(6.5?)	Mean
2,000	1,300	700	620	16.5	19
11,000	6,500	4,500	5,260	23.9	
20,000	10,000	10,000	10,750	21.5	

As is evident from these figures, on the addition of a small quantity of agglutinin it is absorbed (nearly) totally; $B_{obs.}$ is found to = o. Indeed, the method of observation does not permit the observation of values of B below $B = 1$. On the further addition of agglutin the absorbed fraction decreases continuously until at the end the absorbed fraction is not greater than the non-absorbed fraction. It has been hitherto often assumed that the agglutinin was chemically bound in the bacteria. To carry out this idea consequentially, we must suppose that the resulting

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compound is a highly dissociable one. For otherwise the oft-observed washing out of the agglutinin from this compound in the bacterium would be inexplicable. And, furthermore, the absorption of the agglutinin ought to be total until saturation was obtained, and thereafter only a very slight increase (due to the physical absorption) ought to be observed.

But even if we suppose the compound to be dissociable to a high degree, we might expect that the bound fraction of agglutinin (C) should increase to a limit value with increasing concentration of B (and T). As Eisenberg and Volk remark, no such limit can be observed in the results of their experiments. Even the assumption that the agglutinin contains many different kinds of "agglutinin" of different combining powers does not extricate us from this difficulty.

In favour of the hypothesis that the agglutination is the consequence of a chemical combination, Joos has adduced some experiments on the effect of partial additions of agglutinin to a suspension of typhoid bacilli. He determined the least dosage of agglutinin that is able to agglutinate all the bacteria in the test. Then he added a part of this quantity to a similar suspension; the agglutination was incomplete. He centrifugated the solution and in this way segregated the agglutinised bacteria and removed them. Then he added a new portion of agglutinin to the solution, and so forth, until all the bacteria had been agglutinated. The total quantity of agglutinin added in the several fractions in this manner was found to be equal to the quantity necessary to agglutinate all the bacteria when added at once. Considering the great experimental errors

in such observations, the conclusion does not seem to possess a very high degree of accuracy. And even if this were the case, it is very probable that the quantities of bacteria separated out in the first fractions were rather small, and under such circumstances it was evidently to have been expected that the total quantity of agglutinin in the two experiments would be nearly the same.

Now in regard to the figures of Eisenberg and Volk, it is evident that a relation exists between the absorbed quantity of agglutinin C and the free quantity B. This relation may be expressed in a very simple mathematical formula, namely : —

$$C = KB^{\frac{2}{3}}.$$

With the aid of this equation the calculated figures $B_{calc.}$ are found. They agree very well with the observations, within the errors of observation, as Dr. Eisenberg also has stated. The only observation in the second series which gives an unsatisfactory agreement between the observed and calculated values is where $B_{obs.} = 80$ and $B_{calc.} = 27$. Eisenberg and Volk themselves state in their original memoir that this observation must be influenced by some occasional error.

The physical interpretation of the above formula is very simple. It states that the agglutinin molecules are divided between two solvents, the bacterial cells and the surrounding medium, and that of two molecules of the free agglutinin are formed three molecules of the absorbed agglutinin.

In the experiments of Ransom cholesterin is a solvent for saponin, and the existence of cholesterin in the red blood-corpuscles causes the entrance of the hæmo-

lytic substance saponin into them, following which the red blood-corpuscles are poisoned, so that they give up their colouring matter, the hæmoglobin. Now in a similar manner the bacterial cells contain some substance that is a good solvent for the corresponding agglutinin, which is thereby caused to enter the bacteria to a preponderating extent. The molecular weight of the agglutinin in the bacterial solvent is only two-thirds of the molecular weight of the agglutinin in the surrounding fluid, the physiological salt solution.

This behaviour of the agglutinin molecules in two solvents recalls vividly the behaviour of benzoic acid in two different solvents, water and benzene. According to determinations of the freezing point of solutions of benzoic acid, this has in aqueous solution the molecular weight 122, corresponding to the formula C_6H_5COOH; but in benzene its molecular weight is double that. Therefore, if we dissolve benzoic acid in water, and shake this solution with benzene, the concentration of the aqueous solution C_a is related to the concentration of the benzene solution C_b as indicated in the following formula : —

$$C_a = KC_b^{\frac{1}{2}}$$

where K is a constant factor. Nernst verified this equation by experiments on the distribution of benzoic acid between water and benzene.

The great velocity of absorption is in good agreement with our interpretation as stated above.

A difficult thing to explain is the specificity of the agglutinins. The agglutinin produced by injecting typhoid bacilli into the blood of an animal is only absorbed by

typhoid bacilli and not by other bacteria, for instance not by cholera vibrios, and *vice versa.* Probably the cell-membranes of typhoid bacilli are only permeable to typhoid agglutinins, but not to other agglutinins. Normal sera contain different agglutinins against bacteria and red blood-corpuscles; by shaking them with the corresponding bacilli or cells, it is possible to separate the different agglutinins. Thus Malkoff mixed goat-serum, that agglutinates red blood-corpuscles from man, rabbits, and pigeons, with red blood-corpuscles from rabbits. The centrifugalised serum had lost its agglutinating power for rabbit's erythrocytes, but not for the two other varieties.[1]

The agglutinins lose their agglutinating power spontaneously, but much more rapidly at high than at low temperatures (cf. pp. 87 and 91). Treatment with different chemical agents, as hydrochloric and other acids, bases, formol, and urea, weakens them. The details of these circumstances have not been closely investigated.

Just as agglutinins are absorbed by bacteria and red blood-corpuscles, so in the same manner other different substances are absorbed by these cells, and probably analogous regularities are manifested in these cases. Thus tetanolysin, ricin, and the different immune bodies are absorbed by red blood-corpuscles; and it seems to be a general law that only such substances as are absorbed by these cells exert an influence upon them. Whether the cells are living or dead, seems, as we observed regarding the absorption of agglutinins, to be quite immaterial if the killing of the cells has been cautiously accomplished, so that no notable chemical changes have occurred.

[1] Malkoff: *Deutsche med. Wochenschrift,* 1900.

Morgenroth and I examined the absorption of an immune-body, prepared by the injection of red corpuscles of ox-blood into the veins of a rabbit. Another series of Morgenroth's experiments was carried out with inactivated (heated) serum from a goat injected with sheep's erythrocytes. Different solutions of the immune-bodies — their strengths in an arbitrary unit are tabulated under T — were treated with a constant quantity of erythrocytes from ox or sheep for about one hour at low temperature and then centrifugalised. The centrifugated liquid was examined for its concentration, B, of immune-body, by the addition of normal serum of the guinea-pig, and measuring the hæmolytic power of the hæmolysin produced. The difference, C, was absorbed by the red blood-corpuscles. Beside the observed figures, $B_{obs.}$, for the quantity of free immune-body, are written figures for $B_{calc.}$, calculated by the aid of the same formula as that found to be valid for agglutinins. The constants, K, of the formulæ were calculated to be 18.3 and 39.5 respectively.

ABSORPTION OF IMMUNE-BODIES BY RED BLOOD-CORPUSCLES

SERUM FROM A RABBIT INJECTED WITH BOVINE ERYTHROCYTES				SERUM FROM A GOAT INJECTED WITH ERYTHROCYTES FROM SHEEP			
T	C	$B_{obs.}$	$B_{calc.}$	T	C	$B_{obs.}$	$B_{calc.}$
250	226	24	39	200	189.5	10.5	10.5
330	275	55	57	400	374	26	28.8
670	500	170	151	800	723	77	78.4
1,330	850	480	376	1,600	1,384	216	211
2,700	1,710	990	942	3,200	2,400	800	550
5,000	3,070	1,930	2,050	6,400	5,230	1,170	1,420
10,000	5,800	4,200	4,800	12,800	9,420	3,380	3,570
16,700	7,820	8,880	8,870				
33,000	13,900	19,100	19,700				

As will be seen from the figures, the agreement between the observed and the calculated figures is just as good as for the agglutinins, and wholly within the possible errors of observation. The same physical explanation evidently holds good for both phenomena.

In some recent investigations bearing upon the absorption of coli-agglutinin in the bodies of the *Bacillus coli communis*, Dreyer has found that not only the constant K, but also the exponent n in the equation $C = KB^n$ may turn out different in different experiments. n always falls near unity, sometimes it exceeds it, *e.g.* in one case n was found to be 1.25. A closer investigation regarding the cause of this variability seems very desirable.

This case has a certain theoretical significance. Quite recently the opinion has often been ventured that the absorption of agglutinin by bacteria might be analogous to the so-called adsorption of dissolved substances by charcoal, or of colouring matter by organic tissues. Bordet was the first to make this assumption, which has recently been upheld by Wilh. Biltz. Biltz now states that in the theoretically hitherto little elucidated adsorption-process n is always lower than 1; for absorption by charcoal it is 0.25, according to Schmidt. If therefore n is sometimes found to exceed 1, as in Dreyer's work, we have to abandon the adsorption hypothesis. Biltz, Much, and Siebert for two hours shook typhoid agglutinin with the following colloidal bodies : silicic acid or hydroxids of iron, zircon, and thorium. It was found that silicic acid had a noticeable and the three other substances a much greater destructive action on the agglutinin. This seems to indicate that we have here to deal with a real chemical influence, which is not

astonishing, as many different substances destroy agglu-
tinins. For absorption it is, on the contrary, generally
possible to show that the absorbed bodies (*e.g.* dyes) exist
on or in the absorbing substance, from which they may
often be washed out. The authors have tried in vain to
poison animals by the injection of hydroxid of iron which
had been shaken with diphtheria poison or tetanospasmin,
which, just as agglutinins, are attenuated by such shaking.
If these poisons had been absorbed like agglutinins by the
bacteria, *i.e.* in a reversible way, then a strong poisonous
effect should have manifested itself in the injected animals.
But not a trace of the expected effect was observed. Biltz,
Much, and Siebert were then led to the conclusion that
the hypothesis of absorption is not tenable.

They have therefore taken up an idea incidentally sug-
gested by Nernst for the explanation of the neutralisation
of toxins by their antibodies. This idea is not very differ-
ent from that of Behring. (Cf. p. 29.) Let us suppose
we have finely divided colloidal platinum (Bredig's "an-
organic ferment") and hydrogen peroxid. The peroxid
condenses upon the fine metal particles and thereafter it is
decomposed. This would correspond to the condensation
of a toxin, *e.g.* ricin, on the colloidal particles of its anti-
body, antiricin, and its subsequent decomposition. The
antiricin itself should be slowly attacked by the ricin,
just as the platinum, if it were oxidisable by the hydrogen
peroxid. This explanation is incompatible with the fact
that the ricin can be recovered after it is "neutralised,"
therefore the neutralisation cannot depend upon its de-
struction. It seems that the advocates and adherents of
this idea (the schools of Nernst and of Ehrlich) had an

intuition that it would conflict with experience.   Evidently it agrees with the experiments in the shaking of poisons with colloidal hydroxids (except in that these substances are not chemically attacked by the poisons), but it does not harmonise with our experience with the reactions of bacteria to their agglutinins.

It may also be emphasised that the solutions of antitoxins do not behave as suspensions, as is seen in their diffusibility in jelly, which is not observed with suspended particles.

There is another phenomenon which we encounter in the use of some kinds of agglutinins, *e.g.* such as have been weakened by acids, or other chemicals, or by heat. These modified agglutinins display an increase in their agglutinating power until a maximum of agglutination is reached.   Thereafter new additions of agglutinin diminish the effect and at a high concentration of the agglutinin the agglutination ceases.   A similar behaviour is characteristic of many salts.   Without the presence of some salts in the solution, no agglutination takes place, and the same is also true for very high concentrations of the salts.   With increasing concentration of the salt (above the optimum value) a greater quantity of agglutinin is necessary to produce agglutination, and at a concentration of the salt above a certain limit value the agglutination fails. On small additions of salts the agglutinated quantity seems to be proportional to the added quantity, which might be expected on the basis of most of the hypotheses regarding the nature of agglutination, and not only from the chemical one, as Joos supposes.

In the theory of immunity we find many analogous

cases where maxima or minima of a certain effect are observed at a certain concentration of the reacting substance. One of the most startling of these phenomena is observed on injection of a mixture of the botulismus-poison produced by Bacillus botulinus, and its antibody, obtained from the blood-serum of animals that have been injected with this poison. Madsen used, for instance, a mixture of 0.1 c.c. with 0.0013 c.c. of its antitoxin. The injection of ten such doses in a guinea-pig (of 250 g. weight) had no effect; two doses gave just a trace of illness characterised by a peculiar flaccidity of the animal; one dose caused a flaccidity lasting four to seven days. Injections of between 0.013 and 0.5 doses had a lethal effect, and the maximal toxicity was shown by a 0.1 dose, the animal dying after only two days; 0.01 dose was no longer lethal, but caused flaccidity lasting seven days; and 0.003 dose only a trace of flaccidity lasting only one day.

Another similar instance with a minimum and a maximum of effect is shown in hæmolysis by means of saponin (Madsen and Walbum), an extract from the roots of Saponaria; 8 c.c. of a suspension of 1 per cent of erythrocytes from horse blood was added to the following quantities of a 0.02 per cent solution of saponin with water up to a total quantity of 10 c.c. This mixture was placed for three hours at 37° C. and thereafter cooled on ice and the degree of hæmolysis in per cent measured on the following day after the unattached erythrocytes had subsided.

Quantity of saponin in c.c. =	1	0.7	0.5	0.4	0.3	0.25	0.2	0.17	0
Hæmolysis in per cent =	35	16	18	30	36	36	**45**	41	0

Similar observations were also made with mixtures of sa-

ponin and cholesterin, which acts as an antitoxin against saponin.

Even tetanolysin sometimes gives such maxima of effect for a certain concentration, if the time of action is relatively short. After a prolonged action the maximum disappears. This maximum seems therefore to be related to the velocity of the reaction and not to the final equilibrium. Probably the same would occur also with the saponin if its action could be prolonged for a sufficient time.

Reactions of this kind are often explained as due to the presence in the mixture of two substances of inverse effect. Thus it is supposed that agglutinin treated with acids, etc., contains, besides the real agglutinin, a substance called agglutinoid, which hinders the agglutination. At higher concentrations the bacilli are supposed to absorb chiefly the agglutinoid and not the agglutinin ; at lower concentrations both are supposed to be absorbed. This explication seems to me to have no advantage, to mean no more than the relation of the simple fact itself, besides being much more difficult to remember. Furthermore, it seems more simple to suppose that the reacting substance exerts two different actions on the cells, of which the one, appearing at higher concentrations, hinders the other, prevailing at lower concentrations. Such a behaviour is not rare in common chemistry. Thus, for instance, the addition of alkali to a solution of aluminium chloride gives a precipitate of aluminium hydrate, which is dissolved on further addition of alkali.

In a brochure dealing with the properties of colloids Biltz[1]

[1] Biltz: Göttinger Nachrichten, math.-phys. Klasse 1904, I, *Zeitschr. f. ph. Ch.* **48.** 615 (1904).

recalls attention to the oft-observed fact that in general positive colloids (which wander in the electric field in the same manner as cations) precipitate negative colloids (which wander in the same direction as anions under the influence of the electric current). This reminds one somewhat of the specificity of agglutinins. As we shall see later on, Henri accepted this idea, but later he found it disproved by experiments on agglutinins. Biltz found, further, that in the precipitation of colloids optima are found. Thus, for instance, the addition of 1.62 mg. oxid of zircon gave a more abundant precipitate with 1.4 mg. gold in colloidal solution than greater or less quantities; 3.25 mg. ZrO_2 gave no precipitate at all. In the same manner behaved 4 mg. thorium oxid (as colloidal hydrate) with a colloidal solution containing 5.5 mg. SO_2S_3.

In this point also the colloids present analogies with common dissolved inorganic substances.

Analogous effects are observed as especially characteristic of precipitins, and we will return to this special question when we later consider them.

Regarding the influence of salts upon agglutination, we possess a thorough investigation of Bechhold. He used typhoid bacilli, which were cultivated in bouillon and thereafter killed with formalin and washed by repeated suspension in distilled water, and followed by subsequent centrifugation. These bacteria were in some experiments used in their natural state. One c.c. of a suspension was mixed together in a test-tube with 1 c.c. of the salt solution under investigation. The test-tube was placed for 24 hours in an incubator at 37° C. and its content then examined. The degree of agglutination was tested by

looking through the tube against a printed paper and expressed corresponding to one of the four following degrees: Liquid completely clear, perfect agglutination; Most bacteria subsided, but liquid not wholly clear, strong agglutination; Some bacteria subsided and agglomerated, no clearing of the liquid, weak agglutination; No appreciable change of the liquid, no agglutination.

In other experiments the bacteria used had been treated with serum containing agglutinin, nitrate of lead, ferric-sulphate, alcohol, acids, or uranium acetate (which all agglutinate them), and thereafter thoroughly washed until the wash-water did not show any reaction of the agglutinating substances. The bacteria treated in these different manners may be called sero, lead, iron, alcohol, acid, and uranyl bacilli respectively. The bacteria had then been altered so that they behaved in a different way to saltsolutions than did the original bacteria. On the addition of sulphuretted hydrogen to the bacteria treated with lead, these were coloured black, which proves that the bacteria had retained the lead in spite of the washing. For a comparison Bechhold[1] examined the behaviour of suspensions of mastic, prepared by adding some drops of an alcoholic solution of this material to water (so-called "α mastic"), or some drops of water to the alcoholic solution ("β mastic"). The experiments on the agglutination of this last emulsion were done at ordinary room temperature, as the suspended matter would dissolve at 37°.

In the first place an influence of the time of reaction was noted. Suspensions of sero-bacilli mixed with 0.05, 0.025, or 0.012 normal solutions of sodium chloride or

[1] Bechhold: *Zeitschr. f. ph. Ch.*, **48**. 385 (1904).

sodium iodide were completely agglutinated in 50, 90, and 120 minutes respectively; normal bacilli are not agglutinated at all by these solutions. On the other hand, sero-bacilli are less affected than normal bacilli by some solutions, for instance 0.0033 n. silver nitrate or 0.005 n. hydrochloric acid. After a time of 15, 50, and 1440 minutes these preparations gave the following results:—

	Normal Bacilli			Sero-Bacilli			
	15′	50′	1440′	15′	50′	1440′	
Treatm. with 0.0033 n. AgNO₃	weak	perf.	perf.	no	weak	perf.	Agglutination
Treatm. with 0.005 n. HCl	weak	perf	perf.	no	weak	perf.	

These and other similar experiments indicate that the agglutination requires time (cf. p. 116). Further, a certain concentration (limit-value) of the salt-solution is necessary to give an appreciable agglutination. The following table gives the limit value in 0.001 n. of the concentration for different salts. The sign ∞ indicates that even the strongest salt-solutions did not agglutinate. The quantities of salt necessary for the first trace of agglutination have the greatest value for α-mastic, then come β-mastic and normal bacilli, and after them sero-bacilli, which are the most sensitive to salts. The salts of alkalies, alkaline earths, and magnesia exert little or no influence on common bacteria. The agglutinating power is greater for salts of trivalent metals than for those of divalent metals. A very strong influence is exerted by the acids, especially the strong ones. The anions seem to exert very little influence.

PREPARATION	α MASTIC	β MASTIC	NORMAL BACILLI	SERO-BACILLI	PREPARATION	α MASTIC	β MASTIC	NORMAL BACILLI	SERO-BACILLI
KOH	∞	—	∞	∞	$MgSO_4$	100	—	∞	2.5
NaCl	1000	10	∞	25	$Mg(NO_3)_2$	100	—	—	—
NaI	—	—	—	25	$CaCl_2$	50	—	∞	4.5
$NaNO_3$	—	—	—	25	CaN_2O_6	50	—	—	—
Na_2SO_4	—	50	—	50	BaO_2H_2	50	—	25	25
PbI	—	—	—	25	$BaCl_2$	50	5	∞	5
$HgNO_3$	1.25	—	1	0.5	BaN_2O_6	50	—	—	—
$AgNO_3$	125	—	25	1	$ZnSO_4$	100	—	10	1
					ZnN_2O_6	50	—	—	—
HCl	10	1.25	1	0.5	$CdSO_4$	25	—	10	1
H_2SO_4	10	—	1	0.25	CoN_2O_6	50	—	—	2.5
CH_3CO_2H	500	—	1	1	NiN_2O_6	50	—	—	2.5
o-Amidobenzoic acid	—	—	5	5	$Ni(C_2H_3O_2)_2$	25	—	25	2.5
$Al_2(SO_4)_3$	0.5	—	0.25	0.25	PbN_2O_6	5	—	2.5	0.1
$Al(NO_3)_3$	0.5	—	—	—	$CuCl_2$	10	—	2.5	1
$Fe_2(SO_4)_3$	0.5	—	0.5	0.1	$CuSO_4$	10	—	2.5	—
$Fe(NO_3)_3$	1	—	—	—	CuN_2O_6	5	—	—	0.5
$FeCl_3$	1	—	—	—	$Cu(C_2H_3O_2)_2$	5	2.5	—	—
$PtCl_4$	10	—	2.5	0.5	$HgCl_2$	∞	—	2.5	0.5

The salts of (univalent) mercury and silver differ rather widely from those of other univalent ions. Probably they form nearly insoluble chemical compounds with the albuminous matter.

In other experiments Bechhold investigated the retarding influence of small additions of gelatin, serum, gum arabic, extract of typhoid bacilli or of leeches on mastic emulsion. Gelatin did not exert such an influence on normal bacilli, sero-bacilli, or lead bacilli. The alcohol, acid, or uranyl bacilli display properties lying between those of normal bacilli and of sero-bacilli, but their agglutination was lowered by the addition of gelatin. Probably the addition of gelatin or serum covers the suspended par-

ticles of mastic with a thin film, after which the particles behave as if they consisted of drops of gelatin or serum.

Recently there have been many efforts made to place in parallel the properties of egg-white and its derivatives, as peptons or albumoses, with those of inorganic colloids, which after all consist of suspensions of particles of ultra-microscopic magnitude. These are generally precipitated by the solution of very small quantities of salts in the suspending water, and they assume an electric charge on contact with the water, whereby they wander to the one or to the other pole of a battery submerged in the solution. The solutions of egg-white and its derivatives seemed at first to behave in the same manner. But as Bechhold says, the albumoses, etc., do not behave otherwise than common solutions. The same is evidently true of different albuminous substances, according to the recent investigations of Pauli.[1] He subjected ox or horse serum to dialysis for a very long time, six to eight weeks. This serum did not migrate with or against the electric current, and it was not precipitated by weak solutions of alkaline salts, nor of salts of zinc, copper, iron, mercury, or lead. It was coagulated by strong heat, alcohol, and strong solutions of alkali salts or zinc sulphate. These sera contain two different kinds of protein, albumin and globulin. These could not be separated by means of the current, so that neither of these substances was carried by the current. The great difference between the so-called organic colloids and the inorganic or true colloids caused Pauli to express the opinion that we should not attempt to deduce the properties of organic from those of the inorganic colloids,

[1] W. Pauli: *Hofmeisters Beiträge*, 7. 531 (1906).

but to confine our studies to the albuminous substances if we wish to understand the processes going on in living matter.[1]

One of the properties of albuminous substances which was regarded as proving their colloidal nature was precisely the migration of these substances in the electric field. Dialysed serum, says Pauli, does not migrate, but if we add an acid, the egg-white follows the positive current; if we add an alkali to the solution, it travels to the positive side. This circumstance is explained by the adherents of the colloidal theory by the assumption that the egg-white absorbs positive H ions or negative OH ions. As Bredig, Freundlich, and Loeb[2] have remarked, this follows at once from the well-known fact that proteins are amphoteric electrolytes, which form salts as well with acids as with bases, just as do the amido-acids, *e.g.* glycocoll, with which substances the albumins are even very closely related according to the recent investigations of Kossel and E. Fischer. It is quite true that, just as ammonia by adding an hydrogen ion forms the ammonium ion, in quite the same manner the amido-group of the albuminous substance adds hydrogen and gives an albumin ion. But there is a very great difference between an ion and the suspended particles (*e.g.* of kaolin) which obtain a positive change through the influence of dielectric forces. That the albuminous substance really so behaves, that it adds a hydrogen ion and gives an albumin ion, — *i.e.* that the addition of an acid to it gives rise to a real neutralisa-

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[1] Pauli : *Naturw. Rundschau,* p. 3 (1906).

[2] Cf. Loeb : *Vorlesungen über die Dynamik der Lebenserscheinungen,* pp. 65–67, Leipzig (1906).

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tion phenomenon like that of ammonia, — is evident from Pauli's investigations. On the addition of acid the wandering of the albumen to the cathode at first increases with the quantity of acid added, and then reaches a maximal value. The stronger hydrochloric acid has a greater action than the weaker acetic acid. Evidently the albuminous substance has the character of a weak base and therefore the formation of salt is greater if we use hydrochloric than if we use acetic acid, and a limit of the wandering is reached when the substance is practically neutralised.

In an analogous manner the sera investigated by Pauli behave with alkalies. The wandering to the anode increases with the quantity of alkali added, until a limit is reached. In this case evidently no hydroxyl ions are annexed to the albuminous substance, but instead its "acid" carboxyl group gives off a hydrogen ion and is thereby itself charged negatively as an albuminate ion.

Pauli found that neutral salts do not give a charge to the serum, but KH_2PO_4, which has an acid reaction, and Na_2HPO_4, Na_3PO_4, $NaHCO_3$, and Na_2CO_3, which have an alkaline reaction, exert an influence like a weak acid or as weak bases. This is quite clear as long as we regard the albuminous substances as amphoteric electrolytes; but if we regard them as colloidal particles, we cannot predict anything with respect to the influence of these substances. It is quite incomprehensible how this latter view has been so often preferred.

The behaviour of albumose, pepton, and of egg-white as bases has been very thoroughly investigated by Sjöqvist,[1]

[1] Sjöqvist : *Skandinavisches Archiv f. physiol. Chemie,* Bd. V., p. 59 (1895).

who employed for this purpose his method of study-
ing the conductivity.   Sjöqvist stated that these substances
behave quite regularly as weak bases.   The "mean"
equivalent weights of these three substances were found to
be about 600, 250, and 800 respectively.   The egg-albumen
was a base about six times weaker than aspartic acid,
but about nine times stronger than urea.   The albumose
(from Schuchardt) was about 1.6 times as strong a base
as the albumin.   Sjöqvist investigated the neutralisa-
tion of these bases by means of different acids as hydro-
chloric, sulphuric, phosphoric, and lactic acid, and found
always a good agreement with the theoretical view.   The
amphoteric properties of these substances have also been the
basis for investigations on the action of pepsin and trypsin
by Sjöqvist, Bayliss, and others (cf. pp. 65 and 79 above).

A property of the antibodies, which recalls the tendency
of inorganic colloids to precipitate only (or chiefly) colloids
of the opposite electric sign, is their specificity.   But
whereas the positive colloid ferric hydrate is attacked by
all the many negative colloids, the typhoid bacilli are agglu-
tinated only by typhoid agglutinin.   Victor Henri has, in
association with his students, M. Malloizel and Mme.
Girard-Mangin,[1] carried out some experiments on agglutina-
tion from this point of view.   He found that red blood-
corpuscles and typhoid bacilli are, as most substances
suspended in water, charged negatively, and therefore con-
cluded that these cells would be agglutinated by ferric
hydrate, as Biltz had predicted.   He found that this really

[1] V. Henri et L. Malloizel : *C. R. de la Soc. de Biol.*, 56. I. 1073 (1904) ;
Mme. Girard-Mangin et V. Henri : *C. R. de la Soc. de Biol.*, 56. II. 866,
931, 933, 935, 936, 974 (1904).

occurs, and that normal serum or even dissolved starch has a somewhat protecting influence against the agglutination. But later on M. Henri and Mme. Girard-Mangin investigated the influence of negative colloids, and found that they had precisely the same agglutinating influence as the positive ones. The "colloidal theory," therefore, has proved of little avail.

That proteins are able to combine with positive ions, not only hydrogen, but even potassium, sodium, or calcium, has, according to the investigations of Pauli and Loeb,[1] an extremely great importance for the physiological functions of the proteins.

We have seen in the chapter on the velocity of reaction that this is for agglutinins proportional to the concentration of the agglutinin and increases with temperature. From this we concluded that the agglutination depends on a chemical reaction of the agglutinin with some content of the bacterium. In his excellent work on microbiology Duclaux[2] shows that this chemical action is really a coagulation. He cites the experiments of Kraus, which indicate that the filtrate obtained by means of a Chamberland filter from cultures of cholera vibrios, typhoid or pest-plague bacilli, give a coagulum with their specific agglutinins. He cites further the experiments of Nicolle, who subjected a filtrate from macerated coli bacilli to an agglutinin obtained by injecting these bacilli into the veins of a rabbit. In a mixture of ten drops of the filtrate with one drop of the rabbit's serum there appeared after some hours at 37° a large number of flocculent bodies that resembled to a very

[1] J. Loeb : "Studies in General Physiology," Part II, 544, Chicago, 1905.

[2] Duclaux : "Traité de microbiologie," T. II, p. 706, Paris, 1899.

high degree the flocculation in a culture containing living coli bacilli treated with the same agglutinin. One might replace the bacilli with some fine inert powder, *e.g.* talcum; this fine powder would then, just like the bacilli, be jammed together by the coagulum in their neighbourhood. Therefore the deposits containing bacilli are more voluminous than those of the fluids from which the bacilli are removed by filtering. The liquid which is coagulated by the agglutinin is according to Nicolle very resistant to low and high temperatures. It is soluble in alcohol and to a certain degree in ether, so that an extract from the bacilli in one of these fluids yields, after evaporation to dryness and dissolution in a weakly alkaline bouillon, a flocculent deposit on treatment with its agglutinin.

This coagulable substance is prepared in the interior of the bacilli which contain it, and it is even partially given up to the surrounding medium, as is indicated by the experiments of Kraus. Agglutinins are contained in normal sera, as for instance in the horse, the blood-serum of which agglutinates cholera vibrios to a high degree, and less effectively cultures of *Vibrio Metschnikovi*, typhoid bacilli, colon bacilli, and tetanus bacilli as well as streptococcus. The normal sera of different animals agglomerate the normal erythrocytes of other animals, as, for instance, the serum from the horse agglutinates erythrocytes from guinea-pigs or rabbits. In these cases, just as with the agglutinins acting upon microbes, it is possible to increase the content of the specific agglutinin in the normal serum by repeated injections of the erythrocytes in question.

Even simple chemical reagents cause an agglutination of bacilli, thus, for instance, typhoid bacilli are agglutinated

by formaldehyde, hydrogen peroxid, or strong alcohol. In this case the reagents often display the phenomenon that a greater dosage does not agglutinate, whereas a lesser dose produces the effect. Acetic acid does not agglutinate some cholera vibrios if it be used in a concentration of 0.1 per cent, but has a strong agglutinating effect in solutions of 10 per cent only to lose it again at a strength of 50 per cent, according to the experiments of Bossaert. Mercuric chlorid agglutinates in a concentration of only 0.3 per cent and safranin or vesuvin are active at a concentration of 0.05 per cent. Even in this case different bacilli are sensitive to a different degree; thus, for instance, typhoid bacilli are more than ten times as sensitive to safranin as colon bacilli.

If the action of the agglutinin be a coagulation of the contents of the bacilli, it is easy to understand how other coagulating substances, such as alcohol, acids, or uranium acetate, may change the properties of the bacilli in nearly the same manner as the specific agglutinin, as results from the experiments of Bechhold cited above. The agglutinins are therefore probably only a special class of precipitins, which they in many cases even resemble in showing optima of action at a certain concentration.

CHAPTER VI

NEUTRALISATION OF THE HÆMOLYTIC PROPERTIES OF BASES AND OF LYSINS OF BACTERIAL ORIGIN

THE simplest hæmolytic agents are the bases and the acids. Of these the bases exert an action on erythrocytes, which is very similar to that of hæmolysins of bacterial origin. It is obvious that if we add an acid to an alkaline solution until it is neutralised, its hæmolytic action will be nullified. In some few cases, as for instance for oleic acid, this is not true, since all the olein derivatives are hæmolytic agents, which salts in general are not.

This case presents the closest analogy to the neutralisation of a lysin, *e.g.* tetanolysin, by means of its antilysin. At first sight there seems to be a difference, since any base is neutralised by any acid, whereas the antitetanolysin is a perfect specificum against tetanolysin and exerts no neutralising action on other lysins. But this difference is more apparent than real, since we know now that the acids all contain hydrogen ions which bind the hydroxyl ions, common to all bases.

It therefore seemed to Madsen and myself to promise much for the elucidation of the phenomenon of neutralisation of lysins by their antilysins to make a comparative study of the common neutralisation of a base, regarded as a hæmolytic agent, with that of a lysin and its antilysin. For this purpose we made a thorough investiga-

tion of the action of bases, acids, and salts on erythrocytes and of the hæmolytic action of a lysin, namely, tetanolysin, in the presence of its antilysin and of different other so-called neutral substances, as salts and different proteins. The tetanolysin was chosen because it shows a great similarity in its neutralisation with that practically most important of all poisons, namely, the diphtheria poison. This investigation convinced us that a complete analogy holds for the two phenomena of neutralisation, that of a base, for instance, ammonia, and that of tetanolysin.[1]

If we add different bases, for instance, ammonia and sodium hydrate, to red blood-corpuscles, we find that the first traces of alkali are without any hæmolytic effect on the cells. This first ineffective quantity seems to be bound by the blood-corpuscles rather strongly, since it is, within the errors of observation, proportional to the quantity of blood used, and the quantities of sodium hydrate and of ammonia bound by the same quantity of blood-corpuscles are chemically equivalent (cf. p. 110). On the addition of greater quantities of alkali a very weak hæmolysis occurs; the fluid is only faint yellow. As the quantity of alkali increases, the hæmolysed quantity of the red blood-corpuscles increases very rapidly and often nearly proportionally to the square of the unbound quantity of alkali. This proceeds until the hæmolysis is total, that is, until all the red blood-corpuscles have given up their hæmoglobin to the surrounding medium. After this a further addition of alkali produces no change in the quantity of hæmolysis, though the velocity of reaction is increased. The state-

[1] Arrhenius and Madsen: *Festskrift*, Copenhagen, No. 3, 1902 ; *Zeitschr. f. ph. Ch.*, **44**. 7 (1903).

ment is valid also for the action of such hæmolysins as tetanolysin. It seems as if also in this case a chemical binding takes place, but the compound seems to be to a higher degree dissociated, so that the limit is not so sharp as for the alkalies.

To give an idea of this process I reproduce here some figures for the hæmolytic action of taurocholate of sodium, saponin, potassium hydrate, and solanin. For potassium hydrate 2.5 per cent suspensions of bovine corpuscles, for the other hæmolysins 2 per cent suspensions of equine erythrocytes, were used. Following the addition of the suspension of cells to the poison, the mixture was shaken and then for one hour placed in an incubator of 37° C. and after this eighteen hours in a refrigerator and then compared with the solutions of hæmoglobin of different concentrations prepared by the hæmolysis of different quantities of the same blood-corpuscles by pure water. The concentration is given for the potassium hydrate in fractions of a normal solution, for the other substances in fractions of the whole fluid (weight of dissolved substance : weight of solution).

Saponin			Taurocholate of Sodium		
Conc. $C \cdot 10^6$	H	$K \cdot 10^{-5}$	Conc. $C \cdot 10^5$	H	$K \cdot 10^{-3}$
40	90	2.35	140	91	6.81
28	40	2.26	100	55	7.41
20	10	1.58	68	18	6.24
14	5	1.60	48	4	4.17
10	3	1.73	32	2.5	4.94
7	2	2.02	20	1.3	5.70

Potassium Hydrate			Solanin		
Conc. $C \cdot 10^5$	H	$K \cdot 10^{-3}$	Conc. $C \cdot 10^6$	H	$K \cdot 10^{-5}$
62.5	27	8.31	40	86	2.32
50	13	7.21	28	70	2.98
37.5	4	5.33	20	4	1.00
31.5	3.5	5.98	14	2.4	1.11
25	2	5.66	10	1.5	1.5

As will be seen from these figures, these poisons do not follow nearly as closely as tetanolysin and ammonia the rule that the square root of the degree of hæmolysis is proportional to the concentration. The quotient of these two magnitudes is tabulated under K. In general the greater the velocity of reaction the more marked is the deviation from the said rule. At about 10 per cent the hæmolysis increases relatively more rapidly with the concentration than at other degrees of hæmolysis. Therefore the measurement of the quantity of poison present by means of the hæmolytic determination has the greatest exactitude in the neighbourhood of this point.

Different strong monovalent bases act in equivalent quantities nearly to the same degree upon the blood-corpuscles. The divalent bases $Ca(OH)_2$ and $Ba(OH)_2$ seem to give some solid precipitate in the erythrocytes, which hinders measurements, at least at higher concentrations. The hæmolysis observed is then nearly independent of the quantity of base added. Ammonia also has nearly the same strength of action as equivalent quantities of the strong monovalent bases. Sometimes (for low concentrations of blood) its action is a little less, in other cases (at

higher concentrations of blood) it is somewhat greater than that of the stronger bases, at least after a prolonged time of reaction (cf. p. 111). This seems to indicate that some compound is formed, so that the equivalent quantities act to the same degree, but that the ammonia compound is to some degree hydrolysed, which causes the deviations.

Acids also destroy the red blood-corpuscles, but this phenomenon has an appearance somewhat different from the hæmolysis by means of bases. Following the action of the alkalies and also of the lysins of bacterial origin, the fluid surrounding the blood-corpuscles takes up their intensive purple colouring matter and assumes the characteristic red colour of blood. The acids, on the other hand, alter the colouring matter, so that the fluid becomes dark brown, and after shaking the foam persists often for forty-eight hours or more. This indicates a coagulating influence. With lower degrees of hæmolysis by acids the fluid has, however, also a reddish tint. At the same time a strong agglutination of the blood-corpuscles is perceptible. At higher concentrations large clumps are formed, reminding one somewhat of the flocculent precipitates of aluminium salts mixed with an alkali. The degree of hæmolysis by a strong acid is about as pronounced as that of three to four times the equivalent quantity of a strong base. Equivalent quantities of different acids (hydrochloric, sulphuric, oxalic, tartaric, citric, and acetic) act nearly to the same degree, the weaker acids (for instance, acetic acid) act a little slower than the stronger; and extremely weak acids, as boracic acid, exert no appreciable hæmolytic action (the same is probably valid for extremely weak

bases), probably because the process goes on with insensible velocity.

For stronger concentrations of the hæmolysins total hæmolysis occurs if they are permitted to act through a long enough time. If the time of action be restricted (as by centrifugation), it is possible to follow the development of the reaction, as has been said above (cf. p. 100).

The presence of salt[1] exerts a strong retardative influence upon the hæmolytic power of the alkalies. Probably this effect depends on a diminution of the velocity of reaction. Especially is this true for the influence of ammoniacal salts upon ammonia. The different salts of the strong bases (KOH, $NaOH$, and $LiOH$) are of the same degree of efficacy in equivalent concentrations; the effect is nearly proportional to the cube root of the concentration; 0.02 n. salt solution lowers the effect in the proportions 1 : 0.4. The salts of ammonia also seem to be very similar to each other in this regard : 0.004 n. NH_3 salt lowers the effect in the proportion 1:0.7; 0.016 n. in the proportion 1:0.25; and 0.06 n. in the proportion 1:0.14.

On the other hand, it is perfectly clear that the addition of the equivalent quantity of hydrochloric acid to a solution of sodium hydrate would completely suspend its hæmolytic power, since in this reaction sodium chloride is formed, which has no hæmolytic effect. Of a sodium hydrate solution (neutralised to 50 per cent) it would be necessary to add the double quantity (a slight correction should be introduced for the lowering influence of the salt, and for the quantity of alkali bound that gives no action) to attain the same hæmolytic effect. We then say

[1] In this case the physiological solution is made of cane sugar.

that the toxicity of the first solution is half as great as that of the second solution.   The effect of the neutralisation of the sodium hydrate by the addition of hydrochloric acid may therefore be graphically represented in the following figure by the line *AB*.   The toxicity is here ordinates, and the quantity of acid added is abscissæ.   This line would be a straight line if the effect of the salt did not produce a perturbing influence.

When we have added the acid necessary to neutralise the free alkali (point *B*), we have still to add a small quantity to neutralise the alkali bound in the erythrocytes, and then further add a small quantity of acid before the solution is strong enough in acid to give hæmolysis again. These portions are represented by the parts *BC* and *CD* in the diagram.   On the addition of more acid to the solu-
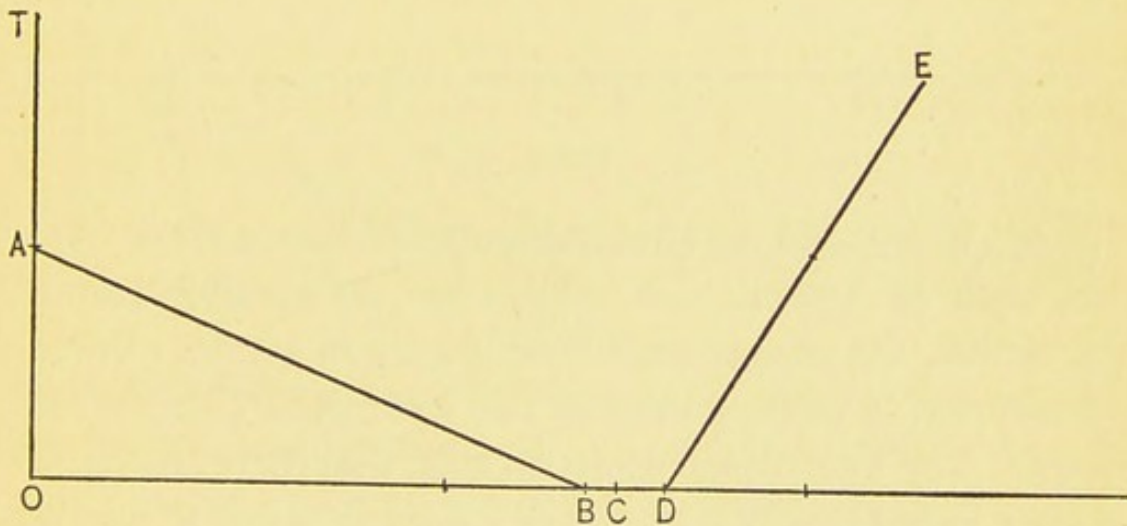
FIG. 1.

tion its toxicity increases nearly proportionally to the quantity of free acid (neither bound to the alkali, nor in the erythrocytes).   This toxicity is represented by the almost straight line *DE*.

In quite the same manner the toxicity changes on the

addition of a strong acid to a solution of ammonia. The line *AB* then deviates a little more, but not very much, from a straight line, according to the relatively strong influence of the ammonium salts. The real curve would lie little below *AB*.
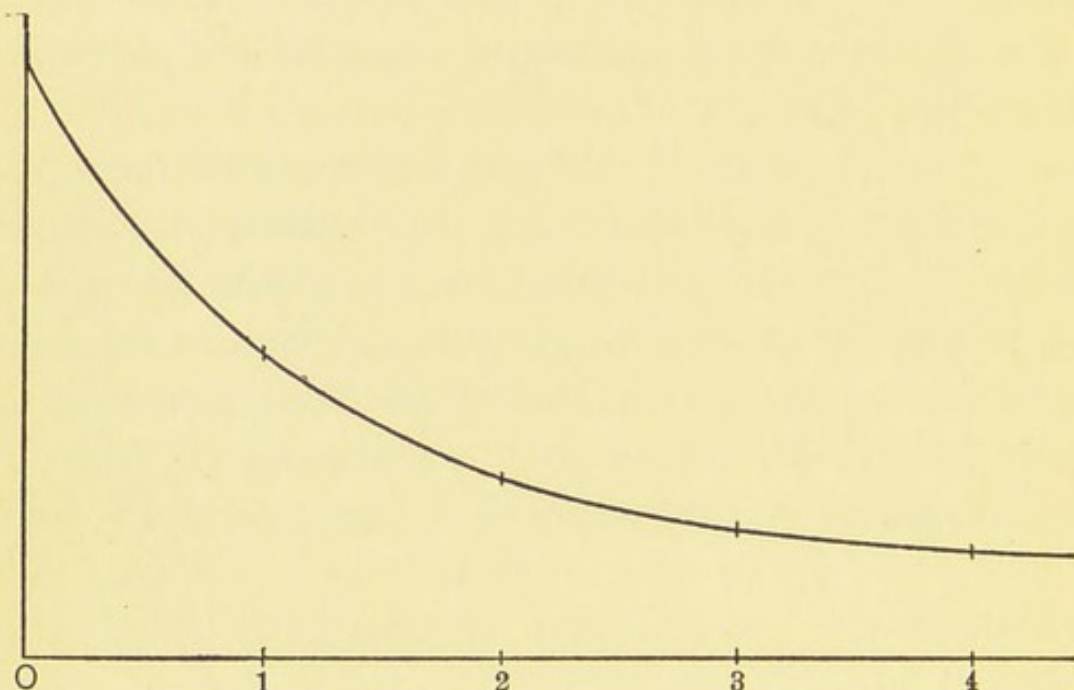
FIG. 2.

Now, if we add to the ammonia-solution a very weak acid, such as boracic acid, which has no sensible hæmolytic action, the phenomenon will behave in a rather different manner, in consequence of the hydrolytic effect of the water. The hydrolysis results in this, that there always remains a certain quantity of free ammonia, even if we add as large quantities of boracic acid as possible (up to saturation). Then the curve representing the toxicity descends as the quantity of boracic acid added increases, but never reaches zero, as is indicated on Fig. 2. The quantity (q) of free ammonia may be calculated according to the equation : —

$$q(n-a+q)=K(a-q)^2$$

where a is the quantity of ammonia present from the beginning, before boracic acid was added, q the quantity of free un-neutralised ammonia, n is the added quantity of this acid, consequently $(a-q)$ the quantity of salt formed, and $\{n-(a-q)\}$ the quantity of free boracic acid. The quantities should be expressed in equivalents, and one molecule of NH_3 is found to be equivalent to one
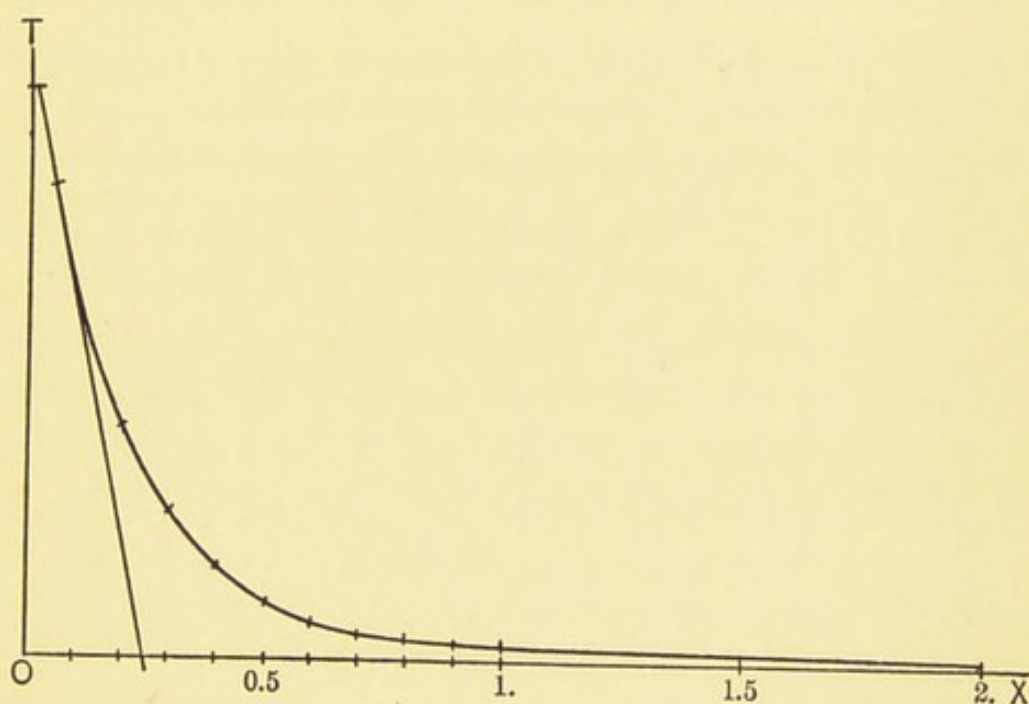
FIG. 3.

molecule of H_3O_3B. The constant K has a value dependent on the temperature. The last equation is a form of the equation expressing the law of Guldberg and Waage. A special theoretical investigation showed that it might be employed in this case.

Now the toxicity is proportional to the concentration of free ammonia, though in this case a correction must be introduced for the lowering action of the ammonium salt, as indicated by experiments on this action. Therefore, if we

carry out experiments on the toxicity of ammonia with the addition of different quantities of boracic acid, this toxicity may be calculated according to the equation cited. On the other hand, this toxicity may be determined directly from the hæmolytic power. In this case we suppose that the quantity of ammonia absorbed by the erythrocytes may be neglected (cf. p. 110). The comparison between the results of observation and calculation ($K=1.02$) is given in the following table :[1]—

TOXICITY (q) OF 0.1 N. NH_3 (1 EQUIVALENT) WITH n EQUIVALENTS OF BORACIC ACID

$n =$	$q_{obs.}$	$q_{calc.}$	$\Delta q_{obs.}$
0	100	(100)	
0.17	85	79	15
0.33	69	64	16
0.67	43	42	$26 : 2 = 13$
1	25	27	$18 : 2 = 9$
1.33	20	18	$5 : 2$
1.67	13	13	$7 : 2$ } 2.5
2	10	10	$3 : 2$

The agreement between the observed and calculated values of q is quite within the limits of the errors of observation. Under $\Delta q_{obs.}$ is tabulated the quantity of ammonia which is neutralised with regard to its hæmolytic power by the addition of the sixth part of one equivalent of boracic acid. The two first additions lower the toxicity by nearly the same amount (16.7 per cent) as a strong acid. The portions between one and two thirds and between two and three thirds have a noticeably lower influence (about $\frac{4}{6}$ and $\frac{3}{6}$ re-

[1] According to a recent investigation by H. Lundén on the hydrolysis of ammonium-borate the constant K is 1.02 in complete agreement with the value given above as results of experiments on hydrolysis.

spectively). The next equivalent of boracic acid added has an effect which equals only about the fifth part of that of the first equivalent.

This behaviour is to a high degree similar to that termed Ehrlich's phenomenon, observed in the neutralisation of a toxin with its antitoxin. The first part of the antitoxin added neutralises, generally speaking, a greater portion of the toxin than does the second equal addition, this a greater one than the third, and so forth. To explain this peculiarity (of diphtheria-toxin) Ehrlich supposes that the toxin is a mixture of many different " partial toxins," which possess different degrees of toxicity in equivalent quantities, and have a different affinity for antitoxin. If antitoxin be added, it at first neutralises that part of the poison which has the greatest affinity, and which also is the strongest poison; thereafter that with the next greatest affinity, which also is the second in toxic strength, and so forth. At the end the very weakest portions appear for neutralisation. Ehrlich designated these hypothetical " partial poisons" with names coined from the Greek language, as prototoxin, deuterotoxin, tritotoxin, epitoxin, etc.

If we apply Ehrlich's views to ammonia, this substance should, according to the experiment of neutralisation by boric acid, be composed of different " partial ammonias," of which the strongest one should be neutralised first, the second strongest next, etc. Of course this complicated explanation cannot possibly be used for ammonia, which we know is a very simple chemical compound of high purity, but it was by Ehrlich and his pupils applied to other poisons quite generally, *e.g.* to diphtheria-poison

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and tetanolysin, which, on neutralisation, as we shall soon see, behave in a manner very similar to ammonia.

The following figures, found for the toxicity of tetanolysin after the addition of different quantities of antitoxin, may serve as an example. It is illustrated by Fig. 3. This curve has a tangent at its beginning, which cuts the x-axis in the point $x = 0.276$, indicating that if the neutralisation proceeded according to the same laws as hold for the neutralisation of strong acids by strong bases, *i.e.* if the quantity neutralised was proportional to the antitoxin added until total neutralisation was reached, then 0.276 parts (c.c.) of the unit of antitoxin used would neutralise completely the quantity of toxin used (2 c.c. of a 2 per cent solution). In other words, these quantities are equivalent. By the aid of this value the quantities (n) of antitoxins are calculated in the following table in equivalents (n_1) of the toxin present taken as unit :—

TOXICITY (q) OF TETANOLYSIN AFTER ADDITION OF n C.C. OF ANTILYSIN

n	n_1	$q$obs.	$q$calc.
0	0	100	100
0.05	0.18	82	82
0.1	0.36	70	66
0.15	0.54	52	52
0.2	0.72	36	38
0.3	1.09	22	23
0.4	1.45	14.2	13.9
0.5	1.81	10.1	10.4
0.7	2.54	6.1	6.3
1.0	3.26	4.0	4.0
1.3	4.35	2.7	2.9
1.6	5.44	2.0	2.5
2.0	6.52	1.8	1.9

The values calculated are derived with the aid of the same equation as given above for ammonia, with the constant 0.115. The experiments were so executed that the mixture of toxin and antitoxin were left at 20° C. for two hours; thereupon they were mixed with the suspension of erythrocytes (2.5 per cent horse blood) and for one hour held in a thermostat at 37°. The degree of hæmolysis was determined as described above (cf. p. 15). The constant 0.115 is therefore valid at 20° C. In these and similar experiments it is assumed that the absorption of the poison in the erythrocytes may be neglected.

The antitoxin used was a solution containing 0.0025 per cent (per c.c.) of the content of antitoxin in a standard solution. This fact indicates that the original blood-serum containing the antitoxin derived from a horse injected with tetanus poison contained in one c.c. nearly 5800 times as much antitoxin as that which was equivalent to the quantity of poison contained in one gram of the dried tetanus preparation prepared by precipitation from a strong tetanus bouillon with ammonium sulphate. From this figure it is easy to see that the injected horse held in its blood (about 50 l.) many (about 15) million times the equivalent quantity of the injected poison (about 20 g.; sometimes this quantity may be still less). This circumstance deters us from accepting an idea which seemed at first rather probable — it was suggested by Behring — that the antitoxin is a derivative of the injected toxin. To similar results have led the experiments on the production of diptheria-antitoxin. If the antitoxin produced did not exceed many times the poison used for its production, the preparation of antitoxin would evidently have no practical value.

The curve which represents the experiments of Madsen
on tetanolysin resembles very much that representing the
neutralisation of ammonia by means of boracic acid.   It is
convex to the *x*-axis, which it never reaches.   This prop-
erty indicates that the greater the quantity added, the
less is also the neutralising power of the same quantity
of antitoxin.   But there is no reason to explain this
peculiarity by the hypothesis of Ehrlich, that in the toxic
solution exist side by side a large number of poisons.

The equation which we used for the calculation of the
results, and which coincides very well with the experiment,
has the following form : —

(Quantity of free lysin) × (quantity of free antitoxin) $\rightleftarrows$

$$K \text{ (quantity of bound toxin}^2).$$

According to the laws of physical chemistry this equa-
tion indicates that of one molecule of toxin and one
molecule of antitoxin there are formed two molecules of
the reaction-products.

This reaction is therefore rather similar to that of alco-
hol and acid to give ester and water, one molecule of each
substance entering into the chemical equation of reaction.
In this case, if equivalent quantities of the reagents are
used, two-thirds of them are transformed to ester and
water, when the equilibrium is reached.   The constant K
is in this case $K = 0.25$, about the double of that found for
tetanolysin.

The constant K of the equation of equilibrium is altered
with temperature.   When Madsen and I investigated this
phenomenon for the first time, we found a very great
increase in the proportion of $1 : 4.7$ for the interval of tem-
perature 20 to 37.3 degrees.   Later experiments have

given a much smaller value of the increase, which amounts only to the proportion 1 : 1.91 between 16 and 37° C.[1] A great difficulty inherent in the determination of the constants of tetanolysin, as well as of other poisons, lies in the circumstance that the constant of equilibrium is rather different for different preparations of the poison.   Fresh specimens of tetanolysin seem to give lower constants than old ones.   In general the constant of fresh tetanolysin seems to lie rather near to 0.12 at 20° C. (room temperature).

From the variation of K with temperature it is possible to calculate the heat of reaction which is developed in the combination of one grammolecule of tetanolysin with one of antitoxin to form two of the reaction-products.   A change in the proportion of 1 : 1.91 in the interval between 16 and 37° C. corresponds to a development of 5480 calories.

As we have seen above (cf. p. 41), tetanolysin is rapidly decomposed in temperatures in the neighbourhood of 50°.   An elevation of the temperature of 3.7 degrees increases the constant of velocity in the proportion 16.8 : 1. At 49.8° C. the rate of destruction is of such a magnitude that a poisonous solution loses half its strength in 62 minutes.   From these it is easy to calculate that at 20.2 and 5.4 degrees it will require 6.6×10^9 and 5.3×10^{14} hours (*i.e.* 7.5×10^5 and 6.2×10^{10} years) respectively to descend to half its strength.   Now it is often observed that solutions of tetanolysin weaken very rapidly, thus, for instance, it may lose about five-sixths of its hæmolytic power within five days

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[1] Madsen and Arrhenius: *Medd. fr. Vet.-Ak : s. Nobelinstitut,* 1. No. 3, 5 (1906).

at 20° C. (as Madsen and I observed in 1902); and dried toxin lost in two years on storage in a cold room (of about 6° C.) two-thirds of its hæmolytic power. This destruction is evidently of a wholly different nature from that observed at 50° C., which would be entirely imperceptible at 6 and 20° C. respectively. In the solutions at room temperature it is perhaps bacteria (*e.g. Bacillus pyocyaneus*) or the influence of dissolved glass which cause the rapid destruction; in the dried tetanolysin other slow chemical processes may be responsible for the loss of hæmolytic action.

The peculiar observation is here made that during this destruction the power of binding antilysin does not decrease at the same rate as the hæmolytic activity. Sometimes no decrease of the antitoxin-binding faculty is observed at all, so that solutions of lysin and antilysin, that were equivalent immediately after their union, also remain equivalent after the deterioration of the poison. On this ground Ehrlich concluded that the lysin is transformed into an innocuous, or nearly innocuous, modification, which retains the properties of neutralisation of the antitoxin characteristic of the original poison. This substance is termed by Ehrlich the toxoid. The toxoid not only seems to be equivalent with the poison, but also to possess nearly the same constant of equilibrium. And even one of the reaction-products of the toxoid with the antitoxin seems to be identical with one of the products of the corresponding reaction of the toxin. Only in this manner is it possible to explain that the neutralisation curve of the attenuated toxin is very similar to that of the original toxin. It may, on the other hand, be recalled that the equilibrium-constant of old tetanolysin preparations

has been found to have a very high value and to change greatly with temperature, so that evidently here greater transformations take place during the course of time.

In order to explain this peculiarity of the toxin, Ehrlich originated his so-called side chain theory, which has played a great rôle in these matters, especially in the German literature. Organic chemistry teaches us that certain properties of different substances, for instance the property of giving coloured solutions, depend upon the presence in these substances of the same group of atoms; in this special case this group is called the chromophoric group. The other parts of the molecule may be rather different in the different substances with the same property, and therefore this function is considered to be, so to speak, located in the common group. Now the poisons possess the two common properties of being poisonous and of binding their antitoxins, and these two properties do not vary with each other; as we have seen, the poisonous attribute diminishes more rapidly than the other in a solution of tetanolysin, and the same is the case with diphtheria-toxin, the study of which led Ehrlich to his conceptions. Ehrlich therefore expresses this peculiarity in the following manner. The two said properties belong to two different groups, called the toxophoric group, which is poisonous, and the haptophoric group, which binds anti-toxin. In the molecule of the poisonous substance, which we suppose to be composed of very many atoms, the two groups lie rather far from each other, so that chemical changes may take place in the one group — the toxophoric — without influencing sensibly the function of the other group. To express this Ehrlich supposes that the two

groups are bound to the central part of the molecule as side chains, known from the chemistry of the benzene derivatives. If now, as is often observed, no other change of the poison takes place than that its toxicity diminishes in a certain ratio, *e.g.* to 50 per cent, then to explain this peculiarity, Ehrlich must assume that all the partial poisons are weakened to the same degree, which seems very improbable. From our point of view we might explain the same fact by saying that the constant of equilibrium in the above equation has not been " sensibly " altered by the change in the toxophoric group. This is quite possible, although experience with the constant of equilibrium, the so-called dissociation-constant, in acids teaches us that it is rather sensible to changes in the molecule. But the great distance between the different groups in the poisonous molecule may have the effect that such an influence is in this case insensible.[1]

There is another possible explanation of the phenomenon. As will be seen in Chapter VIII, many poisons are compounds of two different substances. It is possible to suppose that even the so-called simple poisons are compounds of two substances, of which the one corresponding to the haptophoric group is bound by antitoxin. If this antitoxin binding property of the poison is relatively stable and present in great excess, and if the poisonous compound is formed of one molecule of each constituent, and is a highly dissociable substance, its quantity will be proportional to the concentrations of the two constituents. Then, evidently, the constant of equilibrium would remain unaltered if the group that does not bind antitoxin slowly disappears,

[1] Cf. Ostwald: *Zeitschr. f. ph. Ch.*, **3**. 374 (1889).

and we would expect just the phenomenon actually observed. In order to avoid unnecessary changes we may, until future experiments decide the question, employ the hypothesis that the poisonous molecules possess two groups, the one toxophorous and rather labile, the other haptophorous and more stable.

It is not necessary that the compound poison should exist to a sensible degree. We have, for instance (cf. p. 74), seen that the velocity of coagulation of casein is proportional as well to the rennet present as to the concentration of the calcium ions. As has been made probable by Fuld and Spiro, the "antirennet" contained in normal horse-serum acts so that it binds the calcium ions; before this research, however, it was supposed that the serum neutralised (bound) the rennet. In this case it is possible to destroy the rennet by heating to 60° C. At lower temperatures (30° or so) it weakens slowly; but the calcium ions, we may suppose, remain unaltered. The rennet evidently corresponds to the toxophorous group, the calcium ions to the haptophorous group of a toxin, and the serum to the antitoxin. Evidently the binding of the calcium ions by the serum will be independent of the quantity of rennet present. Therefore we may say of the combination, rennet-calcium-ions, that its antitoxin binding property remains unchanged, while its toxic (coagulating) property diminishes with time. Such a point of view has obviously a great advantage over that of Ehrlich; but in favour of the continuous development of the science, it seems reasonable to retain for this case the nomenclature of Ehrlich, provided that no far-reaching theoretical developments are based upon it — at least until the decomposition of toxins

into their two constituents has been proved for more cases
than the coagulating action of rennet (and other casein-
coagulating substances, lactoserum included, cf. Chapter
IX).

Just in the same manner as tetanolysin behave other
lysins of bacterial origin. For streptolysin produced by
streptococcus Madsen and Walbum have found the follow-
ing figures of the toxicity (q), after the addition of n c.c.
of antitoxin to a given quantity of poison. The calcu-
lated figures are obtained under the assumption that 1 c.c.
of the antitoxin solution used is equivalent to 4.8 times the
used quantity of lysin. The constant of equilibrium is
$K=0.13$ at 20° C., very nearly like that for tetanolysin.

TOXICITY (q) OF STREPTOLYSIN AFTER ADDITION OF n C.C. OF ITS ANTILYSIN

n	n_1	$q_{obs.}$	$q_{calc.}$
0	0	100	100
0.025	0.12	88.7	88.2
0.05	0.24	76.1	76.9
0.075	0.36	64.8	66.3
0.1	0.48	55.9	56.4
0.125	0.60	47.5	47.5
0.15	0.72	40.2	39.8
0.175	0.84	34.6	33.4
0.2	0.96	28.3	28.2
0.225	1.08	23.6	23.6
0.30	1.44	15.0	15.2
0.338	1.62	11.5	13.1
0.375	1.80	8	11.2
0.45	2.16	<6	8.6

The observed figures are mean values from three dif-
ferent series of observations. The agreement between the
observed and the calculated values is nearly perfect until
n_1 is about 1.5. At high values of n_1 the observed toxicity

is somewhat smaller than the calculated action. This accords with the observations on tetanolysin. The experimental method was quite the same in the two cases.

In a lecture held before the British Medical Association at Oxford in July, 1904,[1] Madsen has given curves representing the toxicity of vibrio, staphylo and streptolysin on the addition of increasing quantities of their specific antitoxins. These curves indicate clearly the fact that the Ehrlich phenomenon is quite apparent in all of them, inasmuch as the right branch tends to bend asymptotically to the abscissa.

In all experiments with the lysins the temperature, which corresponds to the equilibrium, is that at which the mixed fluids, toxin and antitoxin, are held during 1 h. to 2 h. before the blood cells are added. In this moment the volume increases to so high a degree that the velocity of reaction may be regarded as practically suspended.

Cholesterin displays a neutralising influence upon tetanolysin, as is seen from the following table, indicating the attenuation of 5 c.c. of a broth of tetanus bacilli on the addition of n c.c. of a 10^{-6} normal solution of cholesterin and so much salt-solution that the total volume was 10 c.c., which reacted upon each other during 3 hours at 37° C. After this time parts of this mixture were added to 8 c.c. of horse blood suspension (2 per cent), and the action observed. The equation of equilibrium indicates that 1 molecule of reaction-products results from 1 molecule of lysin, and 1 molecule of cholesterin. Of the cholesterin-solution 1.43 c.c. were equivalent to 5 c.c. of the broth, which therefore had the concentration $2.86 \cdot 10^{-7}$ normal.

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[1] Madsen: *British Medical Journal*, Sept. 10, 1904, p. 12.

NEUTRALISATION OF TETANOLYSIN BY n C.C. 10^{-6} n CHOLESTERIN AT 37° C. (MADSEN AND WALBUM)

n	$q_{obs.}$	$q_{calc.}$
0	100	100
0.5	67.5	66
0.75	51	49.5
1	34.5	34
1.2	27	23
1.4	15	14
1.6	8	9
1.8	5.5	6.2
2	3.4	4.2

If we choose a concentration in which 5 c.c. of the tetanus broth are contained in 10 c.c. as the unit concentration, we find a value 0.021 for K in the equation:

(Conc. of tetanolysin) × (conc. of cholesterin)=

$$K \text{ (conc. of compound).}$$

Even in this case the observed values of T for high values of n are a little less than the calculated ones.

Even some so-called neutral substances exert an influence upon the hæmolytic action of tetanolysin. The presence of salts in large doses increases the action. In this case the erythrocytes are suspended in a physiological solution of cane sugar. If to this so much salt is added that the solution is 0.04 normal, the hæmolysis (during 1 hour at 37° C.) increases to about the double value. A 0.01 normal solution is without appreciable influence. Different salts in the same concentration seem to exert the same influence. This influence is probably due to an acceleration of the velocity of reaction. For this reason solutions of lysin seem to be more poisonous in a physiological salt-solution than in one containing cane sugar.

The presence of protein, as egg-albumen or normal serum, protects the erythrocytes from the attack of bases and much more still from that of lysins of bacterial origin. Probably this action consists chiefly in a diminution of the velocity of reaction. If great quantities of normal serum are added, they act as an antitoxin.

In mixtures containing heavy doses of poison and of antitoxin, the velocity of reaction is very much retarded, probably because of the presence of large quantities of protein which as impurities accompany the preparations of poison and of antitoxin.

The antitoxic influence of normal serum was first observed by Ehrlich,[1] who employed horse-serum against tetanolysin. Neisser and Wechsberg [2] found a similar influence of horse-serum or human serum on staphylolysin. Madsen and I [3] observed a similar action of chicken egg-white on tetanolysin; still greater is the influence of egg-white from duck's eggs, according to P. T. Müller.[4] Marshall and Morgenroth [5] made similar observations on the action of different sera on different compound hæmolysins (cf. Chapter VIII). The normal serum of the horse, rabbit, and man are the most efficient, that of pigeons, mice, and geese less, while that of sheep is nearly insensible. According to P. T. Müller, the antitoxic action against tetanolysin is probably due to the presence of cholesterin in the horse-serum or egg-white. Against the generalisation of this view it may be pointed out that, ac-

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[1] Ehrlich : *Berl. Klin. Wochenschrift,* No. **12** (1898).

[2] Neisser and Wechsberg : *Zeitschr. f. Hygeine,* **36**. 314, etc. (1901).

[3] Arrhenius and Madsen : *Festskrift,* III. 37 and 43 (1902).

[4] P. T. Müller : *Centralbl. f. Bakteriologie,* I. **34**. 567 (1903).

[5] Marshall and Morgenroth : *Zeitschr. f. klin. Medicin,* **47**. fasc. 3 and 4.

cording to Kyes and Sachs, staphylolysin is not acted upon by cholesterin.[1] Madsen has even found that other constituents, and not the cholesterin alone, protect erythrocytes against tetanolysin (still unpublished).

The action of different anti-bodies on their corresponding poisons is supposed to consist in a simple neutralisation, molecule for molecule. That the process is followed by another phenomenon is indicated by the circumstance that at high concentrations of antitoxin, where it is in excess beyond the toxin present, the calculated values are often found to exceed remarkably the observed ones. Another phenomenon also, discovered by Danysz,[2] indicates that an excess of antitoxin exerts a perturbing influence. Danysz investigated the toxicity of mixtures of ricin with antiricin. He observed that a mixture of a parts of ricin with b parts of antiricin is less toxic if the two constituents are mixed at once, than if a fraction of a, say one-half, is added to the b parts of antitoxin, and after a time the rest of the toxin added to the mixture. The same phenomenon was observed in diphtheria poison by von Dungern, and[3] with tetanolysin, staphylolysin, and rennet by Sachs.[4] On the other hand, the cobra poison does not display this behaviour.

To elucidate this remarkable phenomenon, which seems to indicate that the same quantity of poison may bind different quantities of antibody, a large number of experiments were carried out on tetanolysin by Madsen, and I

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[1] Kyes and Sachs: *Berl. klin. Wochenschrift*, Nos. 2–4 (1903).
[2] Danysz: *Ann. de l'Inst. Pasteur*, 1902.
[3] V. Dungern: *Deutsche med. Wochenschrift*, Nos. 8 and 9 (1904).
[4] Sachs: *Centralbl. f. Bakteriologie*, 37. Part II, 251 (1904).

afterward calculated the results.[1]   Preliminary experiments showed that the effect increased with the quantity of anti-toxin present and that the dilution of the reacting sub-stances exerted no influence, so that the prevailing chemical process must be a monomolecular one.

One c.c. of a bouillon containing tetanolysin, was added to 0.8 c.c. of a solution containing antilysin of which 0.18 c.c. were equivalent to 1 c.c. of the solution containing the lysin. This mixture was kept at 37° C. for a certain time, t, and afterward mixed with 3 c.c. of the poison and the whole held at 37° C. during 30 minutes.   The toxicity, q, was de-termined in the ordinary way and found to increase with the time, t, so that q converged against a maximum value G_∞.   If q_o valid for the time 0 is taken as a unit, the dif-ference $q - q_0$ is a measure of the effect investigated.   The difference $q_\infty - q$ indicates the progress of the effect with time.   This quantity is treated as corresponding to a mono-molecular process with the constant of reaction K.   The results are given in the following table : —

THE PROGRESS OF DANYSZ'S EFFECT FOR TETANOLYSIN WITH TIME

t (hours)	q	$q - q_0$	$q_\infty - q$	$\log (q_\infty - q)$	K
0	1.00	0	0.60	0.778 − 1	—
0.167	1.04	0.04	0.56	0.748 − 1	0.180
0.5	1.14	0.14	0.46	0.663 − 1	0.230
1	1.24	0.24	0.36	0.556 − 1	0.222
2	1.33	0.33	0.27	0.431 − 1	0.173
4	1.47	0.47	0.13	0.114 − 1	0.168
6	1.57	0.57	0.03	0.477 − 2	0.217
∞	1.60	0.60	0		Mean 0.197

[1] Madsen and Arrhenius: *Medd. fr. Vet. — Ak : s. Nobelinstitut*, 1. No. 3 (1906).

In the same manner K was found at 19.7° to be 0.067 and at 27° C. to be 0.105. This corresponds to an increase of the velocity of reaction in the proportion 1.86 : 1 and 1.87 : 1 respectively for an elevation of the temperature of 10° C. This reaction belongs, as the constancy of K indicates, to the order of monomolecular reactions.

In other experiments the quantity of antilysin, A, was varied, and the following values of $q_\infty - q_0$ were found. The quantities of lysin, L, were always the same, namely, 1 c.c. in the first and 3 c.c. in the second fraction, and the experiments were otherwise executed as indicated above.

THE MAGNITUDE OF THE EFFECT OF DANYSZ AS DEPENDENT UPON THE QUANTITY OF ANTITOXIN USED

FIRST FRACTION	$q_\infty - q_0$	
	Observed	Calculated
0.2 c.c. A + 1 c.c. L	0.05	0.02 (0.02)
0.4 c.c. A + 1 c.c. L	0.23	0.21 (0.22)
0.6 c.c. A + 1 c.c. L	0.39	0.40 (0.41)
0.8 c.c. A + 1 c.c. L	0.60	0.60 (0.63)
1.2 c.c. A + 1 c.c. L	0.97	0.99 (0.87)

The calculated values are found under the assumption that the effect is proportional to the excess of antitoxin over that (0.18 c.c.) equivalent to the quantity of lysin (1 c.c.) present in the first fraction. This proportionality is very striking in the figures above. The effect may also be calculated as the quantity of antitoxin bound by the poison, if the experiments are executed as above, minus the corresponding quantity if the total quantity of poison is added at once. The figures calculated in this manner are written in parentheses.

Evidently the effect depends upon some slow molecular change in the antitoxin which is not bound by toxin. This process goes on only in the presence of the neutralisation products of toxin and antitoxin or of free toxin itself, which therefore may be assumed to bind the transformed antitoxin, so that the reaction can proceed further. The circumstance that the effect of Danysz is not observed with cobra poison seems to indicate that it is the poison itself which binds the transformed antitoxin. For in this special case the neutralisation is nearly complete, just as the neutralisation of a strong acid with a strong base ; and therefore, if the antitoxin is present in excess, no sensible quantity of free poison exists in the solution, and hence we should expect that the effect would not be apparent.

Because of the inverse reaction of the reaction-products, new quantities of toxin are always free to bind the transformed antitoxin. This new reaction of (the modified) antitoxin and toxin is much nearer a complete one than the chief reaction between these substances which prevails during the first time of reaction, and which therefore corresponds to the equilibrium studied before. Consequently we find that the bond between toxin and antitoxin "is strengthened with time." Therefore also the toxicity of solutions containing an excess of antitoxin is found to be inferior to the calculated value. In this way it is explicable that mixtures with a very large excess of antitoxin may be practically harmless, although the calculation does not indicate it. This circumstance explains some experiments of Madsen, in which he allowed an "innocuous" mixture of diphtheria-toxin with antitoxin (hence containing a very great excess of free antitoxin) to diffuse into a gelatinous

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solution over which the mixture was placed in a test-tube. If the mixture had been conserved for a certain time (about half an hour at room temperature) there was no indication that free toxin diffused; but if the mixture was used as it was freshly prepared, the toxin diffused downward, so that the lower parts of the solid gelatinous solution contained two lethal doses for guinea-pigs. Such a strengthening of the chemical bonds is very often assumed in the doctrine of immunity, and it corresponds really to the phenomenon of Danysz.

Therefore the strongly toxic solutions in which tetanolysin has been added in fractions slowly lose their abnormal toxicity, and after a time (about 6 hours at 37° C.) they are no more toxic than the corresponding mixtures which have not been fractionated.

The results of the experiments of von Dungern regarding diphtheria-toxin correspond in their general features with those for tetanolysin, so that there is every reason to believe that the cause is identical in the two cases. The same may be said of the other cases in which Danysz's phenomenon has been observed, but the experimental data are extremely meagre.

Portier and Richet observed a peculiar phenomenon, which at first seems rather inexplicable, but which is very similar to the phenomenon of Danysz, and therefore may be interpreted in an analogous manner. They prepared a solution of the poison contained in the filaments of cœlenterates (Actinia or Physalis), which produces the same effects as the poison of Urtica, by macerating these filaments in glycerin and water. The poison was injected into the veins of pigeons or dogs, and caused a deep sleep,

by virtue of which it was called hypnotoxin. In greater doses death results under symptoms of asphyxia. The experimenters hoped to produce an antitoxin in the usual way by injecting increasing doses into the veins of the animals. But they did not succeed, because "if an animal receives in a first injection a parts of the poison and in a second injection b parts, it is killed almost instantaneously after the second injection; whereas an animal in which the dose $a + b$ is injected at once does not show very grave symptoms of intoxication, from which it soon recovers."[1]

[1] Portier and Richet: *Bulletin du musee océanographique de Monaco,* 25 Dec., 1905, p. 10, No. 56.

CHAPTER VII

NEUTRALISATION OF DIPHTHERIA–TOXIN, RICIN, SAPO–NIN, AND SNAKE–VENOMS

In very nearly the same manner as tetanolysin behaves the practically most important of all toxins, namely, diphtheria-toxin. Through the circumstance of its preparation and standardisation upon a large scale, a great number of experiments have been carried out with it. Unfortunately in most of these experiments but a few (4–6) points in the neutralisation curves have been determined, and no indications are given of the magnitude of the experimental errors. This is, of course, due to the circumstance that we are still in the first beginning of the development of the quantitative side of this question. In a memoir in the *Centralblatt für Bakteriologie* (1903), p. 630 *et seq.*, Madsen reported a greater number of measurements of several preparations of this toxin than had been generally done. As illustration may be given the values for poison No. 471 in February-March, 1902 (5 months old), and in November of the same year (14 months old). The letters n and q correspond, as for tetanolysin above, to the quantity of antitoxin added and toxicity.

As will be seen from these figures, the addition of the first quantities of antitoxin seem not to diminish the toxicity of the poison, the observed toxicity remains constant, and a decrease of $q_{obs.}$ is not apparent until the quantities 0.05 respt. 0.18 of antitoxin have been added. From this Mad-

POISON 471. FEB.–MARCH, 1902			POISON 471. NOV., 1902		
n	$q$obs.	$q$calc.	n	$q$obs.	$q$calc.
0	50	67	0	35	67
0.05	50	58	0.06	35	56
0.1	45	48	0.12	35	45
0.15	40	40	0.18	35	36
0.2	30	31	0.24	18	25
0.25	20	23	0.3	14	15
0.3	15	15	0.36	8	8
0.35	10—8	9	0.4	7	5
0.4	6	5	0.48	3	3
0.45	3	3	0.56	1	2
			0.6	1	1

sen concluded — in agreement with Ehrlich [1] — that a fraction of the antitoxin-binding substance is not poisonous, and that it binds antitoxin before the toxin itself. Such a substance is called prototoxoid by Ehrlich. As the quantity, n, necessary to determine a decrease of the toxicity is greater with the old than with the fresh poison, the conclusion was drawn that the quantity of prototoxoid increases with time, and perhaps as the preparation was quite fresh it may at first have contained no prototoxoid at all.

This was in complete agreement with Ehrlich's ideas, but on another point regarding the existence of so-called toxons or epitoxins Madsen differed from Ehrlich. As will be seen from the preceding table, the phenomenon of Ehrlich is very prominent, the same quantity of antitoxin producing a much less marked reduction of the toxicity at the beginning of the neutralisation than later on. This is explained by the chemical equilibrium between toxin and

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[1] Ehrlich: *Deutsche med. Wochenschrift*, No. 38 (1898); Aug. 31, Sept. 7 and 14 (1903).

antitoxin on the one hand and the products of their reaction on the other hand, as the calculated figures show. These are calculated from the equation valid for the neutralisation of tetanolysin, only the constant of equilibrium here is about eight times lower than there; in other words, the reaction proceeds farther in the binding of diphtheria-toxin than in the binding of tetanolysin.

When the toxicity sinks below one, the animals under experiment do not die after the injection of the quantity of poison employed (0.1 c.c.). By administering greater doses it is still possible to kill the animals. But if the quantity of antitoxin exceed a certain amount, the animals are not killed (in short time, eight days), but show other symptoms of the disease; after an incubation time of more than a week paralysis occurs. Now Madsen and Dreyer [1] showed that such paralysis is sometimes observed also after the injection of a quantity of pure diphtheria-poison less than the lethal dose. The free poison injected subcutaneously gives other symptoms, namely, necrosis and alopecia at the site of injection. Ehrlich and his pupils now contend that with mixtures of toxin and antitoxin which do not kill the animals but produce paralysis, the local effects at the site of injection are much less than after the injection of the corresponding quantity of free poison, whereas the paralysis is much stronger with the use of the mixture than with the free poison; and therefore the paralytic result is ascribed to a poison, toxon or epitoxin, which remains after the neutralisation of the true toxin. According to Madsen the difference observed depends upon the following circumstances. The greater part of

[1] Dreyer and Madsen: *Festskrift*, Copenhagen, No. 5 (1902).

the free poison is bound near the area of injection. The remainder on entering the circulation is therefore relatively harmless. The mixture behaves, however, in a wholly different manner. If the greater part of its free poison is bound near the area of injection, the diffusing mixture on entering the circulation gives off free poison through dissociation of the toxin-antitoxin compound and therefore causes a much stronger paralysis than does the smaller quantity of originally free poison. It must also be conceded that if, as in the case of poison No. 471, in the one case less than one lethal dose, and in the other case about forty lethal doses, together with a large quantity of antitoxin, are injected, the animal will have much more difficulty in freeing its body of the poison in the second than in the first case. For in the first case it has only to neutralise and eliminate less than one lethal dose; while in the second case, after the neutralisation or elimination of one lethal dose, new quantities of poison are set free to be eliminated from the animal's body. In the meantime large enough quantities of poison to cause a marked paralysis may diffuse to the nervous organs and disturb their functions. From this point of view the long period of incubation is also easily understood. There is therefore no adequate ground to assume the presence of a poison such as the toxon or epitoxin of Ehrlich, different from the lethal poison in the diphtheria poison. In recent times a great number of investigations have been done to strengthen the probability of the existence of "toxons." Morgenroth[1] has made a very elaborate study of the action upon guinea-pigs of pure diphtheria-toxin and of mixtures with antitoxin. He con-

[1] Morgenroth: *Zeitschr. f. Hygiene*, **48.** 177 (1904).

trasted the difference between the action of pure toxin and of a mixture of toxin and antitoxin, so that there is no doubt upon that point. But there is also no doubt that this difference may be as well explained by the presence of reaction-products of toxin and antitoxin in the solution as by the assumption of a different poison in the two cases.

There is another experiment by van Calcar,[1] which is cited by the many pupils of Ehrlich, who attempt to defend his views. v. Calcar believed he had found that it was possible by the aid of diffusion under pressure to separate diphtheria poison from the accompanying "toxon"; the latter, he reported, was not able to pass through a membrane while the real toxin diffused. Römer[2] has repeated the experiment of v. Calcar and found that it was impossible to detect "the least difference in the behaviour of the original diphtheria poison and of its diffusion-products." Furthermore, he subjected the results of v. Calcar to a critical examination and stated that his experiments did not warrant the conclusion that it is possible to separate toxons from diphtheria-toxin. In the laboratory of Madsen, Walbum has made a thorough investigation of v. Calcar's experiment and come to the same conclusion as Römer, so that there seems to be no doubt that "toxon" cannot be separated from diphtheria-toxin by means of diffusion under the conditions employed by v. Calcar.

The "prototoxoid" also seems to be unproved, if we subject the experimental material to a closer analysis. On surveying the material published by Madsen, it struck me that it had not been employed as exhaustively for the

[1] Van Calcar: *Berl. klin. Wochenschrift*, No. 39 (1904).
[2] Römer: *Behrings Beiträge z. exp. Therapie* (1904).

purposes of calculation as might have been. A recalculation in accordance with the rules valid in the exact sciences (cf. page 13) gave a neutralisation curve which did not show the presence of "prototoxoid" in the poison No. 471 in the experiments of November, 1902. I therefore made a recalculation of all the data relative to the poisons examined by Madsen. For the poison No. 471 I obtained the following results : —

For February, 1902			November, 1902			September, 1903		
n	$q_{obs.}$	$q_{calc.}$	n	$q_{obs.}$	$q_{calc.}$	n	$q_{obs.}$	$q_{calc.}$
0	100	100	0	100	100	0	100	100
0.05	74.4	87.5	0.06	79.3	86.5	0.1	75.1	75.1
0.1	72.8	75.1	0.12	76.0	73.2	0.15	62.6	62.7
0.15	57.6	62.7	0.18	64.7	59.8	0.2	47.6	50.6
0.2	49.8	50.6	0.24	50.9	46.6	0.25	45.8	38.6
0.25	32.2	38.6	0.3	39.1	34.0	0.3	25.9	27.3
0.3	28.0	27.3	0.36	23.1	22.4	0.35	17.3	17.5
0.35	17.2	17.5	0.4	14.8	15.7	0.4	9.6	9.9
0.4	11.1	9.9	0.48	8.3	7.0	0.45	5.3	6.0
0.45	5.6	6.0	0.54	2.5	4.5	0.5	3.1	4.1
0.5	1.2	4.1	0.6	2.6	3.0	0.6	1.6	2.6
Abs. toxicity = 4.1			Abs. toxicity = 2.86			Abs. toxicity = 2.14		

The constant of equilibrium remained unchanged (=0.012), in the whole time. No traces of prototoxoids may be detected from the new calculation. This is also the case for two other poisons, A and C, examined previously by Madsen, and in which he supposed large quantities of prototoxoid to be present. If prototoxoid had been present in the poison No. 471, it would have been most evident in the later researches (of September, 1903), since it was then rather old. But just in this case the

agreement between observation and the calculation by
which no prototoxoid is supposed to exist, is very good
for low values of n.   The hypothesis of prototoxoids
seems therefore untenable.   But on the other hand, the
poisonous effects of No. 471 had fallen very remarkably
in the seventeen months — to very nearly half the original
toxicity — without change in the antitoxin-binding property
of the poison.   For this same reason Ehrlich supposed that
the poison is slowly converted into an innocuous or less
poisonous substance with the same antitoxin-binding prop-
erties as the toxin itself (cf. p. 184).   This new substance
is called syntoxoid by Ehrlich.

The several preparations of diphtheria-toxin differ
from each other in showing rather marked differences
in their constants of equilibrium: No. 471, $K=0.012$;
poison A, $K=0.03$; poison C, $K=0.004$.   It will be nec-
essary to make new experiments on this interesting ques-
tion (and similar ones for tetanolysin) before this can be
elucidated.   The observed peculiarity, that K has rather
different values, might be explained by supposing that the
poison is loosely combined with or absorbed by some
concomitant substance, for instance by albuminous sub-
stances contained in the preparations, and that therefore
only a certain fraction of the toxin enters into the equi-
librium with antitoxin.   This fraction is different in differ-
ent preparations of the diphtheria poison, according to
their different content of the reacting proteins, and the
less this fraction the greater would be the constant of
the equilibrium.   This view is supported by the observa-
tion of Madsen that the value of K for another poison,
namely crotalus venom, is different when injected into

rabbits than with guinea-pigs.[1]   But perhaps this difference depends only upon the different mode of injection (intra-venous respectively intra-peritoneal).

In the same manner behaves, according to the researches of Madsen and Walbum,[2] ricin and its antibody, at least in so far as the agglutinating action of ricin on red blood-corpuscles (of rabbits) is concerned.   The agglutinating power of the ricin was measured by observing the limpidity of 5 c.c. of a one per cent suspension of red blood-corpuscles added to 2 c.c. of the solution and thereafter held at 37° C. for 20 minutes.   As example may serve the following figures :—

n =0	0.025	0.035	0.045	0.055	0.065	0.075	0.087	0.1
$q_{obs.}$ =6.7	6.7	3.6	2.1	1.5	1.0	0.8	0.64	<0.5
$q_{calc.}$ =(19.7)	6.8	3.6	2.1	1.4	1.0	0.8	0.63	0.12

One c.c. of the antiricin, prepared by injection into a goat, was equivalent to 29 c.c. of the solution of ricin employed (containing 1 per cent of a ricin preparation from Merck).   The constant of equilibrium was $K=0.0537$.   The formula was the same as that used for tetanolysin.

As is evident from these figures, the prototoxoid phenomenon is very marked.   No sensible neutralisation takes place until about 0.75 equivalents of antitoxin have been added.   But this phenomenon was "very inconstant, and was observed in only half the number of the cases examined."   The authors could not determine what accidental circumstances could have caused this contradictory behaviour of ricin; the so-called prototoxoid effect was

[1] *British Medical Journal*, Sept. 10, 1904, p. 14.
[2] Madsen and Walbum: *Centralbl. f. Bakteriologie*, Abt. I., **36.** 242 (1904).

observed in some experiments, whereas other experiments executed with the same preparations and on the same day (with the same horse blood) did not indicate the presence of a prototoxoid.

As illustration of ricin which does not show the " protoxoid" zone, the following figures of Madsen and Walbum[1] may suffice : —

n	$=0$	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
$q_{obs.}$	$=100$	88	73	63	44	27.3	15.9	11.9	7.1
$q_{calc.}$	$=100$	86	72	58	44	31.3	19.4	11.0	6.4

Here the constant of equilibrium is only 0.014. Madsen and Walbum found that the strength of the antiricin decreased continually, sinking from the value 1 to 0.4 in 47 days and to 0.196 in 42 additional days, which corresponds closely to a monomolecular reaction (cf. p. 37 ff.). During the same time the constant of equilibrium diminished in the proportion 0.537 : 0.0142.

The ricin has another toxic effect. On subcutaneous injection it kills guinea-pigs, even if it is used in very minute amount. Madsen and Walbum determined the lethal dose to be 0.000123 g. The toxicity is therefore expressed by the inverse number, 8130. Madsen and Walbum took a quantity of poison 200 times less (40.7) or 0.005 g. They added different quantities of antiricin — between 0.01 and 0.035 c.c. — to this quantity of ricin, held the mixture for 20 minutes at 37° C. and injected it thereafter into guinea-pigs. They found that the different mixtures contained the following number of lethal doses : —

[1] Madsen : *British Medical Journal*, Sept. 10, 1904, p. 9.

TOXICITY OF MIXTURES OF THE NERVOUS POISON IN RICIN AND ANTIRICIN

$n = $ C.C.	$q_{obs.}$	$q_{calc.}$
0	40.7	40.7
0.01	25	25.6
0.0125	21	22
0.015	17	18.6
0.02	12	12.2
0.0225	9	9.5
0.025	7	7
0.0275	5	5
0.03	4	3.5
0.0325	3	2.7
0.035	2	1

The values used are $p = 40$, that is, 0.025 c.c. of the antiricin is equivalent to 0.005 g. of the ricin. K was found to be 0.00149 and the formula used was: —

$$[(\text{Conc. of free ricin}) (\text{conc. of free antiricin}) = K (\text{Conc. of bound ricin})^2.]$$

The calculated values agree very well with the observed ones. The equation indicates that of two molecules of ricin and two molecules of antiricin are formed three molecules of innocuous substance.

Therefore we must suppose that it is probably some other poison in the ricin preparations that causes the death of animals than that which agglutinates the red blood-corpuscles. Therefore the assertion of Ehrlich that the action of ricin in vitro is of the same order as in the living animal,[1] cannot endure a quantitative examination. That these two poisons are not identical is known, but it was possible that they were always present in a given proportion. That this is not the case is seen from Bashford's experience, which

[1] Ehrlich: *Fortschritte der Medicin*, No. 2 (1897).

shows that normal rabbit-serum interferes with the agglutinating, but not with the neurotoxic action of ricin.[1]

In the same manner as the nerve-poison of the ricin behaves the hæmolytic poison saponin, according to an investigation of Madsen and Noguchi.[2] They investigated its influence upon different quantities of blood-corpuscles. The experiments were executed in the manner commonly employed, so that to a given quantity (2 c.c. of a 2 per cent solution) of poison (saponin) a certain quantity of antitoxin (cholesterin) and so much physiological salt-solution was added that the total quantity was 4 c.c.

NEUTRALISATION OF 0.04 G. SAPONIN BY MEANS OF n C.C. OF 0.1 n CHOLESTERIN

N.	BLOOD EMULSION OF			TOXICITY (mean)	TOXICITY (calc.)
	1.25 %	2.5 %	5 %		
0	100	100	100	100	95.2
0.01	79.5	82.4	79.3	80.4	78.5
0.015	67.5	71.0	71.8	70.1	71.5
0.02	54.6	57.6	62.1	58.1	62.9
0.025	48.1	53.1	56	52.4	55.6
0.03	44	45.4	48.3	45.9	48.3
0.04	34.5	41	40.6	38.7	35.4
0.05	26.7	33.4	29.3	29.8	24.4
0.06	17.7	21.2	18.1	19 0	15.7
0.07	11.1	12.8	9.4	11.1	9.8
0.08	7.8	6.7	4.3	6.3	6.1
0.1	3.7	3.2	2.4	3.1	2.8
0.12	1.7	1.4	1.2	1.4	1.4
0.14	0.67	0.62	0.51	0.6	0.8
0.16	0.34	0.43	0.42	0.4	0.5

Of the cholesterin solution 0.053 c.c. is equivalent to 0.04 g. of saponin, or 1 c.c. to 0.76 g. K is given as $K = 0.0166$.

[1] Bashford: *Journal of Hygiene*, 4. 56 (1904).
[2] Madsen and Noguchi: *Oversigt over d. k. danske Vid-Selsk's Forh.*, No. 6, 457 (1904).

According to this the molecular weight of saponin would be 7600, if 1 gramme-molecule of cholesterin ($C_{27}H_{46}O = 386$) is supposed to be equal to a gramme equivalent, and the same is valid for the saponin, supposed to be pure.

The different blood suspensions (from horse blood), the concentration of which varies in the proportion 1 : 4, give within the experimental errors the same value for the toxicity. This circumstance seems to indicate that the absorption of a part of the poison in the erythrocytes does not disturb the equilibrium. This depends probably upon the circumstance that the quantity of poison absorbed by the erythrocytes is insignificant compared with that in the surrounding medium. The saponin-cholesterin reaction was selected for this special investigation because its velocity of reaction is so high that it cannot be measured with the methods now used. The mixtures of saponin (in 2 per cent aqueous solution) and of cholesterin (in 0.1 n. = 3.86 per cent ethereous solution) were mixed, and held at 37° C. for three hours. The ether which did not evaporate in this time was removed in vacuo, after which so much salt-solution was added that the quantity of saponin in the solution was 1 per cent. Then the quantities of these mixtures to be investigated were placed in test-tubes and so much salt-solution was added that the volume was 2 c.c., whereafter 8 c.c. of the blood suspensions were rapidly poured into the test-tubes and mixed by shaking. The test-tubes were then placed in an incubator at 37° C. for three hours, and then put on ice and examined the next day in the usual manner.

There was therefore an adequate time for the equilibrium to be established after the addition of the blood suspension,

so that we might in this case have expected that if the presence of the erythrocytes has a perturbing influence, this should be very manifest in the present case, in which the quantity of erythrocytes was in one case double that used in such experiments in general.

But no such influence is seen in the figures of the last table, so that we may on this ground well conclude that the perturbing influence caused by the solubility of the poison in the erythrocytes may be neglected in these and similar experiments.

Some experiments with normal serum of horse or ox gave the same result, as may be seen from the following table, in which the calculated values are found under the assumption that 1 c.c. of normal ox-serum is equivalent to 0.006 g. of saponin.

NEUTRALISATION OF 0.002 G. SAPONIN WITH n C.C. OF NORMAL SERUM OF OX BLOOD

n	$q_{obs.}$	$q_{calc.}$
0	100	100
0.1	80.5	79.4
0.15	70.6	71.7
0.2	62.8	64.5
0.3	52.1	52.2
0.4	44.4	42.5
0.5	35.3	34.4
0.6	28.3	27.9
0.8	18.6	18.6
1.0	10.7	13.2

$$K = 0.093.$$

The equation used for the calculation is the same as in the last case.

Madsen and Noguchi have made an investigation [1]

[1] Madsen and Noguchi: *Oversigt*, 1906, No. 4, p. 233.

of the neutralisation of different snake-poisons by their antibodies. These are specific, *i.e.* they do not react except with the particular poison that was used in the injection. The cobra-antitoxin was secured from horse blood, the two others from goat blood. The snake-venoms contain different poisons, of which some are hæmolytic, others react chiefly upon the nervous system and are lethal for animals. The antibodies neutralise the lysins as well as the other poisons.

In the process of neutralisation, the lysins of the snake venoms behave rather like strong bases, the constant of equilibrium being nearly zero. This agrees well with the results of the older experiments of Kyes.

The following tables give the details. The poisons and their antibodies were allowed to react during three hours at 37° C., the total volume being always the same for the same poison. After the reaction they were mixed with 8 c.c. of blood suspension.

NEUTRALISATION OF 1 C.C. OF 0.05 PER CENT CROTALOLYSIN BY MEANS OF *n* C.C. OF ANTIVENIN (5 PER CENT DOG BLOOD)

n	$q_{obs.}$	$q_{calc.}$
0	100	100
0.05	89	91
0.1	77	81
0.15	71	72
0.2	64	63
0.25	54	53
0.3	46	44
0.35	36	35
0.4	24	26
0.45	16	16
0.5	6.3	7
0.55	1.5	0

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The last figure indicates that the quantity of free poison exceeds the calculated one, under the assumption that $K = 0$, and that hence K has a finite value. If we calculate K from this, placing 1 c.c. of antivenin as equivalent to 1.86 c.c. of the lysin, we find $K = 0.0006$, using the same equation as for tetanolysin. In the experiments with cobralysin to each 1000 c.c. of horse-blood suspension were added 8 c.c. of 0.01 n. lecithin.[1]

NEUTRALISATION OF 1 C.C. OF 0.1 PER CENT COBRALYSIN BY MEANS OF n C.C. OF ANTIVENIN (1 PER CENT HORSE BLOOD)

n	$q_{obs.}$	$q_{calc.}$
0	100	100
0.1	84	83
0.15	71	75
0.2	65	66
0.25	56	58
0.3	44	50
0.4	33	33
0.5	16	16
0.6	3.3	0

Here 1 c.c. of the antivenin-solution is equivalent to 1.68 c.c. of the poison. The last observation gives $K = 0.0016$.

[1] Cf. Kyes : *Berl. klin. Wochenschrift*, No. 19 (1904). Kyes finds the following values for cobralysin : —

n	$q_{obs.}$	$q_{calc.}$
0	100	100
0.75	66.7	66.7
1.5	33.3	33.3
2.25	0.1	0

Even here we find a deviation from the calculated value in the direction that a mixture which was calculated to be neutral showed hæmolytic properties.

NEUTRALISATION OF 1 C.C. OF LYSIN FROM ANCISTRODON PISCIVORUS, ACTING ON AN EMULSION OF 5 PER CENT DOG BLOOD

n	$q_{obs.}$	$q_{calc.}$
0	100	100
0.05	93	94
0.1	87	88
0.15	82	82
0.3	77	76
0.25	70	70
0.3	63	64
0.4	53	52
0.5	42	40
0.6	26	28
0.7	17	16
0.8	11	4
1	2	0

Here the deviation from the calculated figures is still greater than in the two preceding cases. K is about 0.006, or a little larger than for the diphtheria-poison, which exhibits the least value of K ($K = 0.004$). From these experiments it follows that the assertion of Kyes and Sachs, that the neutralisation curve for cobralysin is quite rectilinear (corresponding to $K = 0$), is not exact. This assertion does not even agree with the experiments of Kyes himself. The conclusions which these authors have drawn from their not entirely strict observations can be no longer maintained. Even had the curve been quite rectilinear, that would not have allowed them to conclude that a deviation from the straight line indicates the presence of toxoids and toxons (cf. behaviour of ammonia). It is also noteworthy that Kyes has made no experiments with mixtures containing an excess of antilysin, or at least he has not mentioned them.

Regarding the nerve-poisons of the venoms, experiments may be cited in which the poisons were injected intraperitoneally into guinea-pigs. The observations and calculations were carried out as in the case of diphtheria-poison. The following values were obtained : —

NEUTRALISATION OF 0.006 G. OF CROTALUS-POISON BY MEANS OF n. C.C. OF ANTIVENIN

n	$q_{obs.}$	$q_{calc.}$
0	100	100
0.25	75	75
0.5	58	54
0.75	38	43
1.0	29	31
1.25	21	21
1.5	14	15
1.75	12	10
2.0	8	7
2.25	4	4

Here 1 c.c. of antitoxin is equivalent to the 0.006 g. of crotalus-poison. The equation of equilibrium indicates that three molecules of reaction-products are formed from two molecules of toxin and two of antitoxin. The constant is $K = 0.048$.

The cobra-poison was studied in the same manner. It gave the following figures, which are, however, too few to be used for calculation, but still indicate that the poison behaves like the crotalus-poison.

n = 0   1   2   3   4 c.c. antitoxin on 0.003 g. of poison.
q (obs.) = 100   64   36   14   9

Very peculiar results were obtained in experiments on the neutralisation of the poison of the water-moccasin (*Ancistrodon piscivorus*). There the toxicity fell to a mini-

mum and then subsequently rose, as the following figures
indicate : —

NEUTRALISATION OF WATER-MOCCASIN POISON (0.012 G.) WITH n. C.C.
ANTITOXIN

n	$q_{obs.}$	n	$q_{obs.}$
0	100	8	19
2	56	9	24 (?)
4	44	10	19
5	33	20	36
6	24	40	65

The measurements seem to indicate that the antitoxin
itself injured the guinea-pigs, in whom it was injected in
rather large quantities (10 c.c. in the last experiments).
The results recall those obtained in the neutralisation of
a base with an acid (cf. p. 173). The great loss of
antitoxin prevented the experiments from being carried
further.

As will be seen in the next chapter, cobra-poison unites
with lecithin to form a compound, cobra-lecithid, of a very
high hæmolytic action. Kyes[1] has isolated this cobra-
lecithid and thereafter prepared an antitoxin against it by
injections of this poison into the veins of a rabbit. As
normal serum of rabbit is itself a little antitoxic to cobra-
lecithid, but loses this property after heating for 30
minutes to 64° C., the immune-serum was heated during
30 minutes to 64° C., in which treatment the specific anti-
toxin remains unaltered. With these substances, cobra-
lecithid and its antitoxin, Kyes did some experiments on

[1] Kyes: *l.c.*, p. 8.

ox erythrocytes, the results of which are given in the following table : —

NEUTRALISATION OF 0.4 C.C. SOLUTION OF COBRA-LECITHID WITH n C.C. OF ITS ANTITOXIN

n	n_1	$q_{obs.}$	$q_{calc.}$
0	0	100	100
1	0.4	65.7	64.8
2	0.8	39.2	41.1
3	1.2	28.2	27.6
4	1.6	21.2	20.0

$$K = 0.25$$

The calculated figures, which agree very satisfactorily with the observed ones, were obtained by means of the equation found for the neutralisation of tetanolysin. The high value of the constant shows that the combination is far from complete; in other words the neutralisation curve differs widely from a straight line.

Kyes and Sachs[1] stated that cholesterin has a strong neutralising influence on cobra-lecithid as well as on cobra-lysin. This action of cholesterin is even manifested against the hæmolytic power of tetanolysin and olive oil, but not against that of staphylolysin or arachnolysin.

Morgenroth[2] describes an experiment carried out by him with a mixture of cobralysin containing a little more than the double equivalent quantity of antivenin, in the presence of lecithin. This mixture (in 10 c.c. of physiological salt-solution) was brought into contact with rabbit erythrocytes

[1] Kyes and Sachs: *Berl. klin. Wochenschrift*, Nos. 2–4 (1903).

[2] Morgenroth: "Arbeiten aus dem pathologischen Institut zu Berlin," 1906, p. 11. Cf. the experiments of Madsen and Walbum with ricin, against which Morgenroth uses as an argument his experiment cited above.

for 3 hours at 37° C. and thereafter the fluid separated from the erythrocytes by centrifugalisation and its content of cobra-poison determined. As is so common, no indication is given of the magnitude of the experimental error, which we may estimate as very low, 5 per cent. Morgenroth found that "absolutely" (!) no cobra-poison had been absorbed by the erythrocytes. The correct conclusion would evidently have been that less than 5 per cent of the poison was removed. A calculation from the figures given above ($K = 0.0014$) shows that about 0.1 per cent of the poison was free in the liquid. If now the erythrocytes had absorbed ten times this quantity, it would have been five times less than the least quantity which Morgenroth would have been able to detect. Perhaps the erythrocytes absorbed even the antitoxin in this case.

It is to be regretted that Morgenroth has chosen this particular compound to determine whether a partial dissociation occurs, since it was well known from Kyes' experiments that it is dissociated to an extremely low degree (Kyes asserted that it was not dissociated at all); and also that Morgenroth used such a large excess of antitoxin to still more suppress the insignificant degree of dissociation. Morgenroth might well have used much more dissociated compounds in his experiments without still being able to detect the dissociation-products with the means employed by him in this case.

Biltz[1] has investigated the neutralisation of arsenious acid (As_2O_3) by means of "freshly precipitated ferric hydrate." This hydrate has a physical constitution like that of gel, and behaves just as a colloid in its absorption

[1] Biltz: *Ber. d. deutschen chem. Ges.* **37**. 3138 (1904).

power. Biltz finds that on adding increasing quantities of arsenious acid to a given quantity (200 c.c.) of water in which are suspended 10 c.c. of the hydrate, the quantity absorbed increases much more slowly than the concentration of the liquid. The increase of the arsenious acid absorbed by the hydrate is only proportional to the fifth root of the concentration of the liquid. This observation is in good concordance with other experiments on analogous subjects (for absorption by means of charcoal Schmidt found the fourth root, which seems to be the most general case in so-called adsorption processes).

Biltz finds that there is a very close analogy between this phenomenon and the neutralisation of toxins by means of antitoxins. It is very astonishing that Biltz has not tested his idea on a practical example, for instance on the observations regarding the neutralisation of tetanolysin or diphtheria-toxin published at that time. If I have understood Biltz aright, the toxin would be analogous to the arsenious acid in true solution and the antitoxin would be in the colloidal state and correspond to the ferric hydrate.

From this point of view I have examined the observed figures cited above regarding diphtheria-poison, tetanolysin with antilysin or cholesterin, and saponin with cholesterin or ox-serum. In the two first cases, where the reaction-products enter into the equation of equilibrium to the second power, the formula of Biltz —

$$\text{Conc. of free poison} = K \, (\text{conc. of poison in the antitoxin})^p,$$

gives a value of p which rapidly sinks with increasing concentration of the antitoxin. For the tetanolysin with antilysin, p sinks from the value 4.5 through the values

2, 3, and 1.8 and reaches at the end the value 1.3. For the diphtheria-poison No. 471 (September, 1903), the corresponding values are 150, 11, 7.3 and 4.3. For the neutralisation of tetanolysin with cholesterin, p sinks from an infinite value through 11.5 to 5.1. For the neutralisation of saponin, p goes through a minimum. With cholesterin it gives for p-values, 6.5, 3.1, and 5; with ox-serum the values 5, 1.2, 2.8, and 4.1. There can therefore be no possibility that p may be regarded as a constant, and therefore the hypothesis of Biltz must be regarded as untenable.

On the other hand, we may sum it up as our experience, that we are entitled to regard the formula of Guldberg and Waage, —

(Conc. of toxin) × (conc. of antitoxin)

$$= K \text{ (conc. of reaction-products)}^p,$$

where $p = 2$ or 1.5, or sometimes 1, as valid in all the examined cases of neutralisation of poisons by antitoxins.

CHAPTER VIII

THE COMPOUND HÆMOLYSINS

Since the earliest times the injection of the blood of animals into the veins of the human sick has been practised for therapeutic purposes. It was always known that these experiments with "transfusion" of blood were dangerous, and it was later determined that the serum from animals exerts a "globulicidal" action on the erythrocytes of human origin. Only the blood of the same species (isoserum) is innocuous.[1]

The normal serum of an animal contains a substance which hæmolyses the erythrocytes of animals of other species, and this substance was called alexin (protecting substance) by Buchner, who determined also that the alexin is rapidly decomposed at a temperature of 55° C. or more.

The hæmolytic properties of the blood-serum of an animal are increased to a high degree if the animal be immunised by the injection of erythrocytes of another species. The hæmolytic action is in this case specific against erythrocytes of the variety employed in the injections. This observation was made by Bordet,[2] who found also that the hæmolytic properties vanish after heating for thirty min-

[1] Landois: "Die Transfusion des Blutes," Leipzig, 1875, cited from Hans Sachs: "Die Hämolysine" in Lubarsch-Ostertag's Ergebnisse d. pathol. Anatomie, Vol. 7, 1902. This memoir contains a review of the literature on this subject up to that time, and of the results attained.

[2] Bordet: *Ann. de. l Inst. Pasteur*, 12 (1898).

utes to 55 ° C., but may be restored by the addition of normal serum.

The action of such an immune-serum is augmented by the addition of fresh serum, as Ehrlich and Morgenroth[1] found, and the hæmolytic power of the heated immune-serum may after the addition of normal serum exceed many times that of the original immune-serum ·before being heated. These observations led to the opinion, as was said above (p. 20), that the immune-serum contained two substances, the one the so-called immune-body (or amboceptor), stable at 55° C., and another, the alexin (complement), present even in normal serum, labile and destroyed at 55°.

Moreover, the immune-serum contains, as Bordet found, a substance which agglutinates erythrocytes of the injected variety. This agglutinin resists heating to 60° C., but loses its agglutinating properties at 70° C.

As is seen from the experiments considered on p. 150, the immune-body is absorbed by the erythrocytes in large measure. If the quantity of immune-body present is not very great, it will be practically completely absorbed by the erythrocytes. Therefore, if erythrocytes are shaken for an hour with their specific immune-serum that has been heated, this serum is afterward innocuous to other erythrocytes, even after alexin has been added. But the erythrocytes which have absorbed the immune-body may, after separation from the serum by centrifugation, be dissolved on adding an alexin to them.

In an analogous manner it is possible to show that the alexin is not absorbed to a noteworthy degree by the ery-

[1] Ehrlich and Morgenroth: *Berliner klin. Wochenschrift*, 1899, No. 22.

throcytes. For if these have been shaken for an hour with alexin and have thereafter been separated from the fluid, they remain intact on the addition of immune-body.

Ehrlich and Morgenroth have performed experiments on the binding of immune-body and alexin that have caused a great deal of discussion, but have not been explained in a satisfactory manner.[1] If the hæmolytic mixture of the two substances is shaken with the specific erythrocytes at low temperature (0–3° C.) for an hour, the immune-body (from goat-serum) is absorbed by the erythrocytes (of sheep), the alexin remaining alone in the solution. The experiment succeeds even at 40° if the time of contact between solution and corpuscles is restricted to ten minutes. If the corpuscles are then suspended in physiological salt-solution a moderate hæmolysis is observed, which is augmented if alexin (normal goat-serum) be added.

Evidently we here observe a case of velocity of reaction, in which the immune-body plays nearly the same rôle as cholesterin in Ransom's experiments with saponin. A difference is that the immune-body is very rapidly and strongly absorbed by the erythrocytes, which is evidently not the case with cholesterin. At low temperatures the velocity of reaction between immune-body and alexin is imperceptible. Therefore no measurable part of the alexin (which to a slight degree is taken up by the blood-corpuscles) becomes bound by the immune-body. But at higher temperatures a sufficient quantity of hæmolysin is formed

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[1] Ehrlich and Morgenroth: *Berl. klin. Wochenschrift*, No. 1 (1899); Gruber: *Münch. med. Wochenschrift*, Nos. 48–49 (1901), No. 2 (1904); Morgenroth: *Wiener klin. Wochenschrift*, No. 43 (1903), No. 5 (1904).

in the blood-corpuscles to cause a trace of laking. As soon as the alexin is bound, new quantities of it diffuse into the blood-corpuscles. Therefore the hæmolysis increases if corpuscles loaded with immune-body are treated with fresh alexin. On the other hand Ehrlich and Morgenroth found that the fluid in which the erythrocytes had been suspended for one or two hours at 0° C. contained alexin. In this manner they showed that even the hæmolysins in normal sera (for instance goat-serum acting upon guinea-pigs' erythrocytes) are formed by the union of an immune-body and an alexin. They also stated that a given immune-body combined with different alexins (normal sera from different animals) gives hæmolysins of very different hæmolytic power, which eventually may be due to many different circumstances.

Ehrlich and Morgenroth supposed that the compound of immune-body and alexin, i.e. the hæmolysin, which is present in the mixture of their solutions, is the active part and that it is chemically bound to the erythrocytes, which are regarded as if they were molecules. To explain the failure of the reaction at 0° C. they supposed that the hæmolysin is nearly completely dissociated at low temperatures, 0° C., but not at 40° C.; this presupposes that the union of immune-body and alexin is accompanied by an extremely great absorption of heat, which is in the highest degree improbable. The membrane of the erythrocytes is evidently to a very slight degree permeable to the hæmolysin; and, therefore, it is only the hæmolysin formed inside their membranes which exercises a poisonous action on the corpuscles, just as in the case of saponin and cholesterin. These experiments do not teach us anything concerning

the presence of hæmolysin in the mixture of immune-body and alexin.

Overton has drawn attention to the fact that the alkaloids are much more toxic to cells of animal or vegetable origin than are their salts. This depends upon the circumstance that the cell membranes are permeable to the alkaloids themselves, but not so to their salts, or, more strictly speaking, to their ions. The salts are poisonous at all only because they are to some extent hydrolysed in their watery solutions; and therefore the salts of the weak acids are more poisonous than those of the strong acids. Under these circumstances an excess of acid diminishes the toxicity of these salts, even to the point of complete suppression of toxic action. On the other hand, the presence of substances with alkaline reaction sets the alkaloid free, and thus increases the toxicity of the solution of the salt of an alkaloid. (Cf. Hoeber, *Physikalische Chemie der Zelle und Gewebe*, 2d ed., 1906, p. 165.)

In some cases, which seem to be rather rare, the absorption of the immune-body by the erythrocytes is by far not so evident as in the ordinary case represented by the figures above (p. 150). Ehrlich and Sachs[1] describe some experiments on the action of a mixture of inactivated ox-serum and normal horse-serum on guinea-pigs' erythrocytes. These are at 37° C. laked by the said mixture within one hour. But if the erythrocytes are suspended for one hour at 37° C. in the ox-serum, and then after centrifugation mixed with the horse-serum, no such result is observed.

Ehrlich explains this observation as due to the circum-

[1] Ehrlich and Sachs: *Berl. klin. Wochenschrift*, No. 21 (1902).

stance that the corpuscle has no affinity for the immune-body until it is bound to the alexin. Evidently this is only a very artificial circumscription, and no real explanation. Bordet, however, later on proved that even in this case the immune-body is absorbed; to his investigation on this subject we shall return later (cf. p. 260).

The first theoretical view of these phenomena was given by Bordet,[1] who regarded the immune-body as a kind of catalytic agent, which "sensibilised" the erythrocytes to the attack of the alexin. The objection that alexin alone does not attack the erythrocytes is evidently untenable. Bordet advanced his experiment on the effect of a fractionated addition of hæmolytic serum (cf. p. 34) to a given portion of erythrocytes as pleading in favour of his idea. This experiment indicated that the binding to the cells does not take place in constant proportion, as Ehrlich tacitly supposed. But Ehrlich replied that the cells might bind more hæmolysin than just the quantity necessary for laking. Since we know now that the immune-body is absorbed by the erythrocytes, this controversy has only an historical interest. In a certain sense we must allow Bordet to have been in the right, the entrance of the immune-body into the erythrocytes is the necessary condition for the attack of the alexin, which, as we shall see later, is really bound to equivalent quantities of the immune-body dissolved in the erythrocytes. Against Bordet the experiment of Ehrlich and Sachs has been cited, but this is easily explained by the view that the immune-body is absorbed.

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[1] Bordet: *Annales de l'Inst. Pasteur*, **12**. 688 (1898), and **14**. 257 (1900).

An experiment of Neisser and Wechsberg[1] seems to indicate that immune-body and alexin, when mixed, really enter into a compound, at least partially. They used different bacteriolysins, which like the hæmolysins are of a compound nature, so that the presence of two different substances, an immune-body and an alexin, is necessary. They used a constant quantity of bacteria and of alexin, to which they added different quantities of immune-body. At first, as we know, the action increases with the concentration of the immune-body, but finally reaches a maximum; and, if this quantity exceeds a certain magnitude the effect then decreases, so that the bacteria may even not be destroyed. This phenomenon has been called the "diversion" (*Ablenkung*) of the alexin. As the experiments with bacteriolysins are not very well adapted to quantitative experiments, the following hæmolytic experiments with ox erythrocytes, immunised rabbit-serum as immune-body, and guinea-pig-serum as alexin, may serve to elucidate the relations. In the following table the quantity of alexin present in 2.5 c.c. of solution is called b, and the corresponding quantity of immune-body is called a. As unit is taken the thousandth part of the quantity contained in 1 c.c. of the two original preparations. The quantity of erythrocytes was 1 c.c. of a 5 per cent suspension of ox blood. The mixture, which had been kept for thirty minutes at 24° C., was allowed to act on the erythrocytes for two hours at 37° C. In this manner I found the following degrees of hæmolysis (complete hæmolysis = 100):—

[1] Neisser and Wechsberg: *Münch. med. Wochenschrift* (1901).

ACTION OF DIFFERENT QUANTITIES OF IMMUNE-BODY ("DIVERSION")

$a =$	$b = 10$	$b = 6$	$b = 4$
1	31	—	—
10	45	37	30
30	100	81	71
50	100	87	65
100	100	92	64
200	100	35	15
300	64	24	7

The effect is very evident. The maximum in the three series occurs at about $a = 80$, 50, and 30 respectively. This observation seems to agree very well neither with the views of Bordet nor with those of Ehrlich. If the catalytic agent were present in greater quantity, we might expect a stronger action, but the reverse is the case if a is greater than 100. Now according to Ehrlich's view we might expect that the active substance, the hæmolysin which is here preformed in the mixture and thus attacks the corpuscles, should not decrease in quantity with increasing concentration of immune-body, and therefore the effect should be just as according to Bordet's theory. Ehrlich has accepted the following explanation given by Neisser and Wechsberg for their experiment, in which the bacteria were not attacked in the presence of an excess of immune-body. "If a is large, there is a marked excess over the quantity of alexin (b), so that all the alexin is combined as lysin (ab) before the mixture is added. The excess of a is then bound to the bacteria (e) and gives the compound ea. If now the affinity of b for a is greater than for ea, b will remain as lysin (ab) in the solution, and

Q

not pass into *ea* to give the compound *eab* which gives bacteriolysis. It is mere chance whether the affinity of *b* for *a* is greater than for *ea*, as in this case, or less, in which case hæmolysis would occur." This extremely artificial explanation seems to me quite erroneous. For if we have the compounds *ea* and *ab* and the compound *eab* may be formed, its formation depends wholly upon whether *e* has a greater affinity for *ab* than for *a*.[1] If not, then *eab* is not formed, even if *a* is not present in excess. Therefore, if Ehrlich's idea were right, no bacteriolysis should occur in Neisser and Wechsberg's experiments, and no hæmolysis in the experiments cited above.

The probable cause is the following. If *a* is large, it is not totally absorbed by the cells, but a fraction of it remains in the surrounding fluid, and this part increases rapidly with increasing *a* (cf. p. 150). *a* forms a compound (*ab*) with *b* as well outside the cells as within them. This compound is partially dissociated, so that its quantity increases and the quantity of free alexin *b* decreases with increase of *a* in the liquid. At very high excess of *a*, *b* may become practically wholly bound. This seems to have been the case in Neisser and Wechsberg's experiment (the errors of observation are too great to give a final decision). The membranes of the cells are (cf. p. 221) practically impermeable to the lysin (*ab*), and only the lysin formed from *a* and *b* within the cells is active. As there is now very little (or practically no) free *b* in the

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[1] It is assumed that the amount of *a* exceeds the quantity which is necessary to bind (produce lysis with) the quantities *e* and *b*. This necessary quantity of *a* is rather unimportant, about 2 in my experiments cited above, and even in Neisser and Wechsberg's experiments this condition has probably been fulfilled long before *a* gave a maximum.

solution, b cannot diffuse into the cells during the given time of the experiment and produce a lysis, whereas a is present there in many times the necessary quantity. If a decreases, but not so much so that the quantity necessary for the lytic action is not absorbed by the cells, then b increases in the surrounding fluid and a greater quantity of it diffuses into the cells within a given time. Therefore the quantity of ab present in the cells may increase with decreasing quantity of a, if this substance be present in great excess in the surrounding fluid. The presence of a maximum is therefore quite easy to understand. The very great flatness of the curve of this maximum indicates that the hæmolysin ab is stable only in the presence of a large excess of a and b; and that a binding of strong affinities with sharp discontinuities, as Ehrlich supposes, is excluded. Our view leads furthermore to the conclusion that the effect observed by Neisser and Wechsberg should be more prominent for low values of b than for larger values. The quantity of diffusing free alexin is in the first approximation proportional to b. This quantity is therefore proportional also to the quantity of hæmolysin formed in the given time of action (two hours at $37°$ C. and the time of sedimentation at lower temperature, about $3°$ C.). With large quantities of alexin ($b > 20$ in the present case), the diffusion transports enough of alexin during the time of action to cause total hæmolysis, even at the highest concentrations of a employed. Further, it is evident that as the quantity of free alexin is nearly proportional to b and inversely proportional to a, nearly the same effect will be reached if $\dfrac{b}{a}$ is a

constant. This is actually found to be the case; for instance, the degree of hæmolysis 64 corresponds to the following values of $\dfrac{b}{a}$: $\dfrac{10}{300} = 0.033$, $\dfrac{6}{150} = 0.04$, and $\dfrac{4}{100} = 0.04$. This is evidently true only if the hæmolytic action is of such a magnitude as it is possible to reach with the quantity of b present; and this circumstance indicates that the said rule is only a rather rough approximation. The rule stated leads to the consequence that the maximum appears at a lower value of a for a lower value of b, and the values of a at the maximum are nearly proportional to the value of b ($80 : 10 = 8$; $50 : 6 = 8.3$; $30 : 4 = 7.5$).

It had been observed in different investigations that the quantity of alexin necessary to produce complete hæmolysis is the less the greater the quantity of immune-body that had been used for the "sensibilisation" of the erythrocytes.[1] This matter was subjected to a closer investigation by Morgenroth and Sachs.[2] They found that in one case (viz. hæmolysis of sheep erythrocytes by means of immune-bodies from goat blood and alexin from guinea-pig serum) the quantity (b) of alexin was often nearly inversely proportional to the quantity (a) of immune-body used. In another case (hæmolysis of erythrocytes from ox blood with immune-body from goat-serum and an alexin from serum of guinea-pig or of rabbit or even of sheep) the quantity of alexin necessary for complete hæmolysis was independent (or nearly so) of the quantity of immune-body used. In other cases the same

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[1] v. Dungern: *Münch. med. Wochenschrift*, No. 20 (1900); Gruber: *Wiener klin. Wochenschrift*, No. 15 (1902).

[2] Morgenroth and Sachs: *Berl. klin. Wochenschrift*, No. 35 (1902).

was true except when the quantity a was very low. To explain this very different behaviour in different cases the authors have made use of rather complicated hypotheses, to enter in detail upon which would consume too much space. These experiments were the first quantitative measurements, on a large scale, bearing upon the action of compound hæmolysins, and they therefore merit a certain interest.

Another observation of quantitative nature was made by Morgenroth.[1] He determined the least quantity of alexin that had to be added to produce complete hæmolysis, and the greatest quantity of alexin which could be added before a perceptible hæmolysis occurred. In this case immune-body was present in excess. In other cases alexin was present in excess, and the quantities of immune-body necessary for complete hæmolysis and for the first trace of hæmolysis were observed. Morgenroth found that the two said quantities of alexin were, as the average of 13 combinations, in the proportion 100 to 13.6; and the corresponding quantities of immune-body in the proportion 100 to 14.1 (mean of 10 combinations). If, as is rather probable, the quantity of hæmolysin found is nearly proportional to the quantities of alexin or of immune-body used, and the rule holds that the degree of hæmolysis is proportional to the square of acting hæmolysin, this would indicate that an hæmolysis of 2 per cent is just perceptible, which in some cases may be rather probable.

Quite recently Wilfred H. Manwaring[2] has given a

[1] Morgenroth: *Wiener klin. Wochenschrift*, No. 5 (1904).
[2] Manwaring: *Journ. Biol. Chemistry*, 1. 213 (1906). No definite indication is given of the nature of the preparations employed.

curve representing the degree of hæmolysis by means of different quantities of immune-body in the presence of a great excess of alexin. It may here even be assumed that the quantity of hæmolysin found is proportional to the quantity of immune-body. The following figures are taken from the original curve. *H* is the degree of hæmolysis, *A* the quantity of immune-body.

HÆMOLYSIS BY MEANS OF DIFFERENT QUANTITIES OF IMMUNE-BODY

A	H	$\sqrt{H}$	$\sqrt{H} : A$
1	0.5	0.71	0.71
2	1	1	0.5
3	1.5	1.23	0.41
4	2	1.41	0.35
5	2.5	1.58	0.32
6	3	1.73	0.29
7	4	2.0	0.29
8	6	2.45	0.31
9	9	3.0	0.33
10	22	4.69	0.47
11	35	5.92	0.54
12	47	6.86	0.57
13	58	7.62	0.59
14	68	8.25	0.59
15	79	8.89	0.59
16	84	9.17	0.57
17	86	9.27	0.55
18	90	9.49	0.53
19	93	9.64	0.51
20	97	9.85	0.50
21	99	9.95	0.47
22	100	10.0	0.45

Between the degrees of hæmolysis 35 and 90, *i.e.* within two-thirds of the interval examined, a remarkable proportionality between the square root of the degree of hæmolysis and the quantity of immune-body is observed. With

lesser quantities of immune-body, the hæmolysis is not so great as this rule predicts, and this corresponds to the behaviour of other lysins (cf. pp. 103 and 169), except that the deviation from the rule in this case is evident throughout the wide interval from 0 to 30 per cent, where as for other lysins the deviation is observed only under 10 or 15 per cent.

In the memoirs cited Morgenroth and Morgenroth and Sachs employed,[1] among other hypotheses to explain their observations, this also, that immune-body and alexin sometimes enter into a compound hæmolysin or hæmolysin united with an erythrocyte, which is stable only in the presence of considerable quantities of the components. These different substances participate, according to the Frankfort school, in a chemical equilibrium. On the other hand, the adherents of Bordet, among whom Metchnikoff, Gruber, and Biltz may be cited, regard the hæmolytic action as a phenomenon of absorption. It seemed possible to decide by means of quantitative experiments which of these two ideas corresponds to the facts, and Ehrlich invited me to undertake an investigation of this point in the laboratory of the serum institute in Frankfort-on-the-Main. These investigations led to the very certain conclusion that a chemical equilibrium governs the reaction of immune-body and alexin, but the important reaction takes place within the erythrocytes. It is very probable also that such an equilibrium exists, even in the mixture of immune-body and alexin, as is indicated by the diminution of the hæmolytic effect with increasing immune-body, as noted in some cases

[1] Morgenroth: *Wiener klin. Wochenschrift*, No. 43, p. 5 (1903); Morgenroth and Sachs: *Berl klin. Wochenschrift*, No. 35, p. 8 (1902).

(cf. p. 225). And it is even probable that the hæmolysin enters into combination with the molecules of the protoplasm inside the erythrocytes, just as tetanolysin or alkalies do (cf. pp. 103 and 110); but concerning this reaction we learned very little or nothing from my experiments.

My measurements were founded upon determinations of the degree of hæmolysis exerted by a given mixture of immune-body and alexin acting upon a given quantity of erythrocytes. To this end 1 c.c. of a 5 per cent suspension of erythrocytes (from sheep or ox) in physiological solution of NaCl was mixed with 1.5 c.c. of a fluid containing the desired quantities of immune-body and alexin and additional physiological salt-solution until the total volume was always 2.5 c.c. All the preparations were kindly supplied by the Institute.

After the blood suspension and the hæmolytic mixture had been each mixed thoroughly by shaking (for reasons referred to above, cf. p. 15), the cell suspension was poured into the mixture in a rapid manner, the test-tubes containing the mixture were placed in an incubator at 37° C. for 2 hours, after which time the test-tubes were taken out and set into a refrigerator at about 2° C., where they remained until the next morning (about 17 hours), when they were examined and the degree of hæmolysis determined by comparison with test-tubes containing different quantities of the same variety of erythrocytes, laked in distilled water.

The immune-body and the alexin were left together for about half an hour at room temperature (20–24° C.) before the addition of the cell suspension, but the time of reaction between these two substances seemed not to have a per-

ceptible influence, as special experiments indicated. As has been said, probably the chemical reaction takes place, at least chiefly, in the interior of the red blood-corpuscles, and under such circumstances it is easy to understand that the time of reaction between immune-body and alexin prior to the addition of the blood is of little or no consequence. Since we know the hæmolysed quantity, we wish to calculate from it the quantity of hæmolysin. For this purpose we may employ the rule that the hæmolysed quantity is nearly proportional to the square of the quantity of acting hæmolysin. If this rule for low concentrations of hæmolysin does not lead to quite exact results, this result does little harm, for the chief thing is to find instances in which the quantity of hæmolysin is the same, and this occurs, evidently, as soon as the degree of hæmolysis is the same. Approximately the quantity of hæmolysin is proportional to the square root of the known quantity of hæmolysis. In reality we make use of this rule only in order to set forth the observations in a simple manner.

As illustration I reproduce here a series of experiments with red blood-corpuscles from the ox. The immune-body was prepared by injection of such erythrocytes into the veins of a goat. The alexin was normal serum of guinea-pigs. The arbitrary units employed are 0.001 of a c.c. of the preparations containing immune-body or serum of guinea-pigs. The unit of hæmolysin is a hundredth part of the quantity necessary for total hæmolysis of the fixed quantity of blood-corpuscles. The first of the following series gives the observed hæmolysed quantities, the second the quantities of hæmolysin (square root of the foregoing

multiplied by 100). In parentheses are tabulated the calculated values of the same quantities according to the formula : —

$$(5\,a - x)(20\,b - x) = 90\,x.$$

Where a is the quantity of immune-body added, and b the corresponding quantity of alexin, x is the quantity of hæmolysin formed.

EQUILIBRIUM BETWEEN IMMUNE-BODY FROM GOAT, ALEXIN FROM GUINEA-PIG, AND HÆMOLYSIN FOR OX ERYTHROCYTES

A. HÆMOLYSIS

$a =$	10	30	100	300	900
$b = 60$	16 (21)	—	—	—	—
40	14 (20)	—	—	—	—
25	14 (18)	—	—	—	—
15	15 (14)	—	—	—	—
10	14 (10)	50 (70)	96 (100)	100 (100)	—
6	5 (6)	35 (36)	72 (96)	96 (100)	—
4	4 (4)	20 (19)	56 (43)	67 (53)	—
2.5	—	6 (8)	26 (18)	22 (22)	—
1.5	—	2 (3)	6 (6)	5 (8)	6 (9)
1	—	—	2 (3)	2 (3)	3 (4)
0.6	—	—	1 (1)	2 (1)	2 (1)

B. QUANTITY OF HÆMOLYSIN

$a =$	10	30	100	300	900
$b = 60$	40 (46)	—	—	—	—
40	37 (45)	—	—	—	—
25	38 (42)	—	—	—	—
15	39 (37)	—	—	—	—
10	38 (33)	71 (84)	98 (100)	100 (100)	—
6	22 (25)	59 (60)	85 (98)	98 (100)	—
4	20 (20)	45 (44)	75 (66)	82 (73)	—
2.5	—	24 (29)	51 (43)	47 (47)	—
1.5	—	15 (18)	25 (25)	22 (28)	24 (29)
1	—	—	15 (17)	15 (19)	18 (20)
1.6	—	—	11 (10)	13 (11)	13 (12)

As will be seen from these figures, the agreement between experiment and calculation is very satisfactory and the differences lie within the possible errors of observation. For low values of x, the observed values are generally less than the calculated ones, which may be due to the deviation from the rule of square roots. The time of reaction was long enough to yield nearly the limit value of the reaction, so that it was not necessary to measure the time with great exactitude.

If, now, the immune-body acted like a catalytic agent, we might expect that the hæmolysis, at a constant concentration of immune-body, would increase with the quantity of alexin, as it indeed does if the quantity of immune-body is not very low. But if the quantity of alexin is sufficient for total hæmolysis ($b > 5$), then the reaction should attain total hæmolysis more slowly with low quantities of immune-body and more rapidly with higher quantities.

For small quantities of immune-body the limit of reaction would therefore not be reached before total hæmolysis was attained, as soon as $b > 5$. This does not agree at all with experience. With small quantities of immune-body the hæmolysis shows itself to be nearly independent of the added quantity of alexin as soon as this exceeds a certain quantity ($b > 10$). This can be explained only by assuming a chemical reaction in which the immune-body contributes material to the formation of hæmolysin. With low quantities of immune-body there cannot be formed a greater quantity of hæmolysin than is equivalent to the available quantity of immune-body; the added quantity of alexin may be of any magnitude. This corresponds very well with the observations and the same reasoning is evidently valid

for the alexin. If it be added in small quantity, there cannot be formed more than the equivalent quantity of hæmolysin. This property is also very evident from the measurements.

Much better than all general considerations do the agreements between the values calculated from the formula and the observed values indicate the correctness of the view adopted. This formula indicates also that one unit of the hæmolysin (that is, the hundredth part of the quantity necessary to hæmolyse completely 1 c.c. of a 5 per cent suspension of bovine red blood-corpuscles) is equivalent to the fifth part of a unit of immune-body and to the twentieth part of a unit of alexin. The circumstance that the quantity of hæmolysin is to be subtracted from the quantity of added immune-body and alexin, recalculated to equivalent quantities, shows that both substances are consumed in the formation of the hæmolysin. The form of the equation indicates also that one molecule of immune-body and one molecule of alexin form one molecule of hæmolysin.

Another illustration is red corpuscles from sheep blood attacked by a hæmolysin formed of an immune-body from a goat injected with sheep blood, and alexin in serum from guinea-pigs. The quantity of hæmolysin, x, was calculated with the formula : —

$$(40\,a - x)\,(25\,b - x) = 1900\,x.$$

I give only the series for the observed hæmolysis.

The agreement between the observed and calculated values is, as in the preceding illustration, as good as could be desired, that is, within the possible experimental errors.

HÆMOLYSIS OF ERYTHROCYTES FROM SHEEP BY IMMUNE-BODY FROM GOAT
AND ALEXIN FROM GUINEA-PIG

$a =$	1	3	10	30	100	300	1000
$b = 60$	5 (4)	—	—	—	—	—	—
40	5 (2)	17 (16)	—	—	—	—	—
25	2 (2)	15 (9)	60 (75)	—	—	—	—
15	—	5 (4)	35 (33)	95 (100)	—	—	—
10	1 (0.3)	3 (2)	20 (16)	80 (85)	95 (100)	—	—
6	—	2 (1)	7 (6)	35 (31)	75 (100)	100 (100)	100 (100)
4	—	—	3 (3)	15 (15)	35 (44)	80 (75)	70 (90)
2.5	—	—	—	7 (6)	15 (17)	30 (30)	50 (36)
1.5	—	—	—	—	4 (6)	4 (10)	17 (13)
1	—	—	—	—	—	2 (4)	3 (6)

It may perhaps seem strange that greater quantities of alexin have not been used, but the normal serum of guinea-pigs in itself contains a little hæmolysin, so that it is necessary to introduce a correction for this action; a simple subtraction was used. For greater concentrations of alexin the corrections would be rather great, and as they are always accompanied by some uncertainty, it seemed best not to use such concentrations.

Even in this case the formula indicates that from one molecule of immune-body and one molecule of alexin one molecule of hæmolysin is formed. This is not always the case, as will be seen from the following examples. The indicator used was bovine red blood-corpuscles; the immune-body was prepared from the blood of a rabbit injected with bovine corpuscles; the alexin was normal serum from the guinea-pig. The arrangement of the experimental series was quite the same as in the instances treated above. The values concern the hæmolysis.

Here we meet with the exponent $\frac{2}{3}$, which seems to be common in this domain. The equation indicates that two

molecules of immune-body with three molecules of alexin yield six molecules of hæmolysin.

HÆMOLYSIS OF BOVINE ERYTHROCYTES BY IMMUNE-BODY FROM RABBIT AND ALEXIN FROM GUINEA-PIG

$a =$	0.4	1	10	50	100	300
$= 60$	12 (11)	32 (35)	—	—	—·	—
40	12 (10)	27 (26)	100 (100)	—	—	—
25	5 (7)	18 (17)	70 (83)	—	—	—
15	3 (5)	6 (11)	40 (44)	—	90 (100)	—
10	—	5 (7)	27 (27)	—	70 (59)	—
6	—	2 (4)	12 (13)	40 (22)	22 (26)	—
4	—	1 (3)	4 (7)	10 (10)	3 (12)	10 (14)
2.5	—	—	2 (3)	3 (5)	2 (5)	5 (6)

The equation used for the calculation has the form :—

$$(100\,a - x)^{\frac{2}{3}}(10\,b - x) = 1.8x^2.$$

HÆMOLYSIS OF ERYTHROCYTES FROM OX BY MEANS OF COBRA-LECITHID

LECITHIN =	2	3	10	50	100
Cobra = 250	88 (94)	—	—	—	—
150	80 (57)	100 (100)	—	—	—
100	32 (37)	72 (79)	—	—	—
75	32 (28)	64 (59)	—	—	—
50	20 (20)	36 (39)	100 (100)	—	—
35	10 (13)	32 (28)	88 (87)	—	—
25	8 (9)	32 (20)	66 (62)	100 (100)	—
15	—	8 (12)	36 (38)	72 (84)	—
10	—	4 (8)	—	60 (56)	100 (100)
7.5	—	—	—	40 (42)	68 (96)
5	—	—	—	36 (28)	64 (64)
3.5	—	—	—	4 (20)	40 (45)
2.5	—	—	—	—	40 (32)
1.5	—	—	—	—	32 (16)
1	—	—	—	—	24 (13)

In all these cases the immune-body and the alexin are

consumed to a sensible degree in the formation of the hæmolysin. Therefore in the formulæ *x* is always subtracted from the equivalent quantity of immune-body and alexin. In another similar case, namely, the formation of the hæmolytic substance cobra-lecithid from cobra-poison and lecithin, the quantity of hæmolysin seems always to be so small that it makes no difference if it be subtracted from the quantities of cobra-poison and lecithin or not. The experiments were carried out with a 0.1 per cent solution of cobra-poison (1 c.c. = 10,000 units) and a 1 per cent solution of lecithin (1 c.c. = 1000 units). The arrangement of the table is the same as in the foregoing.

The calculated values were obtained with the aid of the formula: —

$$C\,(L - 1.5)^{\frac{2}{3}} = 6.67x^2 = 6.67\,h,$$

where *C* represents the quantity of cobra-poison added, *L* the corresponding quantity of lecithin, *x* the quantity of hæmolysin formed, and *h* the observed quantity of hæmolysis tabulated above. As will be seen from the equation, no hæmolysis occurs until 1.5 units of lecithin have been added. This quantity was determined by special experiments. The experiments with cobra-lecithid indicate that this poison behaves a little differently than the other hæmolysins. With these a limit of the reaction was practically reached on standing for two hours in an incubator at 37° C., and afterwards for seventeen hours in a refrigerator. But in the case of cobralysin, hæmolysis continued in the sedimented blood-corpuscles after the observations cited above were ended, so that the result was wholly different after an additional twenty-four hours, during the

chief part of which time the temperature was 2° C. The hæmoglobin that had passed out in the last twenty-four hours had not had time to diffuse throughout the liquid, but remained in the lowest strongly coloured stratum. These observations suggest strongly that the hæmolytic substance was absorbed by the erythrocytes. The quantity, 1.5, of lecithin must in some manner be bound by a foreign substance in the blood suspension, so that it is not available for the cobra-poison.

In this case we cannot see from the formula that cobra-poison and lecithin are consumed for the formation of the cobra-lecithid. This must therefore be dissociated in solution to an extremely high degree, and the least quantities of it must be sufficient to produce hæmolytic actions. But precisely for this case Kyes has made it probable that a compound — cobra-lecithid — is formed, that even in very small quantities produces hæmolysis.[1]

The power $\frac{2}{3}$ for the quantity of lecithin in the last formula is the same which is sometimes found for the immune-body, but never for the alexin. This seems to indicate that the lecithin plays the same rôle as the immune-body in the formation of the hæmolysin, and that the cobra-poison corresponds to the alexin. This opinion seems corroborated by the fact that the alexin is regarded as the properly poisonous substance of the two, and it seems more natural to suppose that the cobra-poison is the carrier of the poisonous properties, than that the innocuous lecithin acts hæmolytically.

Quite recently there has been a vivid discussion, if lecithin exerts an influence on the hæmolytic action of a

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[1] Preston Kyes: *Berl. klin. Wochenschrift,* Nos. 42 and 43 (1903).

very simple substance, namely, mercuric chloride.[1] As this process is rather perspicuous and indicates the mode of action of lecithin, we will take it under consideration for some moments.

In weak doses mercuric chloride acts hæmolytically; in greater concentration it agglutinates the erythrocytes and the hæmolysis is weak or insensible. At a certain concentration, therefore, the degree of hæmolysis passes through a maximum. The disappearance of the hæmolytic action at higher concentrations is regarded as due to the hardening of the protoplasma and especially of the membranes of the erythrocytes, whereby the passage of hæmoglobin through it is hindered. As Sachs has shown,[2] it is possible to provoke the hæmolysis of such hardened erythrocytes by treating them with solutions of potassium iodide or hyposulphite of sodium or of albumen, which all take away a deal of the mercuric chloride from the erythrocytes, which have been in contact with a solution of this substance.

The observed maximum, therefore, in this case depends upon a double action of the mercuric chloride, one destructive, whereby the erythrocytes give away their hæmoglobin to the surrounding fluid, and one hardening, whereby the permeability of the membranes of these cells is checked. It is well possible that other similar cases, in which maxima are observed, as for instance with the botulismus-poison or with mixtures of saponin and

[1] Detre and Sellei: *Berl. klin. Wochenschrift*, No. 30 (1904); *Wiener klin. Wochenschrift*, Nos. 45 and 46 (1904); No. 30 (1905). Sachs: *Wiener klin. Wochenschrift*, No. 35 (1905).

[2] Sachs: *Münchner medicinische Wochenschrift*, No. 5 (1902).

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cholesterin, may be due to a secondary action, whereby the velocity of the process is retarded, concurring with the chief action of the poison.

Detre and Sellei had observed that the hæmolytic action of mercuric chloride is diminished by the presence of lecithin. Against this Sachs stated that lecithin accelerates the action of mercuric chloride. Sachs rightly holds the opinion that no poisonous combination of mercuric chloride and of lecithin exists, but that we here observe an action of the lecithin on the erythrocytes, whereby these are rendered more accessible to the mercuric chloride. Here we find that Sachs takes up the opinion of Bordet regarding the sensibilisating action of certain substances on erythrocytes. Evidently the lecithin enters into the cell-membranes and increases their permeability to the hæmoglobin or to the mercuric chloride.

According to this observation it seems obvious to suppose that the action of lecithin on erythrocytes treated with cobra-poison is due to a similar circumstance. The slowness of the hæmolytic action in this case speaks in favour of this opinion. If the chief part of the lecithin remains in the fluid, the absorbed part in the membranes of the erythrocytes is proportional to the $\frac{2}{3}$ power of the quantity of lecithin, diminished by the chemically bound part of it. The results given above regarding the action of cobra-poison in presence of lecithin will then be understood if we make the very plausible hypothesis that the hæmolytic action is proportional to the concentration of the cobra-poison, and further that the permeability of the cell-membranes is proportional to the absorbed quantity of lecithin.

As we have seen before, the immune-bodies in the rabbit treated with ox erythrocytes, and in the goat treated with sheep erythrocytes are almost completely absorbed by the red blood-corpuscles (cf. p. 150) in weak solutions. The same is probably the case for the immune-body from a goat treated with ox erythrocytes. The formation of hæmolysin is, without doubt, effected only in the red blood-corpuscles themselves, for these absorb the immune-body before a reaction takes place (cf. p. 220). On the other hand, they dissolve very little of the alexin, but this is combined with the immune-body, so that new alexin enters and forms the poisonous hæmolysin. The circumstance that the alexin always enters to the power 1 would then indicate that the alexin has the same molecular weight in the blood-corpuscle and in the surrounding fluid, so that a constant fraction, probably very little, is always contained within the blood corpuscles. In the equation of reaction this fraction should probably be introduced, but if instead of that we, as in the formulæ above, write the whole quantity of alexin, that has no other effect than that the constant of equilibrium changes in a certain proportion. The hæmolysin formed probably remains for the greatest part absorbed in the red blood-corpuscles.

As for lecithin, it probably enters very easily into the red blood-corpuscles, and therefore plays the rôle of an immune-body.

The action of the compound hæmolysins is therefore, like that of the simple hæmolysins, based upon their presence in the red blood-corpuscles, which are thereby so changed that the membrane becomes permeable to hæmoglobin.

As we have seen before, the immune-body at higher

concentrations is not wholly absorbed by the erythrocytes, and therefore gives a compound with the alexin, even outside the erythrocytes. Thereby we explain the seeming anomaly that a less degree of hæmolysis may be produced by a greater quantity of immune-body (cf. p. 224). It is evident that equations like those given above are not able to reproduce such a phenomenon. The same effect may even exert a diminishing influence, though to a lesser degree, on the action of the immune-body. It would therefore be conceivable that this effect is responsible for the exponent $\frac{2}{3}$ for the quantity of the immune-body; and that in reality, if this disturbing effect did not occur, the first power of the said quantity would result from the experiments. This opinion seems confirmed by the fact that the exponent $\frac{2}{3}$ occurs precisely for the combination of immune-body from the rabbit treated with ox erythrocytes and alexin from guinea-pigs, which in another series of experiments — with different preparations — gave a very pronounced diversion of the alexin. But this explanation is not applicable to the action of lecithin in the experiments with cobra-lecithid.

My experiments show that even in this case a remarkable regularity governs the binding of immune-body and alexin. Therefore it has not been necessary for me to make use of a large number of different methods of explanation, as Morgenroth and Sachs did. To explain the results of their measurements they suppose that the immune-body is bound to the erythrocytes in different manners, that the affinity of immune-body for alexin is altered to different degrees by the influence of the erythrocytes, and that the immunised sera contain a great number

of different immune-bodies. " We observe that the different phenomena which we have found correspond to relative quantities of immune-bodies and alexins (at complete hæmolysis) may have very different causes, but that they, if we regard all these factors related above, may be explained in an unconstrained manner." [1] On the other hand, I have found that the observed phenomena may be explained by assuming that the law of mass action governs the equilibrium of reaction between immune-body and alexin, and that no special hypotheses are necessary for the special cases.   It may even be regarded as very probable that a closer investigation of the combinations examined by Morgenroth and Sachs would have led them to a more simple explanation than that which they have proposed.

We have stated above that after repeated injections of a poison, *e.g.* ricin, into the veins of an animal, *e.g.* a rabbit, the serum of this animal contains an antitoxin, in this case antiricin.   If we inject this antibody into the veins of another animal, *e.g.* a guinea-pig, this animal is said to be passively immunised against ricin, *i.e.* its blood-serum contains the injected antiricin, which slowly disappears (cf. p. 4) from the blood.   On the other hand, the animal produces an antibody against the antiricin, as may be shown by experiments with mixtures of ricin, antiricin, and the anti-antiricin.   Such experiments were executed by Bashford,[2] who even says that some of his experiments indicate analogous properties of blood from animals injected with diphtheria antitoxin, or with antitetanolysin.

[1] Morgenroth and Sachs: *Berl. klin. Wochenschrift*, No. 35, p. 8 (1902).
[2] Bashford: *Journ. of Pathology and Bacteriology*, 9. 192 (1903).

The experiments do not succeed if the passively immunised animal is of the same species as that which has produced the antitoxin.

Probably the new antibody, the anti-antiricin, binds the antiricin, just as ricin does, so that the antiricin becomes divided between the ricin and the antibody; and therefore the ricin acts as if a less quantity of antiricin than that used in the experiment had been employed.

In the same manner it is possible by intravenous injection of a hæmolysin (*i.e.* an antierythrocytic substance) to produce corresponding antibodies. Thus, for instance, after the injection of immune-body from the blood-serum from a rabbit which had been actively immunised against bovine erythrocytes into the veins of a guinea-pig, this animal presented in its serum an anti-immune-body, specific against the injected immune-body. Such experiments were first carried out by Bordet,[1] who called the new antibody "anti-sensibilisatrice." Nearly simultaneously Ehrlich and Morgenroth[2] produced what they called "anti-immune-bodies," and later on "anti-amboceptors." Pfeiffer and Friedberger[3] prepared anti-immune-bodies against cholera-serum from a goat, by injecting such serum into the veins of a rabbit.

Now, a compound hæmolysin contains immune-body and alexin and hæmolysin; it may therefore seem possible to obtain antibodies against these three different substances. To determine if the antibody be anti-immune-

[1] Bordet: *Ann. de l'Inst. Pasteur*, **14**, 270 (1900).

[2] Ehrlich and Morgenroth: *Berl. klin. Wochenschrift*, No. 31 (1900); Nos. 21 and 22 (1901).

[3] Pfeiffer and Friedberger: *Berl. klin. Wochenschrift*, No. 1 (1902).

body or antialexin, Ehrlich and Morgenroth proceeded in the following manner. Immune-body is added to the antibody, and if this is an antialexin, it leaves the immune-body free, which thereafter may be extracted with erythrocytes, that may afterward be hæmolysed through treatment with alexin. If it be an anti-immune-body, it binds the immune-body present, which thereafter cannot be extracted by erythrocytes.

Evidently the antihæmolysin, if such an antibody exists, behaves in this case as the antialexin. The specificity is not very prominent. Normal serum contains anti-immune-bodies and antialexins, and after its injection into animals these produce antialexins. According to Ehrlich and Morgenroth, inactivated serum heated to 56° C., which ought not to contain alexin, still gives an antialexin after injection. (The antialexins resist a temperature of 55–60° C.; usually they are heated before using in order to free them of perturbations through the presence of alexins.) Another peculiarity was found by Ehrlich and Morgenroth, namely, that serum from a goat previously injected with serum from a rabbit contains an antialexin, not only against the alexin in rabbit-serum, but also against the alexin contained in guinea-pig-serum. The same antialexin is even contained in the serum of a goat treated by injections of horse-serum.

The antialexins have been regarded as especially important because they bind the alexins, which are — according to the views of Bordet as well as of Ehrlich — the effective parts of the hæmolysins. Therefore the chief part of the work on the antihæmolytic substances has been concerned with investigations of antialexins,

which were generally prepared simply by the injection of the normal serum of one animal into the veins of another animal. As will be seen from the following accounts of the results of experiments, the sera thus prepared seem to contain anti-immune-bodies as well as antialexins.[1] And regarding the relative probability of the production of these two antibodies, it must be said that as the immune-bodies are very much more rare than the alexins, the organism will more easily produce the necessary quantity of anti-immune-bodies than of antialexins. Moreover, the serum of the animal which is exposed to dangers of a hæmolytic nature contains in its blood-serum generally an alexin which, united with the proper immune-body, hæmolyses its own erythrocytes. Against this alexin the animal evidently produces no antibody, for otherwise the alexin would be of no use against foreign substances which enter the blood.

Morgenroth and Sachs[2] have carried out an investigation on the quantities of antialexin which are necessary to completely suppress the action of a mixture of immune-body and alexin which is just able to produce complete hæmolysis of the erythrocytes used in the experiment. The results are given in the following table, where a denotes the quantity of immune-body (given as number of c.c. of the preparation used), b the corresponding quantity of alexin, and c the quantity of antialexin necessary to inhibit the hæmolysis. The erythrocytes were present in the amount of 1 c.c. of a five per cent suspension. The alexin and the antialexin were in contact for thirty minutes

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[1] This circumstance has been observed already by Bordet (*l.c.* p. 273).

[2] Morgenroth and Sachs: *Berl. klin. Wochenschrift*, No. 35 (1902).

at 37° C. before the erythrocytes and the immune-body were added.

EXPERIMENTS OF MORGENROTH AND SACHS ON ANTIALEXIN

1. SHEEP BLOOD ERYTHROCYTES

Immune-body, from goat treated with sheep erythrocytes; alexin, normal serum of guinea-pigs; antialexin, immune-serum from goat, treated with rabbit-serum

a	b	c	$c:b$	$c:a$
0.3	0.006	0.35	58	1.2
0.05	0.006	0.1	17	2
0.01	0.01	0.075	7.5	7.5
0.005	0.05	0.015	0.3	10

2. Like 1, but the antialexin is from a rabbit which has been treated with guinea-pig-serum

a	b	c	$c:b$	$c:a$
0.2	0.0035	0.005	1.4	0.025
0.1	0.025	0.04	1.6	0.4

3. BOVINE ERYTHROCYTES

Immune-body, from rabbit treated with bovine erythrocytes; alexin, normal serum of guinea-pigs; antialexin, from goat, treated with rabbit-serum

a	b	c	$c:b$	$c:a$
0.2	0.05	0.75	15	38
0.004	0.1	0.1	1.0	25

4. ERYTHROCYTES FROM HUMAN BLOOD

Immune-body, from a rabbit, treated with human erythrocytes; alexin, normal serum from a rabbit; antialexin, from goat, treated with rabbit-serum

a	b	c	$c:b$	$c:a$
0.2	0.05	0.075	1.5	0.38
0.1	0.05	0.035	0.7	0.35
0.05	0.1	0.025	0.25	0.5

Only in the case 2 is there the approximate proportionality between c and b which was expected by the authors. Evidently if the alexin is bound by the antialexin without dissociation, the quantity of antialexin necessary for neutralisation will be proportional to the quantity of alexin used, just as is the case in this experiment. The authors call attention to the remarkable fact that in this case the

antialexin was produced by the injection of the alexin used, which was not the case in the other series. It seems quite possible that this method is the only one adapted to yield a specific antialexin with a strong affinity for the injected alexin.

If the affinity be not very marked, and therefore the reaction between alexin and antialexin to a high degree incomplete, we would not expect to find a proportionality between the neutralising quantity of antialexin and the quantity of alexin present. (It may be remarked that it is here not a question of an absolute neutralisation, by which the quantity of hæmolysin would sink to zero, but only of a reduction of the quantity of hæmolysin to about 14 per cent of the value exhibited in the absence of antialexin (cf. p. 229).)

What complicated phenomena may be encountered in such cases is evident from the following example, in which it is supposed that the formula valid for the equilibrium has the form, $(5\,a-x)(20\,b-x)=100\;x$, which quite closely corresponds to the combination of the immune-body (a) from a goat treated with bullock erythrocytes and with guinea-pig serum as alexin (b) to hæmolysin (x). I have supposed that an antialexin of the same affinity for the alexin as that of the immune-body is added, so that the same formula may be used for the two cases of equilibrium. From this it follows that the proportions of alexin bound to immune-body and antialexin are identical with the proportions of these two substances themselves. The following table corresponds directly to those of Morgenroth and Sachs. b is the quantity of alexin necessary for complete hæmolysis in the presence of the quantity a of im-

mune-body. c is the quantity of antialexin necessary to reduce the quantity of hæmolysin to the eighth part of that necessary for total hæmolysis, which nearly corresponds to the limit of observable hæmolysis.

INFLUENCE OF ANTIALEXIN ACCORDING TO THE LAW OF MASS-ACTION

a	b	c	$c:b$	$c:a$
1000	5.1	7130	1400	7.1
300	5.4	2270	450	7.6
100	6.3	885	141	8.9
70	7.0	690	99	9.9
50	8.4	606	72	12.1
30	15	670	45	22.3
25	25	950	38	38.1

Here we observe that for a certain quantity of immune-body ($a = 40$; $b = 10$), when the immune-body and the alexin are present in equivalent quantities, c passes through a minimum; for higher values of a, c increases with a and tends to reach proportionality with a as a attains very high values; for lower values of a, c increases with b. In other words, the addition of a given quantity of antialexin has a maximal influence when $a = 40$, and the hæmolytic action is reduced to a lesser degree for values of a above or below 40. The general behaviour will be the same even if the affinity of the immune-body for the alexin is not equal to that of the antialexin for the alexin, only then the minimum of c is displaced.

If we now compare this last general table with those of Sachs and Morgenroth, we observe that all of them may very easily, within the errors of observation, be regarded as special cases of the general table; while they conclude

that from their observations "it must of necessity (!) be concluded, that the different relations of the affinities cannot give a sufficient explanation." "We must therefore," they say, "make use of another factor, namely, *the plurality of the alexins and antialexins*, for the explanation." This conclusion shows clearly how necessary it is to be cautious in regard to theoretical deductions in this discipline of bio-chemistry. The results of similar deductions led Bashford[1] to introduce one of his memoirs with the words: "On immunity, especially, investigation is rendered difficult by the habit of pushing conclusions farther than the facts really warrant; impartial work and judgment too often lead to the conviction that the 'generally accepted facts' are but flimsily supported hypotheses."

The relations are still more complicated by the possibility that the velocity of absorption for immune-body and antialexin may be rather different in different cases.

In two masterly memoirs Bordet[2] has considered the properties of antisera, procured by injection of normal and immune sera into the veins of a non-related animal. Bordet found that serum from the guinea-pigs which had been treated with erythrocytes from a rabbit caused after injection into a rabbit, the production of substances which counteracted the immune-body contained in the immune-serum of guinea-pigs, as well as the alexin in normal serum from guinea-pigs. This antialexic action not only protected the erythrocytes of rabbit, treated with the said immune-serum, from being hæmolysed, but even cholera vibrios,

[1] Bashford: *Journ. of Pathology and Bacteriology*, **8**. 52 (1902).
[2] Bordet: *Ann. de l'Inst. Pasteur*, **18**. 593 (1904); Bordet and Gay: *ibidem*, **20**. 467 (1906).

treated with bacteriolytic cholera-serum, from being de-
stroyed by guinea-pig-serum. This antialexic action was
in so far specific that it did not protect against other alexins
than that from guinea-pigs. Bordet made a large number
of experiments with the following combination : *erythro-
cytes*, from bullock; *immune-body*, serum of rabbit injected
with erythrocytes from bullock; *alexin*, normal serum from
a guinea-pig; *antiserum*, serum from a guinea-pig treated
with normal rabbit-serum. The immune-body and the
antiserum were freed of alexin by heating to 55–56° C.
for thirty minutes. Bordet separated the active sub-
stances from a large number of other substances present
in the sera by letting the erythrocytes absorb them, then
only those substances that were specific to the erythrocytes
were able to act. That the erythrocytes absorb the im-
mune-body almost completely if it be not present in great
excess, we have seen above; and this method has long
been used for the extraction of immune-bodies from sera.
Bordet has shown that erythrocytes charged with immune-
body also absorb alexins, so that a serum may in this way
be freed from its content of alexin, which is indicated by
the fact that it has lost its hæmolytic power against ery-
throcytes treated with immune-body. The alexin is, on the
other hand, not absorbed by normal erythrocytes, those
that do not contain immune-body. Further, Bordet in an
analogous manner proved that erythrocytes, charged with
immune-body, absorb the antiserum and thereafter have
lost their power of absorbing alexin. Evidently the im-
mune-body is neutralised by the antiserum, which seems
to have a stronger affinity for the immune-body than has
alexin. This observation is confirmed by the fact that a

certain quantity of antiserum can bind only a given equivalent quantity of immune-body, so that erythrocytes protected from hæmolysis by the absorption of a certain quantity of antiserum may yet be hæmolysed on the further addition of immune-body and alexin. Normal rabbit-serum heated to 56° C. contains a substance which does not act as an immune-body against bovine erythrocytes, but has the faculty of binding antiserum, so that it can produce the hæmolysis of erythrocytes that are loaded with the compound of immune-body and antiserum. This hæmolytic action is weaker if the compound has lain for a long time in the erythrocytes, than if the preparation is fresh. This peculiarity seems to indicate that the compound, just as the analogous hæmolytic substances such as tetanolysin and probably also the compound hæmolysins, is slowly bound by the protein substances in the erythrocytes. The normal rabbit serum evidently contains some substance which competes with the immune-body in binding antiserum.

Bordet and Gay made an observation confirming a discovery of Klein.[1] The immune-bodies and alexins contained in sera are absorbed to a much higher degree by the erythrocrytes if these are suspended in a physiological salt-solution, than if they are suspended in a natural serum. Thus a mixture of 0.4 c.c. of normal horse-serum and 0.4 c.c. of erythrocytes from the guinea-pig was treated with 1 c.c. of ox-serum without showing agglutination of the erythrocytes to a notable degree. If, on the other hand, 0.6 c.c. of physiological salt-solution had been present for some time in the said mixture, agglutination was very pronounced. In an analogous manner we may explain an-

[1] Klein: *Wiener klin. Wochenschrift*, No. 48 (1905).

other observation of Bordet. 0.2 c.c. of bovine erythrocytes loaded with immune-body were mixed with 0.6 c.c. of antiserum and physiological salt-solution. After a time the erythrocytes were separated from the liquid by centrifugalisation. To the erythrocytes was then added a mixture of 0.2 c.c. of alexin from the guinea-pig with 0.6 c.c. of normal guinea-pig-serum heated to 56° C. Another experiment was quite similar, but instead of the 0.6 c.c. of guinea-pig-serum, 0.6 c.c. of physiological salt-solution was added. In the first experiment no hæmolysis was observed, but the second gave hæmolysis during the course of one hour. The absorption of the alexin was much greater in the presence of the physiological salt-solution than in the presence of normal serum. The absorbed alexin competes with the antiserum absorbed in the erythrocytes, so that a certain quantity of compound hæmolysin was formed, enough to yield hæmolysis.

This action of the physiological salt-solution which causes the absorption of, *e.g.*, the immune-body and the alexin from horse-serum in the experiment of Bordet and Gay, speaks very much in favour of the view that an absorption and not a chemical binding of the immune-bodies takes place in the erythrocytes.

Through absorption experiments Bordet proved that the same antiserum protects bovine erythrocytes against the immune-body contained in serum from a rabbit treated with bovine erythrocytes, and chicken erythrocytes against rabbit-serum treated with chicken erythrocytes. The same antiserum may therefore neutralise two entirely different immune-bodies, which are produced by the same species of animal (here rabbits). Therefore Bordet rejects the

proof given by Ford and Wassermann,[1] that the agglutinin to chicken erythrocytes, which is found in normal rabbit-serum, is identical with that contained in serum from rabbits treated with the said erythrocytes, because both are neutralised by serum from chicken treated with rabbit erythrocytes.

Bordet makes some remarks of great theoretical interest bearing upon the results of his experiments. Ehrlich and Morgenroth[2] found that in the said combination of erythrocytes and immune-body it was possible to use alexin from goat-serum, although it had a weaker hæmolytic action than that from guinea-pigs. They then made experiments with the neutralising action of an antiserum produced by the injection of serum from rabbits treated with bovine erythrocytes into the veins of a goat. They found that this serum (in a given quantity) hindered the hæmolysis by alexins from guinea-pigs, but not by that of goat-serum. But as the alexin from goats is much weaker, they used in this special case a much greater quantity of immune-body. Thereby they introduced not alone the immune-body in great quantity, but also the substances contained in normal rabbit-serum which are able to neutralise the antiserum. Bordet explains in this simple manner that the antiserum had no action in this case, and rejects the explanation of Ehrlich and Morgenroth, who assume that the different effect is due to the presence of two different kinds of immune-bodies in the preparation, of which the one gives compounds with the alexin from guinea-pigs and with the

[1] Ford: *Zeitschr. f. Hygiene,* **40.** 363 (1902); Wassermann: *ibidem,* **42.** 267 (1903).

[2] Ehrlich and Morgenroth: *Berl. klin. Wochenschrift,* Nos. 21 and 22 (1901).

antiserum, the other with alexin from goats but not with the antiserum used. Evidently this proof is without validity. This is now conceded by Ehrlich, who believes, however, that other proofs are still valid.[1]

Another remark of Bordet touches the side-chain theory of Ehrlich. Ehrlich conceives the formation of antibodies in the following manner: If a foreign substance is injected into the body of an animal, it may be "anchored" to some cells in the tissues of the animal. A chemical affinity localised on a "receptor" of this cell is thereafter bound, and hence the cell is hindered in one of its functions. The cell then produces a new "receptor" to fill the place of that seized by the foreign substance. "According to a law of Weigert's, the regeneration does not only compensate the defect, but overcompensates it." (This so-called "law" has no standing whatever.) The excess of receptors thus produced is given off to the blood and forms the antitoxin. In our case, therefore, the immune-body, according to Ehrlich, is a receptor which is able to bind an erythrocyte. This receptor is called amboceptor, because it may even bind a molecule of alexin at the same time as the erythrocyte. Now, we have seen that the immune-bodies are probably not bound by the erythrocytes, but only absorbed by them. Therefore, in its old formulation, the side-chain theory probably has no application to this special case. Bordet, on the other hand, accepts the theory of a binding process like that by which dyes are bound to fibre. He, therefore, seeks another proof that the theory of Ehrlich, as it is used, cannot be correct. To explain the action of antisera Morgenroth

[1] Ehrlich and Sachs: *Berl. klin. Wochenschrift*, Nos. 19 and 20 (1905).

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supposes that they, as formed by the injection of an immune-body, consist of receptors which bind the same affinity of the immune-body that otherwise might bind the erythrocyte. (Evidently it would be much better to suppose, as Ehrlich does in his reply to Bordet, that the antisera may replace the alexins, and therefore do not attack the affinity with which an erythrocyte may be bound.) Then, Bordet retorts, every substance capable of binding the antiserum ought to bind erythrocytes. As we have seen, normal rabbit-serum contains a substance which combines with the antiserum, but not with bovine erythrocytes. Even immune-serum from the rabbit treated with bovine erythrocytes, which serum had previously, by being shaken with ox erythrocytes, been deprived of substances that bind these erythrocytes, showed an affinity for antiserum. Further, the same antiserum binds immune-bodies which are absorbed by different erythrocytes, as bullock's and hen's, and which are specific for them.

Hence the attack of Bordet on the side-chain theory in the form of its original application to this phenomenon was quite correct. But this theory possesses a high degree of elasticity, and against the new formulation given by Ehrlich and Sachs in their reply to Bordet, according to which the antiserum competes with the alexin in binding the immune-body, no objection is to be made.

We might, perhaps, therefore look for a new method of elucidation of this theoretical question. But, as Bordet remarks, Ehrlich has, in his later publications, modified the side-chain theory to such a degree that the differences between Bordet's and Ehrlich's opinions almost disappear,

and are now more of a formal than of a real character. Evidently, if an animal reacts to injection of a foreign fluid substance, the simplest way to explain this is to assume that the injected fluid attacks some cell of the animal chemically. In common language, we say that some substance in the fluid has been bound by the cells. The animal retaliates with the production of some substance, the so-called antibody, which, as we have seen, partially binds the reacting substance in the fluid ; if the reacting substance be a cell, the antibody enters into similar cells, and in many cases causes chemical alterations in the contents. As now the reacting substance is bound chemically by the cells of the inoculated animal, as well as by the antibody produced by it, we may, with Ehrlich, for the sake of simplicity, suppose that the antibody consists of just that part of the cells which are attacked by the foreign fluid. But it is not necessary to make this supposition. On a closer inspection, the side-chain theory seems to be little more than a circumscription of the definition of the conception " antibody," under the further supposition that we are dealing with chemical processes. If, as for the immune-bodies, no proof has been given of their chemical action, the side-chain theory finds, in its present state, no application.

Bordet also makes an attack upon Morgenroth's[1] proof that immune-body and alexin bind each other in solutions containing both. As Morgenroth,[2] and likewise Ehrlich and Sachs,[3] have later on conceded that the criticism of Bordet is well founded, we will not here enter upon this

[1] Morgenroth: *Centralbl. f. Bakteriologie*, **35.** 501 (1904).

[2] Morgenroth: *Arbeiten aus dem pathologischen Institut zu Berlin*, p. 6 (1906).

[3] Ehrlich and Sachs: *l.c.*, p. 16.

criticism. We only remark that the compound is very easily dissociated, so that it was only with the help of quantitative measurements that a proof of its presence could be given (cf. p. 224). As this compound occurs in the erythrocytes, it would seem very improbable that it should not exist also outside of the erythrocytes, although dissociated to a high degree.

As we have seen above, Ehrlich and Sachs made experiments with erythrocytes from guinea-pigs, normal bovine serum heated to 56° C. (which they regarded as immune-body), and alexin from horse blood. They found that the hæmolytic agent from the bovine serum was not absorbed by the erythrocytes, for it did not lose its activity after having been shaken with such erythrocytes. Hence they concluded that not the immune-body, but its compound with alexin, is absorbed by the erythrocytes. This conclusion is not corroborated by the investigation of Bordet and Gay, for they found that if they used alexin from guinea-pigs instead of alexin from horses, hæmolysis occurred, but not after the immune-body had been separated from the bovine serum by treatment with erythrocytes from guinea-pigs. There must therefore be another explanation of the experiments of Ehrlich and Sachs. After a thorough investigation, Bordet and Gay conclude that the normal horse-serum, used by Ehrlich and Sachs, furnishes not only the alexin, but also the immune-body to the erythrocytes. The compound hæmolysin of these two substances is too weak to cause a perceptible hæmolysis, but it is strengthened by the presence of some substance contained in the bovine serum, which they call "colloide de bœuf." This colloid is indeed

active in other similar cases. The proof of Ehrlich and Sachs that an immune-body is not itself soluble in (able to be bound by) erythrocytes, but only after combination with alexin, is henceforth untenable.

I have made some experiments on the action of antialexins and found that cases occur in which the influence of the antialexin has even a minimum value for a medium concentration of the immune-body, and not a maximum, as in the theoretical example cited above. In one case the erythrocytes were from sheep (1 c.c. of a 5 per cent suspension); the immune-body (a), from a goat, treated with erythrocytes from sheep the alexin (b) was guinea-pig-serum; and the antialexin (c) was from a goat injected with serum from a rabbit. The quantity of alexin was of such a magnitude that three-fourths of it would be just sufficient to produce complete hæmolysis. The quantities are given in centimetres of the preparations used. The experimental method was the same as that used by

ACTION OF DIFFERENT QUANTITIES OF ANTIALEXIN

c (c.c.)	SER. 1 $a = 0.1$ c.c. $b = 0.004$ c.c.	SER. 2 $a = 0.01$ c.c. $b = 0.015$ c.c.	SER. 3 $a = 0.001$ c.c. $b = 0.04$ c.c.	SER. 4 $a = 0.0005$ c.c. $b = 0.1$ c.c.
0	100	100	100	100
0.025	67	100	100	100
0.035	48	100	100	90
0.05	22	100	100	81
0.075	14	100	54	36
0.1	9	90	4 (?)	13
0.15	6	28	4	8
0.25	8	12	6	8
0.35	8	7	6	9
0.5	10	8	8	9

Morgenroth and Sachs. The total quantity was 2.2 c.c. The tabulated quantity is the degree of hæmolysis.

The action is the least in Ser. 2, after this comes Ser. 3, then Ser. 4, and last Ser. 1. It seems difficult to explain this peculiar behaviour, which was controlled by other measurements, so long as we suppose that the "antialexin" entered into combination only with the alexin present. The observations may be understood if we assume that either an anti-immune-body or an antihæmolysin was present. If we suppose that the preparation *c* contained anti-immune-body as well as antialexin and their affinities are strong, then small quantities of *c* may be sufficient for the neutralisation to a remarkable degree if small quantities of either the alexin (Ser. 1) or of the immune-body (Ser. 4) are present. The explanation is analogous (the result of the partial dissociation) if we suppose the presence of an antihæmolysin in the solution *c*.

In any case the reactions in the presence of antialexins or anti-immune-bodies are rather complicated and difficult of survey.

CHAPTER IX

THE PRECIPITINS AND THEIR ANTIBODIES

In many instances the reaction-products of the ferments are solid bodies, and such ferments are called precipitins. Generally these solid substances contain a great deal of water, like albuminous substances in general; and this circumstance has in recent times led to the opinion that the act of precipitation might consist only of a coalescence and subsidence of the "colloidal" particles of the particular albuminous substance in the system. Thus, for instance, the casein of milk is, according to this theory, present in the state of so-called pseudo-solution. Its smallest particles may be regarded as an extremely fine solid powder of ultramicroscopic magnitude (dimensions less than .0002 millimeter). On the addition of rennet these solid particles coalesce to form larger clumps and subside, just as finely powdered clay sedimentates following the addition of salts or acids to the water in which it is suspended.

In corroboration of this view Duclaux[1] cites the following observation. "In milk, which is turning sour but which is still quite liquid, we observe with the miscroscope, as I have indicated, a precipitate of fine grains, which at the beginning are seen only with difficulty, and are detected only by a faint disturbance of the visional field, but which later on display quite distinct granulations, characterised by the Brownian movement, just as minute particles

[1] Duclaux: *Microbiologie*, Tome 2, pp. 253–339 (1899).

of clay. Shall we suppose now that the coagulation of the casein changes its value rapidly in the same moment that we are able to observe its progress? From this point of view the phenomenon manifests itself to our eyes as a steadily increasing molecular condensation. It displays the behaviour of clay particles which aggregate and subside. . . . We are therefore led to suppose that this regular condensation, which causes the coagulation as far as we are able to observe it, begins already before the microscope can detect it. But however well grounded this induction may seem to us, it would remain unfounded if we could not control it by experiment."

This experimental proof Duclaux finds in the reaction of Tyndall. Ultramicroscopical powders suspended in an absolutely clear fluid reveal their presence by the production of a blue colour on illumination by a ray of light, and this blue reflected light is polarised. This phenomenon is displayed, for instance, by a suspension of fine particles of mastic, prepared by adding a few drops of an alcoholic solution of mastic to water. If we add greater quantities of the solution, the light reflected becomes more pale and white; and at a certain point it is possible by means of the microscope to detect in it small particles that show the Brownian movement. At a still higher concentration of the solution a real precipitate is formed, which is easily observed with the naked eye.

To this argument may be added still another fact. Such submicroscopic particles may be observed by means of the ultramicroscope of Siedentopf. Solutions of proteins generally reveal the presence of such submicroscopic granules, and this observation has been interpreted in

favour of the view that all solutions of proteins may be regarded as pseudo-solutions, *i.e.* consisting of suspensions of finely divided particles. Against this view it may, however, be urged that the presence of some submicroscopic particles does not prove at all that the whole of the quantity of protein present, or even that a considerable part of it, is in this state of pseudo-solution.

Even in this case some salts, especially those of calcium, strontium, and barium, exert a great influence, just as in the case of agglutination, with which the phenomenon of coagulation shows many analogies. (Cf. above, pp. 73 and 159.) Just as salts are of marked influence on the agglomeration of fine particles, as, *e.g.*, of clay or of mastic (cf. p. 159), so in the same way the influence of the salts of Ca, Sr, and Ba on the process of coagulation is so prominent that it is doubtful if coagulation can be realised in their absence. These salts alone, without rennet, give precipitates with milk or solutions of casein. The coagulated matter is more flocculent and does not entangle the fat-drops to so high a degree as the coagulum produced under the action of rennet. But this action of the salts of the metals of the calcium group seems to be rather specific. Next to them come the salts of magnesia, which accelerate also the coagulation by rennet if they are present in greater quantity, though according to Lörcher's observations small quantities of $MgCl_2$ retard the action of rennet, whereas even the least traces of Ca, Sr, or Ba salts accelerate the coagulation by rennet. These salts give without rennet a coagulation with the casein of milk. Salts of the other alkali metals coagulate milk only at high concentrations ; they retard the coagulating action of rennet. In

strong solution even the salts of Ca, Sr, and Ba retard the action of rennet. This action of stronger solutions is probably only due to a change in the solubility, just as the precipitation of casein by means of alcohol, and possesses, therefore, only a secondary interest.

Acids precipitate the casein, even in rather minute quantity. Salts which have an acid reaction accelerate the action of rennet just as acids do. This may be due to the presence of free acid in these solutions. On the other hand, alkalies hinder or retard the precipitation of the casein, and in the same manner behave salts with an alkaline reaction, as the carbonates or bicarbonates of the alkali metals. These last facts speak very much in favour of the chemical theory first advanced by Hammarsten, who regards the casein as an acid, which is very slightly soluble, whereas its salts with alkali metals are soluble in water.

Laqueur and Sackur [1] have determined its equivalent weight to be 1135. The conductivity of the sodium salt in solution seems to indicate, according to a rule of Ostwald, that the acid is tetra- or hexavalent, so that its molecular weight is computed to be 4540 or 6710.

With this last number agrees very well another (6600), found by Hedin, Blum, and Vaubel [2] from a study of the products of the decomposition of casein.

By the prolonged action of weakly alkaline solutions on casein, another stronger acid is formed, termed isocasein, possessing the equivalent weight 960. Its molecular weight is four or six times greater.

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[1] Laqueur and Sackur: *Hofmeisters Beiträge*, 3 (1902); Sackur: *Zeitschr. f. ph. Ch.*, 41. 672 (1902).

[2] Hedin, Blum, and Vaubel: *Journ. f. praktische Ch.*, 60. 55 (1899).

Upon these chemical properties Hammarsten founded his method for the preparation of pure casein. Milk is diluted with three to four times its volume of water, and a precipitate produced by the addition of 0.1 per cent of acetic acid. The precipitate is separated from the solution by filtration through linen and then dissolved in a weak solution of caustic soda or better ammonia. The fat-drops carried down by the precipitate separate upon the top of the liquid, which is thereafter again precipitated and redissolved four or five times. The last traces of fat are removed from the precipitate by extraction with alcohol and ether, and the precipitate is after that dried to a white powder, casein, which is very slightly soluble in water. Its suspensions in water behave as an acid, and it drives the carbonic acid out from the carbonates of alkali metals or calcium, giving clear solutions of its salts with these metals.

If a solution of the calcium caseate is neutralised by the addition of a diluted solution of phosphoric acid, a precipitate of calcium phosphate is formed, and a white liquid remains which resembles very much a milk devoid of cream. Probably we have here a pseudo-solution of casein. It behaves like milk in being coagulated through the action of rennet.

The adherents of the chemical theory of coagulation by rennet assume that this ferment exerts a decomposing action on the casein, just as pepsin on protein,[1] after which

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[1] According to recent investigations of Bang (*Zeitschr. f. ph. Ch.*, **43**. 358, 1905), Hemmeter (*Berl. klin. Wochenschrift, Ewald-Nummer*, 14, 1905), and Schmidt-Nielsen (*Zeitschr. f. physiol. Ch.*, **48**. 92, 1906), pepsin and chymosin — the milk-coagulating substance in neutral rennet — are different substances. Pepsin seems to coagulate milk in acid solution, wherein the results of Pawlow and Sawjalow find their explanation (cf. p. 71).

the digestion-product is precipitated by Ca ions. Some very strong arguments are advanced in favour of this view. The reaction goes on at low temperatures without coagulation, which then occurs almost instantaneously upon elevation of the temperature to over 25° C.,[1] and small quantities of pepton have a strong retarding influence (cf. p. 77). One of the products of decomposition, called para-casein, is supposed to yield the coagulum at higher temperature, whereas other products, for instance serum-albumen, remain in solution. In reality Hammarsten has proved that serum-albumen is an albumos-like substance with characteristic reactions : it is not precipitated by acids (acetic and nitric) nor by diluted solutions of $CuSO_4$, $HgCl_2$, $FeCl_3$, $K_4(CN)_6Fe$, nor $Pb(CH_3CO_2)_2$; it does not give the reaction of Heller ; but responds to the biuret-reaction and the reaction of Millon ; it is precipitated by tannin in acetic acid and by alcohol. Rennet and casein produce serum-albumen even in absence of calcium or barium salts. Duclaux combats this view, which he says leads to the conclusion that these soluble products ought to pass through a Chamberland filter. Now a Chamberland filter restrains casein, but some of the proteins of milk pass through it. Therefore, according to Duclaux, milk filtered through a Chamberland filter should give a filtrate containing more of the filterable proteids after it had been coagulated by rennet, than before this treatment. This, he showed, was not the case. But the conclusion drawn does not seem to be quite convincing. For, just as the casein does not pass through the filter, so the serum-albumen may be held back by it. That the

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[1] This was first observed by Morgenroth (*Archives internationales de pharmacodynamie*, 7. 265, 1900).

casein does not pass through is explained by Duclaux as due to its state of pseudo-solution, which is really only a mode of expression and no real explanation ; and the same property may be characteristic of the serum-albumen, especially if the filter is covered by a gel of casein.

The addition of a solution of an oxalate or of a fluoride to milk inhibits its coagulation. One might suppose this action to be due to the precipitation of the calcium from the compounds of the milk and of the rennet. Duclaux combats this view and states that the coagulation is not inhibited if the oxalate (or fluoride) is not present in excess. He assumes that the oxalate or fluoride has itself a direct influence upon the milk, lowering its tendency to coagulation. From the modern point of view of physical chemistry, it seems quite obvious that for the coagulation a certain degree of concentration of the calcium ion (or barium or strontium ion) is necessary. The more oxalate present, the lower would be this concentration, so that a certain concentration of the free oxalate ion (or fluorine ion) is necessary to prevent coagulation. This concentration probably depends in part upon the temperature.

Another coagulation process of especial interest is the precipitation of blood-plasma by means of fibrin-ferment (cf. p. 91). This process is very similar to the coagulation of milk or casein, but seems to be different in the fact that coagulation of the plasma can occur spontaneously. According to Duclaux, this depends upon the action of the leucocytes present in the blood. In their cytolysis they form a coagulating ferment. Coagulating ferments that act upon blood-plasma are very common in the different tissues, fluids, and organs of the body. Alexander

Schmidt prepared this fibrin-ferment by precipitating blood-serum with 15 to 20 times its volume of alcohol. The precipitate was filtered and dried. This precipitate contains a rather large quantity of fibrin-ferment which coagulates the fibrin contained in blood-plasma.

It is possible to prepare solutions of plasma as well as of fibrin-ferment nearly free of calcium ions, by the addition of oxalates or fluorides to the blood. On mixing these two coagulations occur, although there are oxalate ions present in excess. From this experiment it has been concluded that calcium ions are not necessary in the coagulation of fibrin. The conclusion is not quite binding, for there are always calcium ions present, although in very minute quantity. A more accurate examination of this question would be profitable. On the other hand, it is certain that the ions of calcium, strontium, and barium accelerate to a high degree the coagulation of fibrin. On the whole, the different salts seem to exert upon the coagulation of fibrin an influence similar to that upon milk. The presence of acids is favourable to the plasmatic coagulation.

In the coagulation of plasma (as in that of casein, according to Hammarsten's investigations) two different phases may be observed, according to the opinion of Bordet and Gengou.[1] The one, the transformation of the so-called fibrinogen, contained in the plasma, into fibrin, may proceed as the result of the action of the ferment in the absence of calcium salts; the second process, the coagulation, requires the presence of calcium ions. Hence a perfect analogy exists between the coagulations of plasma

[1] Bordet and Gengou: *Ann. de l'Inst. Pasteur*, **18**. 26 (1904).

and of casein. This duality of the processes recalls the action of the compound hæmolysins in which at first the immune-body is absorbed in the erythrocytes, following which the formation of hæmolysin occurs. According to Bordet's view, the parallelism between hæmolysis and co-agulation is still more prominent.

Bordet and Gengou [1] suggest that the action of fluorides on blood-plasma interferes not only with the action of the Ca ions, which are precipitated from the solution as $CaFl_2$, but also with that of the fibrin-ferment, which is carried down from the solution by the precipitate. The addition of an oxalate does not interfere sensibly with the action of the ferment.

Leo Loeb [2] found a certain specificity between fibrin-ferment and plasma, "in so far as the blood of each species of animal used coagulated more rapidly under the influence of the tissues of animals of the same species or of the tissues of related animals than under the influence of the tissues of more distant animals." The addition of serum of the animal producing the fibrin-ferment increases the action. Even the products of bacteria (especially *Strepto-coccus pyogenes aureus*) are sometimes favourable to the coagulation.

There is a third type of coagulation provoked by a ferment. In certain fruits and even roots of plants occurs a substance called pectin. This substance gives with water solutions of high viscosity; it is precipitated by alcohol. It may be coagulated by means of a ferment called

[1] Bordet and Gengou: *Ann. de l'Inst. Pasteur*, **18**. 98 (1904).
[2] Leo Loeb: *Hofmeisters Beiträge*, **5**. 534 (1904) ; *Journ. of Medical Re-search*, **10**. 407 (1903).

pectase, found in the juice of carrots, beets, etc. The co-agulation is hindered by the presence of oxalate ions, *i.e.* the presence of calcium ions (or ions of barium or strontium) seems necessary also in this reaction. Acids retard the coagulation, and strong mineral acids exert a greater influence than weak vegetable acids.

Coagulation or precipitation plays a very important rôle in the chemistry of antibodies. We have already spoken of the action of agglutinin as probably associated with a coagulating influence upon the cells (cf. p. 164). The agglutinating power of acids for erythrocytes is followed by a very obvious coagulation. Corrosive sublimate produces on erythrocytes both coagulative and an agglutinating action in higher concentrations, and a hæmolytic action in lower concentrations. Hæmolysis and coagulation (which makes itself manifest as agglutination) seem to be so often concomitant in the so-called phytalbumoses — toxins of vegetable origin, as, *e.g.*, ricin or crotin — that Ehrlich has assumed that they are in this case inseparable.[1] But for the bacteriolysins this is not the case, according to Kraus and Ludwig.[2]

If toxins and antitoxins are mixed in higher concentrations, they often yield a precipitate. Thus, for instance, Jacoby[3] observed a flocculent precipitate on mixing ricin and antiricin; and Hausmann[4] made a similar observation for abrin and antiabrin. Bashford[5] found that blood-serum

[1] Ehrlich: "Schlussbetrachtungen" in "Nothnagel's spezielle Pathologie und Therapie," Bd. 8, p. 13, Vienna, 1901.

[2] Kraus and Ludwig: *Wien. klin. Wochenschrift*, No. 5 (1902).

[3] Jacoby: *Hofmeisters Beiträge*, 1. 51 (1901).

[4] Hausmann: *Hofmeisters Beiträge*, 2. 134 (1902).

[5] Bashford: *Journ. of Pathology*, etc., 8. 59 (1902).

from a rabbit, which had been actively immunised against crotin, gave a very dense precipitate with this substance, a precipitate not produced with normal serum. Myers showed that various albuminous bodies (Witte pepton, crystallised egg-albumen, serum globulin) injected into rabbits produced precipitins in their serum which acted upon the corresponding albuminous bodies in experiments *in vitro*.[1]

"If this reaction *in vitro*," Bashford continues, "be comparable to the action in the body, then the injection of a solution of Witte pepton into the ear-vein of such an immunised animal should result in death from embolism." "The injection very slowly, however, of 5 c.c. 20 per cent Witte pepton into the ear-vein" of a highly immunised rabbit "gave no symptoms." "The reactions *in vitro* and *in corpore* are here again different. Also in the case of a rabbit immunised against crotin; direct injection of my albumin-containing crotin-solution had no consequence which I could observe." As we have seen before (p. 205), the equation of equilibrium for ricin and antiricin is not of the same form, if we investigate the action of this poison on red blood-corpuscles *in vitro* and in living animals. From this circumstance we must conclude that there are different poisons acting in the two cases. Ehrlich[2] and Kobert[3] supposed that the two poisons were identical, and used this hypothesis as a basis for theoretical deductions. But now it is well known that this hypothesis is wrong.

Even for tetanolysin and corrosive sublimate Bashford found a different action *in vitro* and *in vivo*. Hæmolysins,

[1] Myers : *Centralbl. f. Bakteriologie*, **28** (1900).
[2] Ehrlich : *Fortschritte de Medicin*, **15**. 41 (1897).
[3] Kobert : *Arbeiten des pharmakologischen Instituts zu Dorpat*, Tome 8 (cited from Bashford).

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as cyclamin, saponin, digitalin, solanin, cobra-venom, and hæmolytic sera, produce, when injected into living animals, hæmolysis, which is manifested by hæmoglobinuria. "It must, however, be stated that the actions of the hæmolysins *in corpore* are in no case limited to the erythrocytes." At all events, it seems prudent not to assume *a priori* that the actions of poisons *in vitro* and *in vivo* are identical.

The best known of all the coagulating substances is rennet (or chymosin). It seems rather probable that the chief action of rennet is analogous to that of peptic digestion, and that the coagulation is a mere accidental property belonging to the products of the digestion at higher temperature (cf. p. 267). Be that as it may, the coagulation is the property employed hitherto for the investigation of the action of rennet. The coagulating power of rennet is diminished by some normal sera, especially serum from horses, as well as by immune-sera produced by the injection of rennet into animals,[1] rabbits being especially adapted to this purpose.

Madsen and Walbum have investigated the neutralising power of this "antirennet" and found that it behaves just as antitetanolysin does against tetanolysin. The experimental method was the following: Mixtures of 4 c.c. of a 1 per cent solution of rennet and different quantities (0.02–1 c.c.) of the antirennet-containing serum and of physiological salt-solution were prepared so that the total quantity was 5 c.c. These mixtures were held at room

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[1] Morgenroth: (*Centralbl. f. Bakteriologie*, 1. *Abth.* **26.** 349, 1899, and **27.** 721, 1900) was the first to prepare antirennet. He immunised goats by the subcutaneous injection of rennet. After repeated injections the serum of the goat contained antirennet.

temperature (in mean 16° C.) during twenty to fifty minutes. Special experiments seemed to indicate that this time of reaction has no notable influence if it only exceeds five minutes, and after this time different quantities of the mixture were added to 10 c.c. of milk and physiological salt-solution added up to 12 c.c. The test-tubes containing these mixtures were placed in a water-bath of constant temperature and the coagulation examined after a given time (two hours). The calculation for the experiment is the same as for tetanolysin. One c.c. of the antirennet was found equivalent to 1.48 times the fixed quantity of rennet. The constant of equilibrium was found to be $K = 0.012$. n is the quantity of antirennet used.

NEUTRALISATION OF RENNET BY MEANS OF IMMUNE-SERUM FROM RABBIT

$n =$	$g_{obs.}$	$g_{calc.}$	Δ	n	$g_{obs.}$	$g_{calc.}$	Δ
0	100	100		0.4	42.6	41.8	± 1.3
0.02	97.4	97.1	± 0.6	0.5	30.2	28.2	± 1.2
0.05	92.3	92.6	± 1.4	0.6	16.5	16.5	± 0.4
0.1	85.9	85.2	± 1.5	0.7	8.2	8.4	± 0.6
0.2	70.4	70.6	± 1.8	0.8	4.7	4.7	± 0.3
0.3	54.3	56.0	± 1.9	0.9	2.8	3.1	± 0.2

The experiments are rather difficult and therefore a great number of observations have been taken. The observed values of the strength of the rennet in the mixture are mean values of not less than eleven different measurements. Thanks to this circumstance, it has been possible to calculate the probable error of each value. This probable error is tabulated under Δ. As is seen from the comparison of $g_{calc.}$ with $g_{obs.}$ the difference between the calculated values and the observed ones are in eight cases

less than the probable error; only in two do they exceed these, and even then not greatly. The agreement may be regarded as striking, and the whole series, which is the condensed result of about seven hundred and fifty tests, — of every mixture there were taken six to eight different tests in as many test-tubes, — may be regarded as a model for further investigation on these difficult subjects.

The concordance of $g_{calc.}$ with $g_{obs.}$ may serve as a very strong proof that the equation used for the calculation is the correct expression of the phenomenon.

As has already been noted (cf. p. 3), Hammarsten and Rodén[1] observed that normal horse-serum contains a substance which is in many respects similar to antirennet. Therefore Madsen and Walbum have examined this antibody in a similar manner, only the time of reaction between the two antibodies was longer, two to four hours. The results of experiments with two different preparations are abridged in the table on opposite page.

This process of neutralisation is not reproduced by the formula valid for the action of rennet upon antirennet, but the equation is the same as that valid for the neutralisation of tetanolysin by means of cholesterin, which indicates that one molecule of rennet and one of the antibody give only one molecule of the reaction-product. The constants are, if we use as unit concentration that of the unneutralised rennet in the first experiment, for the first preparation $K = 0.354$, and for the second $K = 0.138$;

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[1] Rodén: *Upsala läkareförenings forhandlingar*, **22.** 546 (1887). Rodén observed that serum of swine blood is nearly as active as that of horse blood; sera from cattle or rabbits have a much weaker action. Even ascites-fluid has some action. The active substance is destroyed by heating for some few minutes to 70° C. or even by treatment with alcohol.

1 c.c. of the first serum was equivalent to 2 times the fixed part of rennet and 1 c.c. of the second serum corresponded to 2.1 part of the rennet. The observed values in the first series are mean values of three, and in the second of two different series of measurements.

NEUTRALISATION OF RENNET BY MEANS OF NORMAL HORSE-SERUM

n	$g_{obs.}$	$g_{calc.}$	n	$g_{obs.}$	$g_{calc.}$
0	100	100	0	100	100
0.1	80.0	80.2	0.02	93.0	96.3
0.2	64.3	62.1	0.05	87.3	90.9
0.4	51.7	52.1	0.1	71.0	82.0
0.8	33.5	28.4	0.2	63.5	65.3
1.0	24.9	22.2	0.3	54.5	49.5
1.2	21.0	18.1	0.4	34.7	38.3
1.35	16.0	15.8	0.5	29.4	28.9
1.5	13.5	14.0	0.6	22.0	22.2
1.7	10.0	12.2	0.8	19.2	14.4
			1.0	9.4	10.3
			1.3	8.7	7.1
			1.7	3.1	5.0
			2.0	2.9	4.0

The circumstance that the neutralisation of the antibody from normal serum follows quite different laws than those valid for the antirennet produced by immunisation, is a certain indication that these two antibodies are really different substances. This is also probable because the antirennet is much more easily destroyed by heat than the antibody from normal serum.[1] The occurrence of many antibodies in normal sera led Ehrlich to the supposition

[1] This criterion gives alone no absolutely certain indication, for the thermo-stability of a dissolved substance may depend to a rather high degree on the presence of other substances, as salts or proteids (*e.g.* pepton), in the solution. Cf. Biérnacki: *Zeitschr. f. Biologie*, **28** (1891).

that animals generally produce antibodies against different toxins and that this production is only augmented by the experimental injection of toxins into the blood of the animal. In other words, the antibodies in normal sera ought to be identical with those produced after active immunisation. This is evidently not true for the antirennets. There are also many other considerations which speak against Ehrlich's idea, of which Bashford has given a detailed criticism.[1]

In one point I cannot agree with Bashford. He assumes that the toxin, *i.e.* in this case the rennet, is divided between the normal serum and the rest of the fluid. This is quite like the idea held by Biltz. According to Bashford, if for the second serum 0.3 c.c. of the serum takes half of the rennet, then 0.6 c.c. ought to fix two-thirds of it, whereas it actually takes 78 per cent; 0.9 c.c. should fix 75 per cent, whereas the table gives 88; 1.2 c.c. should carry 80 per cent, instead of the 91 per cent observed.

This last series may be calculated according to the scheme of Biltz; it gives a nearly constant value of the exponent p, viz. $p = 2.3$ (cf. p. 216). But the first series for normal horse-serum yields a steadily increasing value of p with increasing n. Between $n = 0.1$ and $n = 0.4$, p is 0.85; between $n = 0.4$ and $n = 1.0$, we find $p = 1.5$; between $n = 1.0$ and $n = 1.5$, p is 2.4; for higher values of n, p is found to be 3.3.

If we apply the idea of Biltz to the fixation of rennet to antirennet, we find that at first the concentration of rennet in the serum is constant until about $n = 0.6$, whereas, that of the rennet in the fluid sinks in the proportion 6 to 1,

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[1] Bashford: *Journ. of Pathology*, **8**. 62 (1902).

which is evidently impossible, p is infinite. Then p passes through the value 12 between $n = 0.6$ to 0.7, and sinks to 5.3 between $n = 0.8$ and $n = 0.9$.

In a very excellent memoir Fuld and Spiro[1] have made it probable that the "antirennet" contained in the normal serum of horse blood is a so-called pseudo-globulin[2] which acts in such a manner that it binds a part of the calcium ions and thereby hinders, or better retards, the coagulation. As we have seen before (cf. p. 74), the influence of the quantity of free calcium ions upon the time of coagulation is as large as that of the quantity of rennet. Therefore a binding of the calcium ions in a certain proportion has the same effect as a neutralisation of rennet in the same proportion. In this case the quantity of calcium salt of the para-casein regulates the velocity of coagulation. The salts of calcium with para-casein and with the pseudo-globulin must be dissociated to a very low degree, and for the sake of simplicity we may suppose that the degree of dissociation of the two salts and of the acids in the presence of a given quantity of calcium ions is such that we may make use of the formula of Guldberg and Waage. This may at least be regarded as a preliminary approximation, which we may employ until the properties of the reacting compounds have been better examined. Then if the quantity of para-caseate of calcium in the absence of

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[1] Fuld and Spiro: *Zeitschr. f. ph. Ch.*, **31**. 147 (1900).

[2] On precipitation with ammonium sulphate, euglobulin and pseudo-globulin separate out. Their aqueous solution is dialysed, then the euglobulin precipitates and the pseudo-globulin remains in solution. The euglobulin has a coagulating influence on casein. The pseudo-globulin retards the coagulation, even if caused by papayotin, cynarase or euglobulin, as well as by rennet.

serum be taken as a unit, this may be regarded as nearly equivalent to the quantity of calcium present, if it does not exceed a certain limit; now n equivalents of pseudo-globulin are added, then at equilibrium we have $(n - x)$ equivalents of pseudo-globulin, $(1 - x)$ equivalents of calcium para-caseate, x equivalents of calcium salt of the pseudo-globulin, and $(a + x)$ equivalents of para-casein, where a is the quantity of free para-casein for $n = 0$. Then the equilibrium follows the equation

$$(n - x)(1 - x) = K \cdot x(a + x).$$

If a is high, i.e. in the presence of much casein, we may regard $(a + x)$ as a constant, and we have the equation with the aid of which the calculated values above have been derived. We may therefore say that the opinion of Fuld and Spiro agrees with the experiments, and it is easy to see how these might be controlled to a still higher degree.

Even against the coagulation of blood-plasma there exist some natural antibodies, one of which, the hirudin, the extract of leeches, has been known for a long time. Fuld and Spiro [1] made the following determinations of the quantity of "free muscle extract" from goose, of which 0.4 c.c. had been taken, in the presence of n c.c. of an extract of leeches, acting on 1 c.c. of goose plasma.

NEUTRALISATION OF MUSCLE EXTRACT FROM GOOSE BY MEANS OF HIRUDIN

n	$\mathcal{E}_{obs.}$	$\mathcal{E}_{calc.}$
0	100	100
0.2	75.0	82.0
0.4	66.7	64.9
0.8	35.7	36.0
1.6	11.1	11.2

[1] Fuld and Spiro: *Hofmeisters Beiträge*, 5. 181 (1904).

For the calculation the formula used was that employed before for the calculation of the influence of horse-serum on rennet. Of the solution of hirudin 1 c.c. is equivalent to the fixed quantity (0.4 c.c.) of the extract, and the constant is $K = 0.09$. The agreement is very satisfying. It therefore seems quite proper to assume that the action of hirudin on the coagulation of blood-plasma is of the same nature as the action of pseudo-globulin on the coagulation of casein.

The circumstance that the substances concerned in biochemistry react not only with their specific antibodies, but also with other compounds, specially with such well-known properties as acids, bases, salts, or even lecithin and cholesterin, is a very promising feature. For if there were no reactions except with the specific antibodies, which we have very little hope of obtaining in the pure state adapted to a detailed investigation, we would have very slight prospects of rapid progress.

Besides the coagulating ferments there is another group of precipitins which deserve this name to a higher degree, namely, those prepared by injection into living animals of fluids containing proteins. Among these the lacto-serum, which is obtained by the injection of milk into animals, has been investigated very thoroughly by P. T. Müller.[1] Other precipitins produced by the intraperitoneal injection of egg-albumen or normal horse-serum into rabbits were studied by Eisenberg;[2] they behaved very like the lacto-serum prepared by the intraperitoneal injection into rabbits and studied by Müller.

[1] P. T. Müller: *Archiv f. Hygiene*, **44**. 126 (1902); *Centralbl. f. Bakteriologie*, etc., **32**. 521 (1902), and **34**. 48 (1903).

[2] Eisenberg: *Bull. de l'Ac. des Sciences de Cracovie*, p. 289 (1902).

Bordet[1] early drew attention to the great difference between rennet and lacto-serum, though both coagulate milk (casein). The coagulation with rennet is much more voluminous and gelatinous than that with lacto-serum, which also gives precipitation at lower temperatures (under 20° C.), very different from rennet. Furthermore, lacto-serum is inactivated only by being heated for thirty minutes to over 70° C., whereas a 2 per cent solution of rennet loses its coagulative power in less than five minutes at 50° C. (cf. p. 87). The lacto-serum is, on the other hand, much more easily precipitated by ammonium sulphate than is rennet. A very important point is that the coagulum of the lacto-serum yields no serum-albumen. A similarity is that in both cases the presence of calcium or barium salts is necessary for the precipitation, so that the reaction is hindered by the presence of oxalates in the milk. Müller investigated other salts, viz. $NaCl$, KCl, NH_4Cl, Na_2HPO_4, $NaCH_3CO_2$, $NaNO_3$, KNO_3, KI, KBr, $KSCN$, and $MgSO_4$, but none of them could replace Ca salts. In this point there is a great distinction from the agglutinations, which are rendered possible by the most widely different salts (cf. p. 159) according to Friedberger.[2] The precipitins examined by Eisenberg do not seem to need salts for their action. Many salts diminish the action in even rather weak concentrations, thus, for instance, $(NH_4)_2SO_4$ in 0.25 normal solution and $MgCl_2$ in 2 normal solution completely check the precipitating action. By heating the milk during some time to 100° C.

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[1] Bordet: *Ann. de l'Inst. Pasteur*, **13**. 241 (1899).
[2] Friedberger: *Centralbl. f. Bakteriologie*, **30**. 341 (1901). Cf. Bechhold: p. 159 above.

or egg-albumen to 78° C. during 60–90 minutes, their precipitability was lost. But Müller found that the precipitation by lacto-serum could be restored by the addition of Ca salts, or even that it would not disappear if the milk contained naturally much calcium. Concentrated solutions of urea or formalin destroy the precipitability of egg-albumen as well as the agglutinability of bacteria.

The precipitate from a mixture of milk and lacto-serum dissolves in a 1 per cent solution of NaCl. This solution can be precipitated again by rennet or lacto-serum, just as a solution of casein. The treatment with rennet also yielded serum-albumen. Evidently the precipitate is somewhat soluble, and in solution partly dissociated into the two components. At high temperature the free lacto-serum is decomposed and new quantities of lacto-serum are successively formed by the decomposition of the soluble fraction of the precipitate until this is wholly reconverted into casein. Müller also isolated the lacto-serum from the precipitate through cautious treatment with acetic acid; the liquid obtained by centrifugation of the precipitate, which had been in contact with the acid solution for two hours, contained a noticeable quantity of precipitin. The compound of lacto-serum and casein may be precipitated by the sudden addition of acetic acid. The precipitate dissolves completely upon neutralisation, but is precipitated by small quantities of a calcium salt. The compound, therefore, probably exists in the solution, but in a partially dissociated state, and gives an insoluble product with calcium or barium salts. Para-casein, prepared by the action of rennet on milk, does not bind the lacto-serum.

The lacto-serum heated to 70° C. for thirty minutes

acquires the peculiar property of hindering the precipitation of casein by means of lacto-serum. In the same manner Eisenberg found that his precipitin for egg-albumen on heating for one hour to 72° C. was transformed into an antiprecipitin. In the same manner the precipitins against cholera and typhoid, prepared by injection of cultures of the corresponding bacteria into the veins of a horse, lose their property of coagulating the corresponding bacterium on being heated for thirty minutes to about 60° C.,[1] and acquire anticoagulating properties on heating to 73° C. (for cholera-serum). Even for the agglutinins, similar observations have been made by Eisenberg and Volk. Through different experiments Müller was led to the conclusion that the antiprecipitin binds the casein, with which it gives a compound soluble in the presence of calcium salts. Antiprecipitin may even dissolve the precipitate, in the same manner as a carbonate is dissolved by a not too weak acid. This was shown in a simple manner by Eisenberg, by preparing in one test a mixture of precipitin and antiprecipitin with egg-albumen, and in a second test a mixture of antiprecipitin and egg-albumen with precipitin. In the second case no precipitate was formed because the egg-albumen was bound by the antiprecipitin, while in the first case precipitation occurred. (The velocity of reaction is evidently rather slow, otherwise the two experiments would give the same result.) Similar experiments were made by Eisenberg with coagulating serum antagonistic to the bouillon of typhoid cultures. The antiprecipitins are derived from the precipitins, for after these have been precipitated from the serum (for

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[1] Pick: *Hofmeisters Beiträge*, 1. 81 (1901).

instance, from lacto-serum by means of milk), it yields no antiprecipitin on being heated; normal serum likewise gives no antiprecipitin.

The binding of precipitin to casein may be judged, on the basis of the insignificant solubility of the compound, to be nearly complete if the two substances are present in equivalent quantities. If one of them is present in the fluid in excess, it is to a large extent carried down by the precipitate. Especially is this valid for precipitin. With the precipitable substance there is another perturbing influence, which is especially prominent with egg-albumen and which has its analogies in the behaviours of agglutinins and serum-precipitins. An excess of egg-albumen dissolves the precipitate, so that it is often observed that a given quantity of precipitin causes a precipitate with a weak but not with a stronger solution of egg-albumen. Even with casein this peculiarity may be observed, as Müller's later experiments indicate. The quantity of the precipitate produced by a given quantity of lacto-serum therefore increases at first with the quantity of casein added, and reaches a maximum in the neighbourhood of the point where the casein added is equivalent to the quantity of lacto-serum, only to decrease thereafter and fall to zero at a point where the quantity of casein added is nearly double that corresponding to the maximum value; the determinations do not seem to be accurate enough to warrant more than an approximative valuation.

Some experiments of Eisenberg afford an idea of the relations between antiprecipitin and precipitin. The quantities of the preparations are given in drops. The antiserum was heated precipitin diluted in the proportion 1 to 5.

A is the quantity of egg-albumen, B the quantity of precipitin, C that of antiprecipitin, D the rest of the fluid given in quantity of physiological salt-solution. The total quantity was always about sixty drops.

ANTAGONISM BETWEEN PRECIPITIN AND ANTIPRECIPITIN

A	B	C	D	OBS.	P
1	0.6	0	58	Prec.	0.6
1	1.2	1	56	No	0.38
1	3	1	55	Prec.	0.6
1	12	5	42	No	0.55
1	30	5	24	Trace	0.75
1	53	5	1	Prec.	0.84
1	44	15	0	No	0.59
3	3	15	39	No	0.27
10	3	15	32	Prec.	0.9

The results may be calculated on the supposition that the antiprecipitin is double as strong as the precipitin, *i.e.* one drop of the antiprecipitin contains double the number of equivalents as one drop of precipitin, and that the egg-albumen is divided equally between the different equivalents. Then the precipitate P formed may be calculated from the formula : —

$$P = \frac{B}{B+2C} \cdot A.$$

I have therefore calculated this expression and tabulated it under P. It will be seen that if P equals 0.6 or is greater, precipitation is observed; if it is less, not.

The precipitate from egg-albumen is soluble in weak solutions of acids or bases, but insoluble even in concentrated solution of sodium chloride. The acid solution yields precipitation on neutralisation. On heating, it coag-

ulates, so that it loses its solubility in weak acids.   In this point it differs from the lacto-serum precipitate and from agglutinated bacteria, which lose their agglutination at high temperatures.   It is soluble in concentrated solutions of urea or magnesium chloride or in formalin.   In this point it resembles agglutinated bacteria.

A great practical importance is attached to the examination of the properties of serum-precipitins, prepared by the injection of serum from one animal into the veins of another animal.   These precipitins are to a high degree specific, and they have therefore been used to determine the origin of blood-flecks for forensic or medico-legal purposes. On this point it may be sufficient here to refer to the investigations of Uhlenhuth,[1] Wassermann and Schütze,[2] and Hamburger.[3]

Recently Hamburger[4] has executed some quantitative experiments on the action of these precipitins.   He measured the precipitate formed in the reaction between a certain quantity of a blood-serum and its antibody produced by the repeated injections of this serum into the veins of another animal.   The reacting fluids were mixed in a funnel-shaped vessel which extended into a capillary tube of uniform calibre and graduated in 100 divisions. By vigorous centrifugation of this vessel for 1.5 to 2 hours he packed the precipitate into the capillary tube, where it reached a constant volume and was measured by the reading of the divisions.

[1] Uhlenhuth: *Deutsche med. Wochenschrift*, No. 30, p. 499 (1901).
[2] Wassermann and Schütze: *Berl. klin. Wochenschrift* (1901).
[3] Hamburger: *Deutsche med. Wochenschrift*, No. 6 (1905).
[4] Hamburger: *Folia hæmatologica*, Vol. 2, No. 8 (1905).

One series of experiments was done with serum (A) from horse blood and serum (B) from a calf which had been treated with horse-serum several times. The horse-serum was diluted with 50 times its volume of a 1 per cent solution of sodium chloride. If to a certain quantity, 1 c.c., of the calf-serum increasing quantities of horse-serum (A) were added, at first no precipitate appeared; then at higher concentrations of A the quantity of precipitate increased nearly proportionally to the quantity of A added until a maximum was reached. Thereafter further additions of horse-serum caused the quantity of precipitate to decrease until at a certain limit the precipitate again disappeared. This behaviour, which is very common in reactions between sera and their precipitins, is clearly apparent from the following figures.[1] In this case the total volume (100 div.) of the capillary tube was 0.04 c.c. The precipitate is therefore in the following table given in units of 0.0004 c.c., corresponding to one division. One c.c. of calf-serum is fixed as 100 units, corresponding to the fact deduced from the experiments that it could yield in maximo 100 units of precipitate, P; and 1 c.c. of the diluted horse-serum is on analogous grounds fixed as 300, so that 1 c.c. of A is equivalent to 3 c.c. of B. This seems to indicate that nearly the whole quantity of the albuminous substances in A, but only a very small fraction of those in B, enter into the precipitate.

The maximum is reached at $A = 100$; that is, on the addition of 0.333 c.c. of horse-serum, the quantity equiva-

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[1] Hamburger and Arrhenius: Proc. of the Meeting of the R. Ac. of Sciences, in Amsterdam, May 26, 1906, p. 33.

lent to the quantity of calf-serum present. (This observation has really led to the determination of the equivalent quantities of the two sera.)

ACTION OF DIFFERENT QUANTITIES (A) OF HORSE-SERUM ON A GIVEN QUANTITY (100) OF CALF-SERUM

A	VOL.	$P_{obs.}$	$P_{calc.}$	A	VOL.	$P_{obs.}$	$P_{calc.}$
4	1.013	0	0.2	79	1.267	51	53.6
8	1.027	3	3.9	88.3	1.294	55	57.1
15	1.05	10	10.3	90	1.3	57	57.5
24	1.08	17	17.8	100	1.333	59	58.9
30	1.1	21	23.6	115.4	1.385	55	57.4
39	1.13	32	29.7	137	1.457	50	51.3
45	1.15	34	34.0	167	1.557	43	41.3
54	1.18	43	40.1	214	1.713	25	26.8
60	1.2	45	43.9	300	2.0	5	5.5
75	1.25	$\begin{cases} 53 \\ 51 \end{cases}$	51.9	500	2.67	2	0

The calculated figures, which within the errors of observation agree with the observed values — the magnitude of the errors of observation may be judged by the difference of the two observations for $A = 75$ — are found in the following manner: The quantity B of calf-serum (in this case $B = 100$) may react with A equivalents of horse-serum. Then a certain amount of precipitate, P_1, is formed, of which p parts are soluble in 1 c.c. and the rest, $P_1 - pV = P$, is packed in the capillary tube (V is the volume of the mixture). Then there are remaining in the solution $A - P_1$ equivalents of horse-serum, and $B - P_1$ equivalents of calf-serum. But experience indicates that a further addition of horse-serum dissolves the precipitate. Let us suppose that there are formed Y equivalents of

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soluble compound, such that one equivalent of it contains one equivalent of precipitate and n equivalents of horse-serum (or in other words $n + 1$ equivalents of horse-serum and 1 equivalent of calf-serum). Then there are present in the solution the following quantities in equivalents of the different substances : —

$$(A - P - pV - (n + 1)Y) \text{ of horse-serum,}$$
$$(B - P - pV - Y) \text{ of calf-serum;}$$

and the following equations of equilibrium are valid : —

$$\{A - P - pV - (n + 1)Y\} \{B - P - pV - Y\} = K_1 p^m V^2,$$
$$\{A - P - pV - (n + 1)Y\} p = K_2 Y.$$

If we determine Y from the last equation and introduce it into the first one, we obtain : —

$$(A - P - pV)\left(1 - \frac{(n + 1)p}{K_2 + (n + 1)p}\right)\left(B - P - pV - \frac{(A - P - pV)p}{K_2 + (n + 1)p}\right) = K_1 p^m V^2.$$

p is found in the experiments to be 2.5. If we put $\frac{K_2}{p} + n + 1 = 3.45$ and $K_1 p^m - \frac{K_2 + (n + 1)p}{K_2} = 130$, we find the calculated values of the total quantity (P) of precipitate. The tabulated values of P give this quantity divided with the volume, V, i.e. the quantity of precipitate in 1 c.c.

The last equation gives $\frac{K_1}{K_2} \frac{p^{m+1}}{0.29} = 130$. If we suppose $m = 1$, i.e. that one molecule of precipitate is formed from one molecule of each of its two components, we obtain $\frac{K_1}{K_2} = 3.08$; if we suppose $m = 2$, we find $\frac{K_1}{K_2} = \frac{3.08}{p} = 0.879$.

These two assumptions regarding m seem the most probable. In the first case the constants of reaction are of the same order of magnitude; in the second case K_2 is of the same order of magnitude as $K_1 p$. As, further, $n = 2.45 - \dfrac{K_2}{p}$, and $\dfrac{K_2}{p}$ has a positive value, n can only be 1 or 2 (if we do not take into consideration fraction numbers). The probability is therefore that the soluble compound of precipitate with the active fraction of the horse-serum is built up of one molecule of the precipitate and one or two molecules of the other reacting substance.

In some cases it is not necessary to assume that the precipitate is dissolved on the further addition of the serum which has been injected. A very interesting instance of this behaviour is given by Hamburger in the action of the serum of a rabbit treated with sheep-serum on the sera of three different animals, viz.: sheep, goat, and bullock, which are so closely related to each other that all yield precipitates with the said rabbit-serum. As is natural, the sheep-serum yielded the most voluminous precipitate; next comes the goat-serum, which animal is the most closely related to the sheep; and the least quantity of precipitate is given by the serum from the bullock, which is less closely related to the sheep than is the goat. The figures are given in the following three tables. The rabbit-serum was always used in the quantity of 0.4 c.c.

The quantity of the precipitate is given in scale-divisions of the capillary tube (100 divisions = 0.04 c.c.). The sheep-serum was normal serum diluted with 49 times its volume of 1 per cent solution of sodium chloride. The sera of goat and bullock used in the following two series of ex-

ACTION OF SERUM FROM A RABBIT, IMMUNISED WITH SHEEP-SERUM, ON SHEEP-SERUM (A)

Quantity of A		V^2	Quantity P of Precipitate	
c.c.	Equiv.		obs.	calc.
0.02	0.8	0.162	1	0.5
0.04	1.6	0.163	2	1.3
0.1	4	0.25	3	3.5
0.15	6	0.302	6	5.3
0.2	8	0.36	7	7.2
0.6	24	1.00	21	21.5
1	40	1.96	35	34
1.5	60	3.61	39	48
2	80	5.76	60	57
3	120	11.56	67	66
5	200	29.2	64	65
7	280	54.8	58	58
10	400	108.2	49	46
15	600	237	10	19
18	720	338	5	3
20	800	416	2	0
1 (+ 1 c.c. aq.)	40	5.76	28	25
5 (+ 1 c.c. aq.)	200	41	57	51
10 (+ 1 c.c. aq.)	400	130	41	32

periments were also diluted in the same manner. The experiments were calculated in the following manner. I c.c. of the sheep-serum is equivalent to 40 units of precipitate. The number of such equivalents of sheep-serum is given in the second column. In the same manner 0.4 c.c. of rabbit-serum contains 120 such equivalents, $i.e.$ 1 c.c. contains 300 equivalents. If now the volume is V, containing originally A equivalents of sheep-serum and 120 equivalents of rabbit-serum, and P equivalents of precipitate have been formed, then the concentrations of the two

sera are $\dfrac{(A-P)}{V}$ and $\dfrac{(120-P)}{V}$, and the formula repre-senting the equilibrium is : —

$$(A-P)(120-P)=K\cdot V^2,$$

where K is a constant, which according to the experiments is 250. With the aid of this equation the calculated values of P have been found and are written beside the observed values. In the three last tests 1 c.c. of water containing 1 per cent of sodium chloride has been added to the mixture of the sera, which circumstance has been taken account of in the calculation of the volume V (in c.c.).

The agreement between the observed and the calculated values may be regarded as very satisfying, especially with respect to the enormous change of A (in the proportion of 1 : 1000) and of V^2 (1 : 2500).

The simple form of the equation of equilibrium depends upon two circumstances: the extremely low solubility of the precipitate in rabbit-serum (not 1 in 3000), and in sheep-serum. Probably these two circumstances are connected with each other. The figures obtained with goat-serum were calculated in the same manner and the results are given below. In this case 1 c.c. of the goat-serum was always equivalent to 40 units of the precipitate, but 0.4 c.c. of the rabbit-serum was equivalent to only 85 units of precipitate.

The constant K is set at 200. Here the agreement is not so perfect as in the foregoing series. The agreement would have been much closer for the latter part of this series if we had taken $K = 160$, but then the first part would have shown greater deviations than it does now;

ACTION OF SERUM FROM A RABBIT, IMMUNISED WITH SHEEP-SERUM, ON GOAT-SERUM (A)

QUANTITY OF A		V^2	QUANTITY P OF PRECIPITATE	
c.c.	Equiv.		obs.	calc.
0.02	0.8	0.162	1	0.4
0.04	1.6	0.163	2	1.2
0.1	4	0.25	4	3.4
0.15	6	0.30	5	5.2
0.2	8	0.36	6	7
0.6	24	1.00	16	21
1	40	1.96	26	32
1.5	60	3.61	30	43
2	80	5.76	35	48
3	120	11.56	40	51
5	200	29.2	50	47
7	280	54.8	52	40
10	400	108.2	34	27
12	480	154	27	18
15	600	237	9	5
18	720	338	8	0
20	800	416	4	0
1 (+1 c.c. aq.)	40	5.76	21	22
5 (+1 c.c. aq.)	200	41.0	48	35
10 (+1 c.c. aq.)	400	130	30	17

perhaps this difficulty depends upon a different solubility of the precipitate in rabbit-serum and in 1 per cent solution of sodium chloride. Perhaps even a difference of temperature in the two parts of the series is responsible for the disagreement; it is really the weak point of these measurements that the temperature cannot be controlled during the centrifugation. But in any case there can be no doubt that the chief part of the phenomenon is represented by the last formula.

ACTION OF SERUM FROM A RABBIT, IMMUNISED WITH SHEEP-SERUM, ON BULLOCK-SERUM (A)

QUANTITY OF A		V^2	QUANTITY P OF PRECIPITATE	
c.c.	Equiv.		obs.	calc.
0.02	0.8	0.162	1	0.5
0.04	1.6	0.163	2	1.3
0.1	4	0.25	4	3.4
0.15	6	0.30	5	5.2
0.2	8	0.36	7	6.7
0.6	24	1.00	16	19
1	40	1.96	20	24
1.5	60	3.61	22	26
2	80	5.76	25	26
3	120	11.56	28	25
5	200	29.2	22	21
7	280	54.8	10	17
10	400	108.2	7	11
12	480	154	5	7
15	600	237	3	1
18	720	338	2	0
20	800	416	1	0
1(+1 c.c. aq.)	40	5.76	16	15
5(+1 c.c. aq.)	200	41	13	16
10(+1 c.c. aq.)	400	130	5	7

This is to a still higher degree valid for the experiments with bullock-serum, reproduced above. One c.c. of this serum contained 40 equivalents, 0.4 c.c. of the rabbit-serum only 35 equivalents. K was found to be equal to 85.

The agreement is very satisfying.

The conclusions which may be drawn from these three series are rather interesting. On injection of sheep-serum into rabbit blood we have obtained an antiserum containing per centimeter cube 300 equivalents of pre-

cipitin against sheep-serum, 212 equivalents of precipitin against goat-serum, and only 90 equivalents of precipitin against bullock-serum. All of these three normal sera contain 2000 equivalents per centimeter cube. In the same manner and in nearly the same proportion the constant of reaction sinks from 250 for sheep-serum to about 170 for goat- and to 85 for bullock-serum. Probably the sheep-serum contains additional substances which occur also in sera of goats and of bullocks, which after injection into rabbits produce antibodies against these sera, although in lesser proportions than the precipitin against the chief substance in sheep-serum, which gives a precipitate with the serum from the inoculated rabbit. The observations lead to the conclusion that we have a mixture of three different precipitins in the rabbit-serum. Otherwise it is difficult to understand that 1 c.c. of all the three normal sera, from sheep, goat, and bullock, contain nearly the same number (2000) of equivalents of the precipitate formed. As 100 equivalents pack a volume of 0.04 c.c., the precipitates given by 1 c.c. of the rabbit-serum would pack a volume of 0.24 c.c., of which probably only a small part is derived from this serum itself, the chief part being derived from the other sera.

The experiments with precipitins lead to the conclusion that they are really bound in the precipitates and do not act as catalytic agents. The action of agglutinins displays a very great similarity to that of the precipitins, so that it is reasonable also for this case to assume a real chemical reaction in stoichiometric proportions and not a catalytic action. Further, we have observed that hæmo-

lytic substances, such as tetanolysin, are bound to the substance acted upon, so that a given quantity of lysin can lake only a given equivalent quantity of erythrocytes (cf. pp. 104, 111). Even for the agglutinins such an equivalence has been observed. It is a general feature of the theory of Ehrlich that he assumes that the action of poisons depends upon a binding, or as he often says an "anchoring," of the poison to the substrate upon which it acts. In this regard Ehrlich goes however a little too far, since he, for instance, supposes that all the immune-body absorbed by an erythrocyte is " anchored " to it.

Furthermore, we have found that on neutralising a poison or an analogous substance with its antibody, a real binding takes place according to stoichiometrical proportions. It may here be observed that in some cases — and these are perhaps rather common (cf. p. 267), as for instance in the coagulation of casein—the reacting substance is really not a single one but two, rennet and calcium ions; the antibody (*e.g.* that from normal horse-serum) binds only the one component of the reacting mass (here the calcium ions), probably leaving the rennet intact. In similar cases it is very easily possible that the one component that is not bound by the antibody acts as a catalysor. This seems very probable for rennet, as the time necessary for coagulation is within very wide limits inversely proportional to its quantity.

Nevertheless, on the whole, we obtain from a closer study of these phenomena the opinion that the catalytic action does not play the chief rôle which has been often assigned to it by different authors. Ehrlich has on repeated occasions rightly laid stress upon the necessity of an

investigation of the relations between toxins and antitoxins according to the general principles of physical chemistry.[1]

In the foregoing pages I have tried to carry through the programme advocated so strongly by Ehrlich. That this has been possible, is due in large part to the great number of quantitative measurements which have been carried out in the last quinquennium, especially by Madsen and his collaborators. This work is still in full progress; and it may therefore be well regarded as very probable that we are here entitled to use the words with which Dr. Findlay[2] closed his lectures in the University of Birmingham, "Considering the very brief period during which physical chemical methods have found application to the study of biological problems, the advance which has been made is very remarkable; and there is every reason to believe that in the future, as the methods become more and more extensively applied, the advance will become more rapid and widespread." Our hope in this direction lies chiefly in the treatment of quantitative experiments on the basis of physical chemistry. It may be confidently expected that the accumulating quantitative work will rapidly give solidity to this discipline of science. I know well that objections have been raised to some of the conclusions that I have here enunciated. But it is clear to me that, if these objections are to deserve a more than momentary

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[1] Cf. P. Ehrlich: *Rapport au 13ᵉ Congrès internationale de médecine*, Paris, 2–9 Août, 1900, *Section de bactériologie;* "Schlussbetrachtungen," "Nothnagel's spezielle Pathologie und Therapie," T. 8, pp. 6–7, Wien, 1901; *Bericht über die Thätigkeit des Instituts f. Serumforschungen zu Sieglitz*, p. 19, Jena, 1899 (G. Fischer); "Ueber Toxine und Antitoxine" in Therapie der Gegenwart, 1901.

[2] Al. Findlay: "Physical Chemistry and its Applications in Medical and Biological Science," p. 68, Longmans, Green & Co., London, 1905.

interest, they must be shown to agree with the quantitative measurements already executed or with other new ones.

In the foregoing discussions it has been shown that the available observations conform to the laws deduced by physical chemistry for common chemical compounds. Therefore discussions as to whether toxins and their antitoxins, being defined as colloids, might be held not subject to these general chemical laws, will possess but little interest until the assumed deviations from said laws are measured quantitatively. Any other method of procedure has a very hypothetical value.

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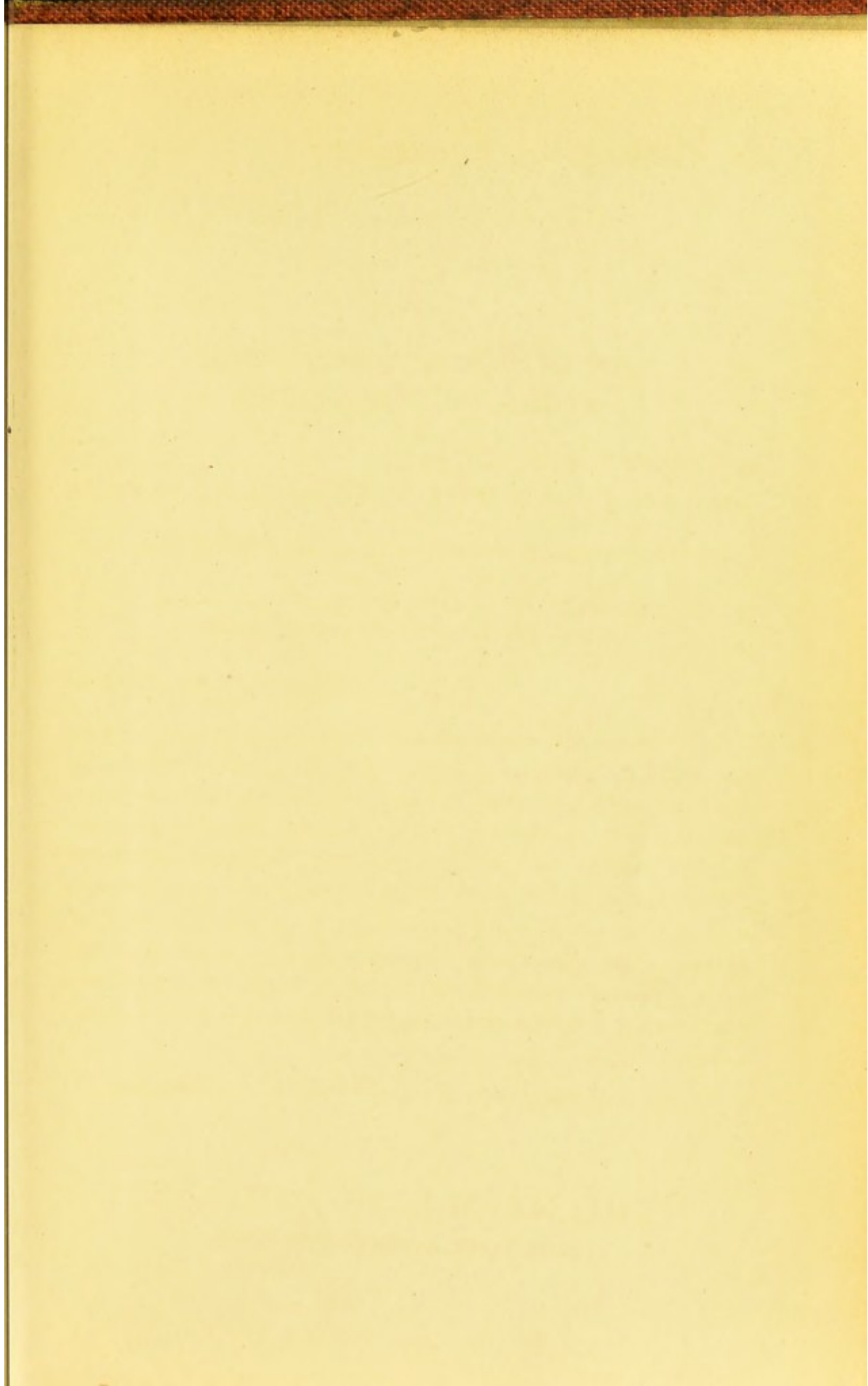
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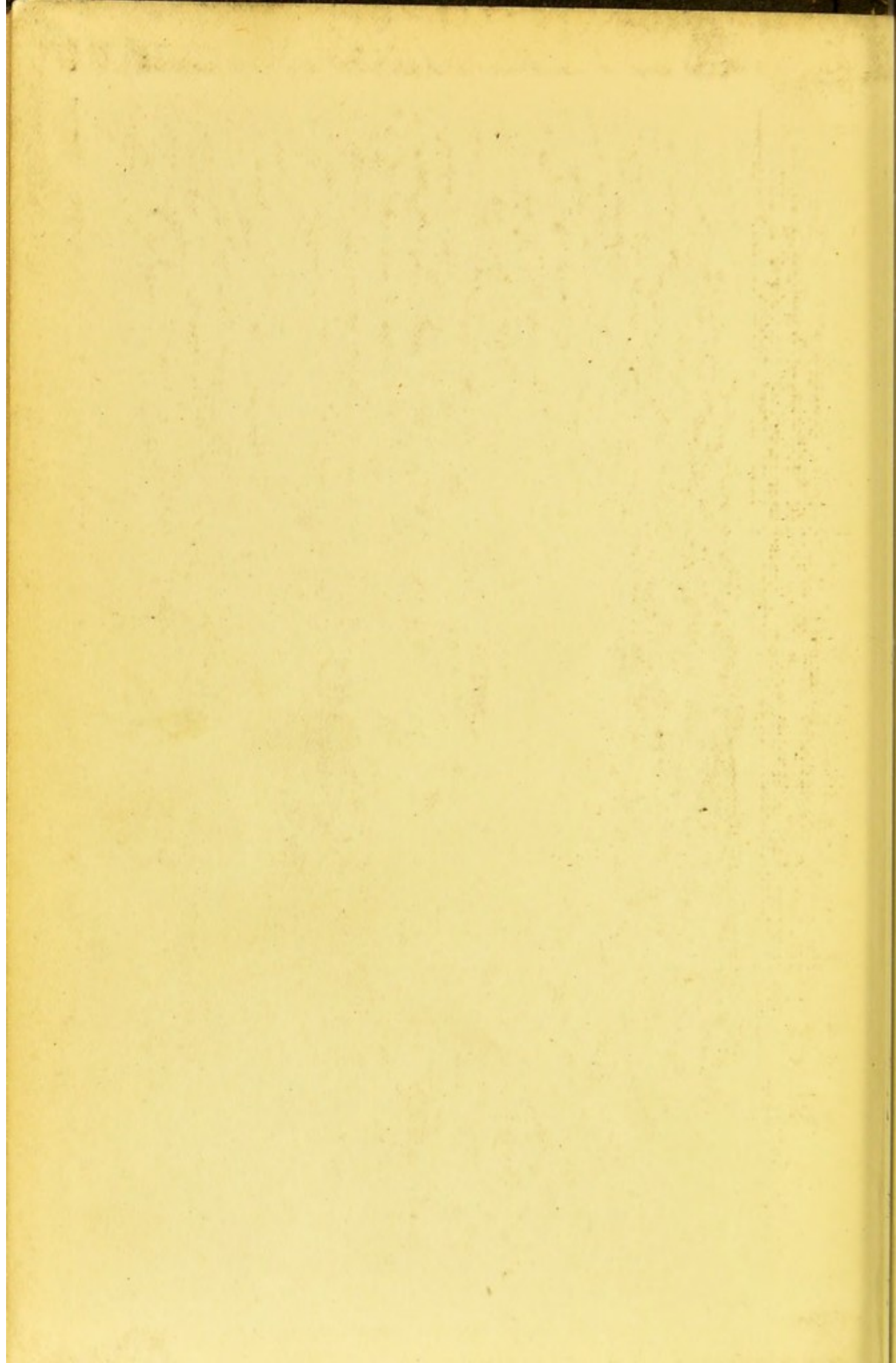

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