The vegetable proteins / by Thomas B. Osborne.

Monographs on Biochemistry

THE VEGETABLE PROTEINS

BY

THOMAS B. OSBORNE, PH.D.

MONOGRAPHS ON BIOCHEMISTRY

EDITED BY

R. H. ADERS PLIMMER, D.Sc.

AND

F. G. HOPKINS, M.A., M.B., D.Sc., F.R.S.

THE subject of Physiological Chemistry, or Biochemistry, is enlarging its borders to such an extent at the present time, that no single text-book upon the subject, without being cumbrous, can adequately deal with it as a whole, so as to give both a general and a detailed account of its present position. It is, moreover, difficult, in the case of the larger text-books, to keep abreast of so rapidly growing a science by means of new editions, and such volumes are therefore issued when much of their contents has become obsolete.

For this reason, an attempt is being made to place this branch of science in a more accessible position by issuing a series of monographs upon the various chapters of the subject, each independent of and yet dependent upon the others, so that from time to time, as new material and the demand therefor necessitate, a new edition of each monograph can be issued without re-issuing the whole series. In this way, both the expenses of publication and the expense to the purchaser will be diminished, and by a moderate outlay it will be possible to obtain a full account of any particular subject as nearly current as possible.

The editors of these monographs have kept two objects in view: firstly, that each author should be himself working at the subject with which he deals; and, secondly, that a *Bibliography*, as complete as possible, should be included, in order to avoid cross references, which are apt to be wrongly cited, and in order that each monograph may yield full and independent information of the work which has been done upon the subject.

It has been decided as a general scheme that the volumes first issued shall deal with the pure chemistry of physiological products and with certain general aspects of the subject. Subsequent monographs will be devoted to such questions as the chemistry of special tissues and particular aspects of metabolism. So the series, if continued, will proceed from physiological chemistry to what may be now more properly termed chemical physiology. This will depend upon the success which the first series achieves, and upon the divisions of the subject which may be of interest at the time.

R. H. A. P.
F. G. H.

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TO

SAMUEL W. JOHNSON

UNDER WHOSE DIRECTION THE AUTHOR

FIRST UNDERTOOK HIS INVESTIGATIONS OF

THE VEGETABLE PROTEINS

PREFACE TO THE SECOND EDITION.

THE prediction in the last paragraph of the preface to the first edition has been so well fulfilled, and so much time has elapsed since the first edition was published, that the author has been urged to revise the text and to extend the scope of this monograph.

During the thirteen years since the appearance of the first edition the progress of biochemistry has made some of the chapters in the first edition less important and others so much more important than formerly that some of these have been omitted and others rewritten. It has also been necessary to introduce several new chapters dealing with subjects which have become important since the first edition was written. As a result of these changes it has been necessary to entirely rewrite a very considerable part of this monograph.

The basic and acid properties of proteins, which were discussed in the first edition, have since been studied by many investigators, among whom Professor L. J. Henderson has been particularly prominent. Since this field has now extended so far beyond the experience of the author, Professor Henderson has kindly contributed the chapter on "The Relation of Proteins to Acids and Bases." The author wishes to express his appreciation of this important contribution to science, and to acknowledge with thanks his co-operation in setting forth our present state of knowledge respecting the relations of the vegetable proteins towards acids and bases.

He also wishes to acknowledge the helpful suggestions and assistance which he has received from Dr. H. B. Vickery and Dr. A. C. Chibnall respectively, in the preparation of the chapters on the rate of hydrolysis of proteins and on the proteins in green plants.

It is with especial pleasure that the author takes this opportunity to acknowledge the debt he owes to Dr. E. H. Jenkins, the Director of the Connecticut Agricultural Experiment Station, for his interest in and support of the investigations with which the author has been engaged during the past thirty-five years.

Whatever may have been contributed by these investigations to progress in this field of science has been in large measure due to the generous financial support which has also been afforded by the Carnegie Institution of Washington and by the kindly interest and encouragement received during many years from Professor R. S. Woodward, formerly Director of this Institution, and his successor, Professor J. C. Merriam.

T. B. O.

PREFACE TO THE FIRST EDITION.

ALTHOUGH the proteins of plants early claimed the attention of many chemists, our present knowledge of them has not yet advanced beyond what is, in fact, a mere beginning of a serious study. The isolation and purification of vegetable proteins present so many difficulties that for a long time the available methods were too crude to enable those who undertook such work to succeed in their task. The development of the methods used by physiological chemists in their investigations of animal tissues, the great development of organic chemistry, and the no less important knowledge of the use of antiseptics and the action of enzymes has only recently made it possible to prosecute a study of the vegetable proteins with a reasonable prospect of success.

A knowledge of the vegetable proteins is in many ways of importance to the animal physiologist, but the latter has had so many rapidly increasing lines of research opened to him during the past few years that he has had little time to give to the literature of the vegetable proteins. The writer has, therefore, thought it more important to devote the limited space of the present monograph to a discussion of the general chemical and physical properties of the vegetable proteins than to give a descriptive account of the individual proteins at present known. It is hoped that by this method of presentation the opportunities offered by the vegetable proteins for obtaining a more definite knowledge of the properties of protein matter in general will be better appreciated, and that in the future the studies of vegetable and animal proteins will be brought into closer relations than in the past.

The knowledge of the chemistry of the carbohydrates has been chiefly founded on studies made with those of vegetable

origin, and it is not at all beyond the range of possibility that further study of the vegetable proteins will result in greatly extending our knowledge of the chemistry of the proteins of both animals and plants.

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CHAPTER I.

HISTORICAL REVIEW.

BECCARI [1745] published in the *Proceedings* of the Bologna Academy an account of his experiments with wheat flour, in which he described the separation of the flour into two parts, one of which, he says, was similar to "those things that are extracted from vegetable substances," and "the other was such that it did not seem possible to extract it except from animal matter." He states that he had already communicated this fact to the Academy in 1728, but this communication appears to have never been published. After giving a detailed description of the method by which he obtained this peculiar substance, which we now know as wheat gluten, he describes at length the experiments by which he compared its properties with products of animal origin and contrasted its behaviour with that of other known substances of vegetable origin. In making these comparisons he used destructive distillation and pointed out that the distillates from vegetable materials were acid, while those from the wheat gluten were alkaline like the distillates from animal substances. A comparison was also made between the products of putrefaction of gluten with those yielded by animal and vegetable matter under similar conditions.

Kessel-Meyer [1759] was the next to call attention to gluten, and gave a brief description of its preparation and of experiments to determine the action of various solvents upon it.

Rouelle [1773, 1] announced that the glutinous matter, which up to that time was known to exist only in the seeds of wheat, was present also in other parts of various plants. This he considered to be the nutritive substance from which the caseous part of milk was derived. He stated that it was insoluble in water and gave rise to the same products as the gluten of flour, and also that it can be changed into a body having the same odour as cheese, as Kessel-Meyer had found to be the case with wheat gluten.

Later, Rouelle [1773, 2] separated this glutinous substance from the juice of hemlock by heating to a moderate temperature and filtering out the coagulum, which had a bright green colour. He also obtained by

fractional coagulation a part which contained nearly all of the colouring matter and, at a higher temperature, a part which was nearly free from colouring matter. The colouring matter could also be extracted by digesting the coagulum with alcohol. The protein nature of this substance was proved by the products of destructive distillation. Rouelle was, therefore, the first to obtain evidence of the wide distribution of protein substances in the different parts of plants.

During the same year Parmentier [1773, 1] described his extensive study of various vegetable substances used for food, and among other questions to which he devoted his attention was the gluten of wheat. He found that this substance, while insoluble in mineral acids, was soluble in vinegar, and that by neutralising its solution with sodium carbonate it was precipitated with apparently unchanged properties. Its solution in vinegar, on evaporation to dryness, left a horny and yellow residue which was not hygroscopic. When the gluten was extracted with spirits of wine some was dissolved, and the yellow solution when evaporated left a transparent residue which burned with a strong odour of burning animal matter. Nevertheless he appears to have regarded this residue as a resin. When the gluten was boiled with water it lost its tenacity and coherence and had evidently suffered a decided physical change. When the gluten was exposed to dry air at a low temperature it left a residue which, when treated with water, regained its original moist weight; from this he concluded that it occurred in the seed in the dry form and on contact with water became hydrated, hence the necessity for kneading dough.

Parmentier [1773, 2] in another paper stated that on drying, gluten lost two-thirds of its weight, and the dried product thus obtained, when triturated with water, was restored to its original glutinous and elastic condition.

In 1776, Parmentier further stated that wheat flour must be very much altered in order to give no gluten, and that this substance completely disappears only when the seed has germinated.

At about this time Berthollet reported that when gluten was treated with nitric acid nitrogen was evolved and the residue became yellow.

Fourcroy [1789] gave an extensive account of the occurrence of coagulable protein in the juices of various parts of many plants, and described the method by which he obtained preparations of what he supposed to be pure plant albumin. This he found to have all the properties of animal albumin, and he gave an account of a comparison of these two substances. Fourcroy was the first to recognise the presence of two kinds of protein in plants.

From 1799 to 1805, albumin was found in the juices of many plants and in the sap of trees by a number of different investigators, who, however, added but little to the information which had been furnished by Fourcroy (cf. Deyeur and Vauquelin [1797], Vauquelin and Brongniart [1798], Jordan [1801], Vauquelin [1801-02, 1802-03, 1803-04], Cadet [1803-04], Proust [1802, 1, 2], Fourcroy [1802]).

Einhof [1805, 2] discovered that a part of the gluten of wheat was soluble in alcohol, and described the existence of similar proteins in rye [1805, 2] and barley [1806, 1]. He, however, assumed that all of the gluten of wheat was soluble in alcohol, and considered this to be a characteristic property of all vegetable proteins except the albumin, which occurred dissolved in the juices of plants. He also undertook an extensive investigation of the constituents of the potato [1805, 1], barley [1806, 1], peas and beans [1806, 2] and lentils [1806, 3], and found that the leguminous seeds contained a form of protein which was not soluble in alcohol or in water. He assumed that this belonged to a distinctly different group of substances, although he recognised that it was related to the gluten found in the seeds of the cereals. His discoveries showed the presence of two new forms of protein matter in plants and laid the foundation for a more extended knowledge of these substances.

Gren [1809] in his *Grundriss der Chemie*, in reviewing the literature of vegetable proteins, stated that gluten contains carbon, hydrogen, nitrogen, phosphorus and calcium, for by distillation it yields products which contain these elements. He further stated that plant albumin contains hydrogen, nitrogen, carbon, sulphur, oxygen and probably calcium phosphate, and cites as his authority the analyses by Fourcroy and a then recent paper by Jordan [1801]. He does not mention, however, the nature of the evidence given by these latter investigators from which this composition of plant albumin was deduced, and as he makes no reference to the original publications by either of these authors, it has not been possible to determine the basis for his statement, which appears to be the first publication in regard to the ultimate composition of vegetable proteins.

During the next ten years little advance was made beyond an addition to the number of seeds in which such substances were found (cf. Braconnot [1813], John [1814], Seguin [1814], Vauquelin [1814, 1817], Link [1815], Boullay [1817], Proust [1817], Vogel [1818]).

Taddei [1819, 1] separated wheat gluten into two well-characterised parts, one of which, soluble in alcohol, he called gliadin, the other,

insoluble therein, zymom. By this observation three distinct proteins were shown to be present in wheat flower.

Gorham [1821] described a protein soluble in alcohol which he obtained from the seeds of maize and to which he gave the name zein. A year later Bizio [1822, 1, 2] stated that zein was a mixture of gliadin and zymom, which Taddei had then recently found in wheat gluten, together with fat, and he regarded this mixture as similar to the gluten of wheat.

Braconnot [1827] described the protein constituents of some leguminous seeds. He named the protein which he obtained from them legumin, and showed that it formed salt-like combinations with acids.

During the next few years little further progress was made in the study of plant proteins (cf. Marcet [1827], Berzelius [1828, 1, 2], Zenneck [1828], Braconnot [1829, 1830, 1831], Hermbstädt [1831], Gay-Lussac [1833]). Up to this time the knowledge of vegetable proteins had extended only to a recognition of their general occurrence in plants and to a more or less crude description of their physical properties and solubility.

Boussingault [1836] published elementary analyses of several plant proteins which marked a new epoch in the development of their study, for these analyses were soon followed by those made by Mulder [1839], and by those made by Liebig and his pupils (Liebig [1841, 1844], Varrentrap and Will [1841], Scherer [1841], Bence-Jones [1841], Heldt [1843], Rochleder [1843, 1844]). Apparently largely on the ground of these analyses, which agreed closely with those obtained with animal proteins, Liebig asserted, in 1841, that the different forms of plant proteins known at that time were identical with the proteins of animal origin which bore similar names. He recognised four such substances, namely, vegetable albumin, plant gelatin, legumin or casein, and plant fibrin.

Throughout the previous history of the development of knowledge of plant proteins and up to the time of Liebig, the idea of their identity with the animal proteins appears to have been universally accepted, and every effort had evidently been made by those who studied them to discover similarities between the proteins from these two sources. In the year following, however, Dumas and Cahours [1842] presented the results of an elaborate study of the elementary composition of a considerable number of animal and vegetable proteins, which formed the foundation of a new advance in the knowledge of protein substances in general and contributed especially to the future studies of

the proteins of plants. By means of their then newly developed method for determining nitrogen, they were able to clearly establish differences in the elementary composition between many of the proteins, and they showed that these differences were particularly great in the case of some of the vegetable proteins. The identity which Liebig [1841] assumed to exist between the vegetable and animal proteins was thus disproved and the further accurate study of the proteins of vegetable origin became a matter of importance.

Little was done during the next ten years materially to advance the knowledge of vegetable proteins, but Hartig [1855, 1856] published the results of his elaborate investigations of seeds, in which he showed that a large part of the reserve protein was present in the cells in the form of crystals and grains of more or less definite structure. This discovery was followed three years later by Maschke's [1858, 1859] announcement that he had succeeded in artificially crystallising the protein of the Para or Brazil-nut, which Hartig had shown to be present in this seed in the form of rhombohedral crystals.

Denis [1859] showed that many protein substances of both animal and vegetable origin were soluble in neutral saline solutions, and thus presented to chemists an entirely new means for isolating and purifying these proteins. Although Denis' discovery has since been of fundamental importance in the modern study of proteins, especially those of seeds, its significance was not appreciated for several years.

In 1860, Ritthausen [1862, 1] began the first serious study of the vegetable proteins, and devoted himself for many succeeding years to the production of preparations of the highest attainable purity, and to accurate determinations of their composition. As a result of these investigations the prevailing knowledge was greatly extended, and it became plain that these substances occurred in many diverse forms in the different seeds. Ritthausen's work, therefore, furnished the first broad foundation for a knowledge of the vegetable proteins, and the service which he rendered in developing this field of knowledge deserves far more recognition than it received during his lifetime.

Weyl [1876, 1877] applied to seeds the method of extraction by solutions of neutral salts which Denis had proposed in 1858, and showed that a large number of different seeds contained protein soluble in saline solutions, and that this protein had properties similar to the globulins of animal origin. These vegetable globulins he divided into two groups, the myosins and vitellins, according as they were insoluble or soluble in saturated solutions of sodium chloride. The views which he advanced in respect to the general character of protein substances

in seeds received immediate and extended recognition on the part of physiologists and of those familiar with proteins of animal origin; and as he claimed that the preparations which Ritthausen had obtained by the aid of weak alkaline solutions were altered products of the original protein constituents of the seeds, the work of Ritthausen soon fell into disrepute and was discredited as not showing the real protein constituents of the seeds which he had studied. Although Ritthausen [1880, 1881, 1, 2, 3; 1882, 3, 5; 1884, 2] soon after showed that many of his preparations were largely or wholly soluble in saline solutions and had for the most part retained their original solubility unaltered, and also that the products obtained by direct extraction of seeds with neutral salt solutions agreed in many cases entirely with those which he had previously described, physiologists continued to overlook and disregard his assertions. At about the time Ritthausen ceased working in this field investigations were begun by the writer and carried on throughout the next twenty years. During this time a few other investigators occasionally dealt with special questions connected with the chemistry of the vegetable proteins but no connected or extensive investigation of these substances was undertaken by any one else. As Ritthausen's researches were far from exhaustive, and left the subject in such a state of confusion that it was impossible to form definite conclusions respecting much that he had described, the writer therefore first directed his attention largely to a reinvestigation of the subjects dealt with by Ritthausen, hoping thereby to clear up some of the uncertainties pertaining to his work.

Ritthausen's efforts were largely directed to attempts to establish the identity of proteins obtained from different seeds and seem to have been inspired by an idea that a comparatively small number of vegetable proteins occurred in nature. The author, on the other hand, recognising that identity of such complex chemical compounds as are the proteins could not be proved by any of the means then available directed his efforts to establishing constant differences between those from different plants. Following this course it soon became apparent that nearly all of the proteins from seeds of unlike species which Ritthausen considered to be alike were in fact different and further investigation showed that no two species of seeds contained proteins which could not readily be shown to be chemically different, except in the case of seeds very closely related botanically. Even the latter have not yet yielded preparations which are certainly alike. Thus the closely related seeds of wheat and rye contain alcohol-soluble proteins which have thus far been proved to differ only in respect to specific

rotation, but this difference appears to be a constant one and to be characteristic of the protein.

Anaphylaxis experiments conducted by Professor H. G. Wells (Wells and Osborne, 1911), using preparations made by the author, have indicated the almost infinite variety of vegetable proteins, and the occurrence of possibly identical proteins only in very closely related species. In this respect animals and plants are alike. After Kossel and Emil Fischer developed methods for determining the amino-acids which proteins yield when completely hydrolysed the vegetable proteins became of especial interest. Analyses of many of them were thus made by the author and his associates as well as by E. Abderhalden and others. The results of these analyses confirmed the wide differences between many of the proteins of seeds which the simpler and less certain methods earlier employed had indicated.

Thus a chemical basis for the differentiation of proteins became available and these substances, long ignored by the organic chemist, began to receive attention. Since many of the vegetable proteins present in seeds commonly used for food by men and animals differed greatly in their amino-acid make-up, not only from one another, but also from those obtained from animal food products, an extensive series of studies of their relative value in nutrition was begun in 1910 by Osborne and Mendel. In 1915, the United States Department of Agriculture undertook investigations of the vegetable proteins under the direction of C. O. Johns and many valuable contributions to this subject have been made by him and his associates.

CHAPTER II.

OCCURRENCE OF PROTEINS IN DIFFERENT PARTS OF PLANTS, AND THEIR GENERAL CHARACTERISTICS.

PROTEINS are found in the living parts of all plants. They occur in the dissolved state in the circulating fluids and in the solutions of the cell vacuoles, that is in the cell sap. In a semi-dissolved state they occur in the protoplasm, and in an undissolved state they are stored as reserve protein in the cells of seeds, tubers, bulbs, buds and roots.

In many cells undissolved protein is found in the form of well-developed crystals of various forms; in irregular, semi-crystalline forms with faces and angles on a part of their surface, and as regular or distorted spheres, all of which several forms are found in aleurone grains; and also in an amorphous, finely granular form, generally designated aleurone. In seeds the reserve protein occurs in the cells together with the non-nitrogenous reserve food materials, starch, oil, etc., which several substances fill the cells, leaving a thin layer of dried protoplasm between them and the cell wall (cf. Hartig [1855, 1856], Radlkofer [1859], Nägeli [1862], Schimper [1880], Schulz [1901]).

In most monocotyledonous plants the cells of the endosperm and embryo occupy distinct parts of the seed. The tissues of the endosperm of such seeds when fully ripe are, therefore, made up of cells which are almost entirely filled with the reserve food substances, since the thin layer of protoplasm next to the cell wall forms a very small part of the contents of the cell.

The tissues of the embryo contain protein associated with a greater variety of subtances than are present in the cells of the endosperm, and are also rich in nucleated cells, in which much of the protein apparently exists in the chromatin substance of the nuclei in special forms of combination with nucleic acid. In this part of the seed the chemical conditions are therefore more complicated than in the cells of the endosperm, since the metabolic processes of the embryo apparently require a greater variety of substances than exist in the cells of the

endosperm of the fully ripe seed, whose chief office is to supply food to the subsequently developing embryo.

In most dicotyledonous seeds the cells containing the reserve protein are distributed among those of the embryo tissues.

In roots, bulbs and tubers the undissolved reserve protein occurs suspended in the cell sap, frequently in the form of crystals.

Little that is definite is known concerning the chemical properties of any of the plant proteins except those of seeds, for the proteins occurring in the physiologically active cells and fluids of plants have been but little studied, owing to the relatively small quantities in which they occur and the difficulty of separating them from each other.

The total protein is contained in several different parts of the seed, namely, in the endosperm cells as reserve protein, in the protoplasm of these cells, and in the cells composing the tissues of the embryo, both in the cytoplasm and in the nuclei. As it is not possible, in most cases, to separate these different parts of the seed by mechanical means, in sufficient quantity to permit a study of the proteins of each, extracts of the entire seed will consequently contain a mixture of proteins from all the different parts of the seed, and may be expected to contain a number of different proteins. Experience has shown this to be the case, as a careful examination of the extracts of all seeds thus far studied has shown the presence of a number of different types of protein. The whole of the protein contained in seeds is, therefore, not reserve protein. Extracts of seeds always contain in addition to a relatively large amount of one or two types of protein, which are manifestly the reserve protein of the seed, a certain small proportion having distinctly different properties. It is probable that most of this latter protein is yielded by the cells of the embryo as well as by the protoplasm of the endosperm cells. With most seeds definite evidence of this has not yet been obtained, but in the case of wheat the embryos are separated by the commercial process of milling in a nearly pure condition, and a study of this product has shown that those proteins which are obtained only in small quantity from the entire seed are present in relatively large amounts in this embryo meal. The proteins of this embryo both in chemical and physical character differ from those of the endosperm and resemble more nearly the physiologically active proteins of animal tissues. These embryo proteins are associated with a large quantity of nucleic acid, so that products similar to the nucleoproteins and nucleins are obtained from this embryo meal.

Thus Osborne and Voorhees [1893] (cf. also Frankfurt [1896] and O'Brien [1895]) found that the flour of the entire kernel of samples of

spring and winter wheat yielded the quantities of the proteins enumerated in the following table :—

	Spring Wheat Per Cent.	Winter Wheat Per Cent.
Glutenin	4·68	4·17
Gliadin	3·96	3·90
Globulin	0·62	0·63
Albumin	0·39	0·36
"Proteose"	0·21	0·43

Osborne and Campbell [1900] obtained from the wheat embryo meal no gliadin or glutenin, 10 per cent. of albumin, 5 per cent. of globulin and about 3 per cent. of "proteose." The embryo meal contains a relatively large proportion of nucleated cells and therefore a large amount of nucleic acid, which was extracted in combination with the proteins, though much remained in the meal residue as an insoluble combination with the residual protein. In view of these facts it is clear that a part of the globulin, albumin and proteose obtained from the entire seed was originally present in the tissues of the embryo, though some is also yielded by the endosperm cells possibly derived from the very small amount of protoplasm which these contained. Similar conditions must certainly exist in other seeds, so that the proteins found only in very small quantity in their extracts may be regarded as derived from the embryo and protoplasm rather than from the reserve protein of the seed. Thus, for instance, the hemp-seed, which yields a large part of its protein in the form of crystalline edestin, also yields a very small quantity of one or two other proteins coagulated by heating the extracts to about 80°, and also a little that is soluble in boiling water.

The proteins of seeds have been the subject of extensive investigation, and we now know much concerning the chemical and physical properties of a number of them from several species. By far the greater part of these belong unquestionably to the reserve proteins of the seeds, and are to be regarded as products of the metabolism of the plant which in the fully ripe seed no longer take part in its physiological processes. They are, therefore, in a sense analogous to excretory products, for, as Pfeffer, has said,[1] "All protoplasmic secretions which appear externally and are lost to the plant or which can take no further part in metabolism are to be regarded as excretory substances."

In many ways the reserve seed proteins bear a relation to the physiologically active tissues of the parent plant similar to that which

[1] Pfeffer, W., *The Physiology of Plants*, p. 131. Second edition, 632 pp. Translated by Alfred J. Ewart. Oxford, 1900.

the animal albuminoids or scleroproteins bear to the physiologically active tissues of the animal, for the reserve protein of ripe seeds has even less connection with the living tissues of the plant which produced it than the albuminoids of hair, horn and hoof have with the living tissues of the animal.

This physiological analogy extends, within certain limits, to the chemical characteristics of these substances. Like the animal albuminoids, the reserve seed proteins are more stable toward chemical and physical agents than are the proteins which form parts of the living protoplasm of animals, and also, like most of the albuminoids, they differ more widely in the proportion of some one or more of the amino-acids which they yield when hydrolysed than do the more physiologically active proteins of either vegetable or animal origin, the protamines excepted. Thus gliadin of wheat and hordein of barley yield more than 40 per cent. of glutaminic acid, while silk fibroin yields 36 per cent. of glycocoll and 21 per cent of alanine. The alcohol-soluble seed proteins yield the basic amino-acids in very small amount as do also some of the albuminoids, e.g. elastin and keratin. No such wide differences have been found among the physiologically active proteins of plants or animals, and in this respect many seed proteins and albuminoids present marked structural differences from the tissue proteins. The seed proteins, moreover, offer an advantage for chemical study which most of the albuminoids do not, as, unlike most of the latter, they can be obtained in a soluble form and very frequently in a well-crystallised state which makes their purification much easier than that of the insoluble albuminoids.

Owing to their relatively great stability most of the reserve proteins of seeds can be subjected to extensive fractional precipitation, and the chemical and physical properties of successive fractions compared. These proteins, therefore, yield more definite and well-characterised preparations than do most of the known proteins of animal origin, and a study of them has resulted in more definite knowledge of the chemistry of protein matter than has been obtained by a study of proteins from any other source, with the exception of casein from cow's milk.

It was one of the earliest observations respecting the proteins of plants that their freshly expressed juices yielded a coagulum, when heated, which consisted chiefly of protein. Beyond numerous observations on the behaviour of the protein in plant cells which have been made by histologists very little has been learned concerning the nature of the protein in the leaves and stems of plants. Osborne, Wakeman,

and Leavenworth [1921] as well as Chibnall and Schryver [1921] have undertaken to isolate such protein, the former from spinach leaves and green alfalfa plants, the latter from cabbage, bean, and spinach leaves.

From these investigations it appears that only a small part of the protein of the clear, expressed juice is in true solution and that most of this is coagulated by adding alcohol or heating. Much the greater part of the protein in the leaf is insoluble in such solvents as are usually employed for extracting proteins.

The majority of seed proteins thus far studied are soluble in neutral saline solutions from which they are precipitated by dilution or dialysis. Those seeds, as yet studied, in which the protein occurs within the cell in the form of crystals or spheroids or of partly crystalline masses yield large quantities of protein precipitable by dialysis in forms similar to, or identical with, those which exist in the seed. Seeds of the cereals which contain the protein in a finely granular condition and without definite form yield relatively small quantities soluble in saline solutions. The proteins isolated from many seeds are apparently obtained with unchanged properties. The crystalline and spheroidal forms exhibited by these proteins within the cells can be artificially reproduced by dialysing their saline solutions.

In those cells of the endosperm in which protein is most frequently found in the form of crystals, or of small spheres, there is usually observed a globule, composed largely of insoluble mineral substance, commonly called a globoid. This globoid is formed from soluble mineral salts contained in the fluid of the cell, and it is possible that, as its constituents pass out of solution, through the formation of an insoluble combination, the concentration of the salts in the cell fluid is correspondingly reduced and the protein thereby precipitated, in much the same way as occurs when salts are removed by dialysis.

As already stated, the proteins extracted from seeds are obtained in various forms which represent distinctly different protein substances. The solubility of the protein matter in different seeds varies greatly, but in general it is found that a part is soluble in water, a part is soluble in neutral saline solutions and a part is insoluble in either of these solutions but soluble in dilute solutions of acids or alkalis, while in the seeds of the cereals a part is also soluble in alcohol of from 70 to 90 per cent. The proteins extracted from seeds by these several solvents will be considered in greater detail later.

CHAPTER III.

CHEMICAL INDIVIDUALITY OF PROTEIN PREPARATIONS.

SINCE proteins have none of those physical and chemical characters which the chemist usually depends upon to show the individuality of an organic chemical compound, their isolation in preparations of assured chemical purity presents unusual difficulties; in other words, proteins have no single physical property by means of which any judgment can be formed in regard to the strict chemical individuality of any one of them. The best that can be done at present is to establish a constancy of the ultimate composition of successive fractional precipitations of the protein under consideration, and to show the constancy of the physical properties and products of hydrolysis of these fractions so far as this is possible. There can be no question, where *differences* are established in composition or properties between successive fractions of a protein preparation, that the substance in question is a mixture. We can, therefore, for the present, treat as individual proteins only those products whose extensive fractionation has given no evidence that a mixture is being dealt with, and we must await new methods of study before any one of these proteins can be definitely accepted as a true chemical entity. Many, however, of the proteins with which we are at present familiar have shown such constancy of composition and properties that we feel justified in now considering them as substances of reasonably definite character.

During recent years, studies of the anaphylaxis reaction have indicated that it may be possible to establish chemical identity by means of this biological reaction, and H. G. Wells (Wells and Osborne 1911) has made extensive comparisons of numerous proteins prepared in the author's laboratory. Several proteins obtained from different species of plants, although so similar in chemical and physical properties as to be indistinguishable, failed to react anaphylactically with one another, while others from botanically closely related species, and presumably chemically identical, reacted as vigorously as did preparations from the same species. This test showed that each seed contains several chemically distinct proteins, and that no two seeds, unless very

closely related botanically, contain chemically identical proteins. At the present time the anaphylaxis reaction seems to be the best means available for establishing the probable chemical identity of protein preparations from different sources.

The conclusion appears to be justified that an almost infinite number of chemically different proteins exist in plants and that it is a hopeless task to attempt to deal with any considerable part of these individually. It is, however, important to have a few well characterised individuals representing different types which shall afford material for studying the physical and chemical characters of this important group of substances. During the past thirty years the author and his associates have concentrated attention on the preparation of a few proteins from different groups of seeds which in physical properties and chemical structure appear to present especial opportunities for future investigation.

In this connection it is worth while to consider the evidence now available which indicates the possibility of separating these proteins from one another, as well as from associated substances, for it has been generally assumed that the difficulties presented in making such separations are so great that there is little hope for success. Most of the seed proteins now known are soluble in salt solutions and can be precipitated by dialysis in the form of crystals or spheroids which are relatively large when compared with the minute particles of an amorphous precipitate. These separate from solution very slowly, and are deposited as dense precipitates which adsorb associated substances to a far less extent than do the amorphous, bulky precipitates of most proteins of animal origin. Good evidence of this is the fact that the chief protein of the castor bean, as Osborne, Mendel, and Harris have found, could be freed by a single reprecipitation by dialysis from all but minute traces of any toxic substance, though the solution from which it separated on the first dialysis contained a large quantity of the extremely poisonous ricin. Ricin, if not a protein, is, at least, so intimately associated with protein that it is almost quantitatively precipitated therewith. The conclusion is, therefore, justified that the separation from the water-soluble protein by a single reprecipitation by dialysis is in a high degree complete. This fact is good ground for considering it possible to separate proteins very completely from one another under such conditions.

Further evidence of this is furnished also by the anaphylaxis reaction. As is well known, extremely small quantities of a protein suffice to sensitise guinea pigs to subsequent doses of the same protein. In many cases Wells and Osborne [1915] found that preparations of

vegetable proteins obtained by repeated precipitation by dialysis, or by dilution, were so completely freed from the water-soluble proteins of the same seed that no sensitisation to intoxicating doses of the latter occurred, even when relatively large quantities of the former were used for sensitising. It thus appears possible to separate very perfectly two individual proteins from one another when these occur in the same solution.

The fact that many vegetable proteins can be easily obtained so free from carbohydrates that their preparations give no trace of reaction with the Molisch test is strong evidence that these can be entirely freed from such non-protein contaminations.

CHAPTER IV.

CLASSIFICATION OF VEGETABLE PROTEINS.

A CHEMICAL classification should be based on definite properties of individual substances, but such a treatment of the proteins is at present manifestly impossible. It is, however, desirable to have some scheme by means of which the proteins can be brought together in an orderly fashion. All attempts, thus far made, to classify them have been based chiefly on their solubility under different conditions. This method of classification has proved in many ways unsatisfactory and inadequate, but seems, for the present, to be the best available. Attempts have been made to establish greater uniformity throughout the world in the classification of the proteins and to attach more definite and generally recognised meanings to the various terms and designations which have been used in describing and classifying them. To this end committees were appointed by societies in England [1] and America [2] for the purpose of agreeing on a scheme of classification for the proteins. These committees have reported plans which involve no serious point of difference. As the scheme of classification of the American committee is more detailed and was prepared to include the vegetable proteins, it is used in this monograph. This scheme provides for the following groups :—

I. The Simple Proteins.
   - (*a*) Albumins.
   - (*b*) Globulins.
   - (*c*) Glutelins.
   - (*d*) Prolamins (Alcohol-soluble proteins).
   - (*e*) Albuminoids.
   - (*f*) Histones.
   - (*g*) Protamines.

[1] *Proteid Nomenclature, Report of Committee.* J. Physiol., 1907, 35, xvii-xx.

[2] *Recommendations of the Committee on Protein Nomenclature.* Amer. J. Physiol., 1908, 21, xxvii-xxx, also Proceedings of the American Society of Biological Chemists, 1908, 1, 142-145.

II. Conjugated Proteins.
  (*a*) Nucleoproteins.
  (*b*) Glycoproteins.
  (*c*) Phosphoproteins.
  (*d*) Hæmoglobins.
  (*e*) Lecithoproteins.

III. Derived Proteins.
  1. Primary Protein Derivatives.
    (*a*) Proteans.
    (*b*) Metaproteins.
    (*c*) Coagulated proteins.
  2. Secondary Protein Derivatives.
    (*a*) Proteoses.
    (*b*) Peptones.
    (*c*) Peptides.

The vegetable proteins belong to groups which were first established in connection with studies of animal proteins, but the definitions of these groups, as usually found in text-books dealing with proteins, must be modified to some extent if they are to include those vegetable proteins which have properties in the main agreeing with those of the animal proteins heretofore assigned to such groups.

All of the proteins of seeds which have been carefully studied belong to the group of simple proteins, and of those supposed to be conjugated proteins none has yet been proved to belong to this group, though it is quite possible that representatives of this group, possibly peculiar to plants, may exist abundantly in the cells of living plants. Thus the greater part of the protein in spinach or alfalfa leaves cannot be extracted by dilute alkali until the tissues are heated therewith for some time, suggesting that the protein moiety is rendered soluble in dilute alkalis or acids only after hydrolysis. Such data as have been secured indicate that this protein may be combined with flavone-like complexes. If true, this would represent a type of conjugated protein hitherto unknown. Conclusive evidence supporting this view has not yet been obtained.

Nucleoproteins have been frequently described as occurring in vegetable cells and it is possible that they do thus occur, but if by nucleoproteins is meant some form of combination between protein and nucleic acid other than as a salt or nucleate, conclusive evidence of their existence has not been advanced. Compounds of nucleic acid and protein have been isolated from the embryo of wheat, but the

products obtained gave no evidence that they were anything other than protein nucleates. Phosphoproteins similar to caseins or egg-yolk vitellins have never been obtained from seeds, although it has not infrequently been asserted that most of the seed proteins belong to this group. Lecithoproteins or glycoproteins are not yet known to be present in plants, but possibly exist in the physiologically active tissues which, up to the present time, have been but little studied from a chemical standpoint.

Of the simple proteins found in plants none has been obtained which in any way resembles the albuminoids or protamines. Some of the seed globulins are similar to the histones in their high content in nitrogen and diamino-acids but there is no other reason for supposing a relation to exist between them.

Most of the globulins from seeds, unlike those of animal origin, are incompletely coagulated by heating their faintly acid solutions and in some cases are not coagulated at all. Their relations to saturated salt solutions are also different, for many of them are not precipitated by saturating their solutions with magnesium sulphate or half saturating with ammonium sulphate. All are, however, precipitated from saline extracts of the seeds by dilution or dialysis, and on the basis of this, the essential characteristic of a globulin, are assigned to this group. The term globulin was originally applied to proteins which are insoluble in water but readily soluble in dilute saline solution from which they are precipitated by dilution or by dialysis. The method now almost universally adopted by physiological chemists of differentiating globulins from albumins by half saturation with ammonium sulphate cannot be applied to the vegetable proteins because many of them which are pre-eminently globulins according to the original definition are not precipitated until their solutions are much more than half saturated. The distinction between globulins and other types of proteins which is founded on solubility in dilute salt solutions is one of the most convenient means of grouping a large part of the known proteins obtained from seeds. It seems unfortunate, therefore, that a method of distinguishing globulins should have been introduced which cannot be applied to one of the best characterised and numerously represented groups of the vegetable proteins. The author thinks that the practice of the physiologists should be abandoned so that the confusion thereby introduced can be avoided.

On the ground of some similarity to proteoses, especially the heteroproteoses, it has been suggested that many of the seed globulins belonged to this group. Experiments with the erepsin-like enzyme of

Penicillium camemberti (Dox, 1909) have shown that while this enzyme rapidly hydrolyses casein it has no action on any of the several seed proteins tested, including some of the typical globulins. It is therefore probable that the seed globulins are constituted similarly to the true or simple proteins of animal tissues and fluids, which are not attacked by erepsin.

A few of the seed proteins have properties characteristic of albumins, that is, are soluble in water and coagulable by heat. These, however, differ from animal albumins in their relations to saturated salt solutions, for some of them are precipitated by saturating with sodium chloride or magnesium sulphate and some also at less than half saturation with ammonium sulphate.

Two groups of proteins are found in seeds which have no representatives among the animal proteins, namely, the prolamins and the glutelins. The former is one of the most definitely characterised group of proteins known, for its members are freely soluble in ethyl alcohol of 70 to 80 per cent, one even dissolving in all proportions in alcohol of 90 to 92 per cent. They are also soluble in many other alcohols. The prolamins are sharply differentiated from other proteins in their constitution, since all that have been hydrolysed with acids yield a relatively large amount of ammonia, glutaminic acid and proline and a very small proportion of arginine and histidine, and very little or no lysine.

The group of glutelins has been provided to include those proteins which cannot be extracted from many seeds by neutral solvents but are easily extracted by very dilute alkalis. The only carefully studied member of this group is glutenin from wheat flour, but evidence of the existence of proteins of similar solubility in many other seeds has been obtained.

I. Simple Proteins.

(*a*) *Albumins.*

After Beccari's discovery of the existence of a protein substance in wheat flour, the presence of coagulable protein was soon recognised in the juices from many parts of different plants. The similarity of this substance to the albumin of hen's egg caused it long to be known as albumin. After advances had been made in the study of the proteins the term albumin was restricted to those proteins which are soluble in pure water and coagulable by heat. The animal albumins are not precipitated by saturating their neutral solutions with sodium chloride

nor with magnesium sulphate. The vegetable albumins on the other hand are, in many cases, precipitated by saturation with one or the other of these salts, and are included in the group of albumins on the basis of their solubility in water at neutral or slightly acid reaction and their coagulability by heat. It is not always easy to decide whether, or not, a vegetable protein actually belongs with the albumins, as it is difficult, in many cases, to determine whether the protein substance in question is soluble in water alone or whether the presence of minute quantities of salts, bases or acids has caused its solubility. Thus, some leguminous seeds contain legumelin, which appears to be soluble in pure water but is partially precipitated by long-continued dialysis. Such precipitates, however, are not soluble in saline solutions and it is probable that they result from a denaturing of the protein. Whether in these cases denaturing occurs before precipitation, or the precipitation results from a complete removal of the salts and denaturing occurs afterwards, is difficult to establish, and doubt exists, therefore, in regard to the solubility relations of such proteins. Owing to the small quantity in which albumins have thus far been obtained from seeds, and the difficulties presented in completely separating them from associated globulins, conclusive evidence in regard to the albumin nature of some of them has not yet been obtained. Such proteins must, for the present, be regarded as albumins, since their properties seem to agree best with those characteristic of this group. Leucosin of wheat has been the most carefully studied vegetable albumin in respect to its solubility in water, and it has been found to be completely soluble in solutions which contain but a mere trace of mineral matter. Most seeds and, probably, most plant juices yield proteins which are as well entitled to be placed in the group of albumins as any of those of animal origin. The best characterised vegetable albumins are:—

Leucosin found in the seeds of
- Wheat, *Triticum vulgare* (Osborne and Voorhees, 1893; Osborne and Campbell, 1900).
- Rye, *Secale cereale* (Osborne, 1895, 1).
- Barley, *Hordeum vulgare* (Osborne, 1895, 2).

Legumelin (Osborne and Campbell, 1898, 5) found in the seeds of
- Pea, *Pisum sativum* (Osborne and Campbell, 1898, 1).
- Horse-bean, *Vicia faba* (Osborne and Campbell, 1898, 3).
- Vetch, *Vicia sativa* (Osborne and Campbell, 1898, 4).
- Soy-bean, *Glycine hispida* (Osborne and Campbell, 1898, 6).
- Lentil, *Ervum lens* (Osborne and Campbell, 1898, 2).
- Adzuki-bean, *Phaseolus radiatus* (Osborne and Campbell, 1897, 5).
- Cow-pea, *Vigna sinensis* (Osborne and Campbell, 1897, 4).

Phaselin found in the seeds of { Kidney-bean, *Phaseolus vulgaris* (Osborne, 1894; Waterman, Johns and Jones, 1923).

Ricin found in the seeds of { Castor-bean, *Ricinus communis* (Osborne, Mendel and Harris, 1905).

Small quantities of albumins are also found in most other seeds but have not been studied or given distinctive names.

(*b*) *Globulins.*

The presence of protein matter soluble in neutral saline solution in seeds was first shown by Denis [1859] and later confirmed by Hoppe-Seyler [1866-1871, 1]. Denis found that such substances were extracted from vegetable tissues by solutions of sodium chloride. Weyl [1876, 1877] examined a large number of seeds and found that all of them contained protein soluble in solutions of sodium chloride. He divided the globulin thus extracted into two groups, the vitellins, soluble in saturated solutions of sodium chloride, and the myosins, insoluble therein. Vines [1879, 1880, 2] soon after investigated the action of salt solutions on the aleurone grains in different seeds and proposed a classification of these based on his observations. The criticism made by Weyl of the results which Ritthausen had obtained by extracting seeds with dilute alkalis led Ritthausen to apply extraction with sodium chloride solutions to a number of the seeds which he had previously studied, and he showed that most of the seed proteins were globulins. The experience of all who have since worked with the proteins of seeds has fully confirmed this conclusion.

Globulins, as here defined, are proteins insoluble in water but soluble in saline solutions. In this group must be included, for convenience, a large number of vegetable proteins which may not strictly conform to this definition. Osborne [1901, 2] showed that preparations of edestin crystallised from sodium chloride solutions contain combined hydrochloric acid, those from sodium sulphate solutions contain combined sulphuric acid. This was demonstrated by suspending the preparation in water and adding potassium hydroxide solution to neutral reaction. During the titration the edestin does not dissolve but yields its combined acid to the alkali. By filtering out the edestin an amount of potassium chloride or sulphate was found in the filtrate closely corresponding to the amount of potassium hydroxide added. It was further found that when the potassium chloride corresponded to 0·7 c.c. of potassium hydroxide per gramme of edestin the preparation was entirely insoluble in water, but when greater, the edestin was soluble in proportion to the excess of combined hydrochloric acid thus

indicated. When this excess was equal to 0·7 c.c. per gramme, corresponding to a total acid content of 1·4 c.c. per gramme, all of the edestin was soluble in water. The free edestin as well as the edestin hydrochloride represented by the lower content of hydrochloric acid is insoluble in water but readily soluble in sodium chloride solution, i.e. these behave like typical globulins. On the other hand, the hydrochloride containing the larger quantity of acid is precipitated from its aqueous solution by adding a small amount of sodium chloride, but redissolves on adding more. It is thus evident that the solubility of this globulin depends on the amount of acid combined with it as well as on the concentration of the salt solution used as a solvent. There are other proteins, e.g. legumin from the pea or vetch, which when precipitated from sodium chloride solutions by dialysis or dilution behave like globulins, but if suspended in water are completely dissolved when dilute alkali is added up to the neutral point to phenolphthalein. Whether or not such proteins form salts with strong acids similar to those formed by edestin has not been definitely determined, but however this may be, they behave like globulins under the conditions under which they are usually obtained. It seems more appropriate, therefore, to assign such proteins to the group of globulins than to the albumins, because it is characteristic of the latter to be soluble in water or in saline solutions irrespective of the presence of acids.

Saturation with sodium chloride was formerly made the basis for a division of the globulins into two groups, myosins and vitellins. Experience has shown that such a distinction cannot well be made, inasmuch as many of the proteins which were designated as myosins are, in fact, albumins, and some of those which were designated vitellins are not soluble in saturated sodium chloride solutions. Thus, the so-called myosin in the seeds of the cereals consists entirely of the albumin leucosin, and the globulin of the castor-bean, which is partially precipitated by saturating its solution with sodium chloride, has been found to consist not of two proteins, one of which is soluble in saturated sodium chloride solution and the other insoluble, but of one protein which is less soluble in a saturated saline solution than in a more dilute one (Osborne and Campbell, 1897, 2).

While the animal globulins are precipitated by saturating their solutions with magnesium sulphate, many of the vegetable globulins cannot thus be precipitated, but saturation with sodium sulphate, at 33°, precipitates all of them as yet thus tested. The vegetable globulins are precipitated by partial saturation with ammonium

sulphate at very different degrees of saturation. Although many of them are precipitated by adding an equal volume of a saturated solution of ammonium sulphate to their solutions, others are not precipitated until their solutions are nearly saturated with this salt.

The animal globulins are all coagulated by heating their solutions to various temperatures. Most of the seed globulins are only imperfectly coagulated by heating their solutions even to boiling, and some of them can be thus heated for a very long time without showing any apparent change.

A number of the vegetable globulins can be readily obtained in crystals; some of them crystallise with difficulty, and nearly all of those which do not crystallise are obtained in the form of minute spheroids. Crystallisation can be effected in various ways. Globulins which crystallise readily are usually precipitated by dialysis in well-formed crystals. Such are edestin from the hemp-seed, excelsin from the Brazil-nut, and the globulins of the squash-seed, the flax-seed, the oat-kernel and the castor bean. The globulin of the castor-bean is commonly obtained by dialysis in the form of spheroids, but frequently mixtures of spheroids and crystals result, although occasionally completely crystalline separations occur. Phaseolin, from *Phaseolus vulgaris*, frequently precipitates on dialysis in the form of minute spheroids, mixed with a few octahedral crystals which sometimes are of unusual size and very perfect form, but completely crystalline preparations of this globulin have never yet been obtained. Globulins which crystallise easily generally can be obtained in crystals by diluting their sodium chloride solutions with water heated to 50° or 60° until a slight turbidity forms. After warming the diluted solution until this turbidity disappears and then allowing it to cool slowly, the protein separates in well-developed crystals.

Schmiedeberg [1877] who obtained crystals of the globulin from the Brazil-nut, by treating its solution with magnesia and evaporating it slowly, considered the crystals of this substance to be the magnesium salt of the protein. This view of the nature of the crystals of this globulin was formerly accepted, and was frequently found in the current literature dealing with this protein, although Osborne [1892, 3], showed that much more perfectly crystalline preparations of this globulin than are described by Schmiedeberg are obtained by simply dialysing its faintly acid saline solutions in running water. These crystals are unquestionably salts of the globulin with the acid of the extract, and are not compounds with magnesia or any other base.

The following is a list of the principal globulins :—

Legumin (Osborne and Campbell 1898, 5), found in the seeds of	Pea, *Pisum sativum* (Osborne and Campbell, 1898, 1). Horse-bean, *Vicia faba* (Osborne and Campbell, 1898, 3). Vetch, *Vicia sativa* (Osborne and Campbell, 1898, 4). Lentil, *Ervum lens* (Osborne and Campbell, 1898, 2).
Vignin found in the seeds of	Cow-pea, *Vigna sinensis* (Osborne and Campbell, 1897, 4).
Glycinin found in the seeds of	Soy-bean, *Glycine hispida* (Osborne and Campbell, 1898, 6).
Phaseolin, crystalline, found in the seeds of	Kidney-bean, *Phaseolus vulgaris* (Osborne 1894 ; Finks and Johns, 1920).
Conphaseolin found in the seeds of	Kidney-bean, *Phaseolus vulgaris* (Waterman, Johns and Jones, 1923).
Conglutin found in the seeds of	Lupines, *Lupinus* (Osborne and Campbell, 1897, 1 ; Osborne and Harris, 1905, 2).
Vicilin found in the seeds of	Pea, *Pisum sativum* (Osborne and Campbell, 1898, 1). Horse-bean, *Vicia faba* (Osborne and Campbell, 1898, 3). Lentil, *Ervum lens* (Osborne and Campbell, 1898, 2),
Stizolobin found in the seeds of	Chinese velvet bean, *Stizolobium niveum* (Johns and Finks, 1918 ; Jones and Johns, 1919).
Canavalin found in the seeds of Concanavalin found in the seeds of	Jack bean, *Canavalia ensiformis* (Jones and Johns, 1916).
Arachin found in the seeds of Conarachin found in the seeds of	Peanut, *Arachis hypogaea* (Ritthausen, 1880 ; Johns and Jones, 1916, 1917, 1).
Acerin found in the seeds of	Maple, *Acer saccharinum* (Anderson, 1921).
Corylin found in the seeds of	Hazel-nut, *Corylus avellena* (Osborne and Campbell, 1896, 5).
Amandin found in the seeds of	Almond, *Prunus amygdalus* (Osborne and Campbell, 1896, 5). Peach, *Prunus persica* (Osborne and Campbell, 1896, 5). Plum ? *Prunus domestica* (Dumas and Cahours, 1842). Apricot ? *Prunus armeniaca* (Dumas and Cahours, 1842).
Juglansin found in the seeds of	European walnut, *Juglans regia* (Osborne and Campbell, 1896, 5). American black walnut, *Juglans nigra* (Osborne and Harris, 1903, 5). American butter-nut, *Juglans cinerea* (Osborne and Harris, 1903, 5).
Excelsin, crystalline, found in the seeds of	Brazil-nut, *Bertholletia excelsa* (Osborne, 1892, 3 ; Weyl, 1877).
Edestin, crystalline, found in the seeds of	Hemp, *Cannabis sativa* (Ritthausen, 1881, 1 ; Osborne, 1892, 3).
Avenalin, crystalline, found in the seeds of	Oat, *Avena sativa* (Osborne, 1892, 1 ; Osborne and Campbell, 1896, 5).
Castanin found in the seeds of	European chestnut, *Castanea vulgaris* (Barlow, 1905).
Maysin found in the seeds of	Maize, *Zea mays* (Osborne, 1897, 2).
Tuberin found in the tubers of	Potato, *Solanum tuberosum* (Zöller, 1880 ; Osborne and Campbell, 1896, 3).
Cucurbitin, crystalline, found in the seeds of	Squash, *Cucurbita maxima* (Grübler, 1881 ; Osborne, 1892, 3).

Globulins have also been isolated in considerable quantity from the following seeds and have been the subject of more or less study, but distinctive names have not yet been given to them :—

Globulin, crystalline, from	Flax-seed, *Linum usitatissimum* (Osborne, 1892, 2).
,, ,, ,,	Castor-bean, *Ricinus communis* (Ritthausen, 1881, 1; Osborne, 1892, 3).
,, ,, ,,	Cocoa-nut, *Cocos nucifera* (Ritthausen, 1880; Kirkwood and Gies, 1902; Johns and Jones, 1920; Jones and Johns, 1920).
,, ,, ,,	Sesame-seed, *Sesamum indicum* (Ritthausen, 1881, 1).
,, from	Cotton-seed, *Gossypium herbaceum* (Osborne and Voorhees, 1894, 2).
,, ,,	Sunflower-seed, *Helianthus annuus* (Ritthausen, 1880; Osborne and Campbell, 1897, 3).
,, ,,	Candle-nut, *Aleurites triloba* (Ritthausen, 1881, 3).
,, ,,	Radish-seed, *Raphanus sativus* (Ritthausen, 1881, 3).
,, ,,	Rape-seed, *Brassica campestris* (Weyl, 1877).
,, ,,	Mustard-seed, *Brassica alba* (Weyl, 1877).
,, α ,,	Mung-bean, *Phaseolus aureus Roxburgh* (Johns and Waterman, 1920, 2).
,, β ,,	
,, ,,	Buckwheat, *Fagopyrum fagopyrum* (Johns and Chernoff, 1918).
,, ,,	Cohune-nut, *Attalea cohune* (Johns and Gersdorff, 1920).
,, α ,,	Tomato seed, *Solanum esculentum* (Johns and Gersdorff, 1922).
,, β ,,	
,, α ,,	Adsuki bean, *Phaseolus angularis* (Jones, Finks and Gersdorff, 1922).
,, β ,,	
,, α ,,	Lima bean, *Phaseolus lunatus* (Jones, Gersdorff, Johns and Finks, 1922).
,, β ,,	
,, α ,,	Georgia velvet bean, *Stizolobium deeringianum* (Johns and Waterman, 1920, 1).
,, β ,,	

Globulins which have not yet been named have also been isolated in small quantity from the seeds of wheat, *Triticum vulgare* [Osborne and Voorhees, 1893; Denis, 1859]; rye, *Secale cereale* [Osborne, 1895, 1]; barley, *Hordeum vulgare* [Osborne, 1895, 2]; rice, *Oryza sativa* [Rosenheim and Kajiura, 1908]; and maize, *Zea mays* [Chittenden and Osborne, 1891-92; Osborne, 1897, 2; Weyl, 1877], and in larger amount from those of the oat, *Avena sativa* [Osborne, 1891, 1892, 1, 1893; Weyl, 1877]. In the wheat-kernel most, if not all, of the globulin is contained in the embryo [O'Brien, 1895; Osborne and Campbell, 1900], and from analogy, it is probable that most of it occurs in this part of the seeds of the other cereals except in those of oats, in which the quantity is relatively so great that, in this seed, it must form a part of the reserve protein.

(*c*) *Glutelins.*

This group includes those proteins which are not dissolved by neutral aqueous solutions, by saline solutions, or by alcohol. The glutenin of wheat is the only well-characterised representative of this group which has yet been obtained. The seeds of other cereals doubtless contain proteins of similar character, but, owing to

the difficulties encountered in extracting them, no preparations have been made which appear to be at all definite. Wheat, rye and barley yield similar quantities of albumins and globulins and also nearly the same quantity of protein soluble in alcohol. Protein matter can be extracted from rye and barley flour by treating the residue, from which the other proteins have been removed, with dilute alkaline solutions, but the preparations obtained are manifestly impure, and, owing to the difficulty of filtering the alkaline extracts, only very small quantities of any of them have been obtained. Since much nitrogen remains in the extracted residue, it is fair to presume that the greater part of it belongs to protein matter, as is the case with wheat. From a by-product of maize starch manufacture, which is known as "gluten," a considerable quantity of protein can be extracted with alkaline solutions after the alcohol-soluble zein has been removed. In its products of hydrolysis this protein, called maize glutelin, differs, both quantitatively and qualitatively, from zein, and such preparations as have thus far been made are probably impure preparations of the glutenin of this seed. A similar type of protein from rice has been described by Rosenheim and Kajiura [1908] under the name of oryzenin, which they state represents the greater part of the protein of this seed.

After extraction with neutral solvents the residues of the seeds of most species usually contain a small quantity of nitrogen which may or may not belong to protein. Alkalis usually extract from such residues a small quantity of impure protein which may be either a protein with the properties of glutelin, or a portion of the proteins which failed to be extracted by neutral solvents, either because this residual protein was contained in unruptured cells which were afterwards destroyed by the alkaline solution or was retained in the meal residue in combination with other substances, such as nucleic acid or tannin, which rendered it insoluble in neutral solution. Although it is possible that proteins of the character of glutelins are widely distributed among the different seeds there is no conclusive evidence that this is in fact so.

The only well-defined glutelins are :—

Glutenin found in the seeds of	Wheat, *Triticum vulgare* [Osborne and Voorhees, 1893].
Oryzenin found in the seeds of	Rice, *Oryza sativa* [Rosenheim and Kajiura, 1908].
Maize glutelin found in the seeds of	Maize, *Zea mays* [Osborne and Clapp, 1908, 2].

(*d*) *Prolamins.*

The group of proteins soluble in relatively strong alcohol deserves a definite name, for it is one of the best characterised groups yet

found in either plants or animals. It has been proposed to call these proteins "gliadins," but as this name has been used to designate a definite protein obtained from wheat, some generic name should be adopted. The writer has proposed [Osborne, 1908, 2] calling this group "prolamins," since all its members which have thus far been hydrolysed yield a relatively large quantity of both proline and amide nitrogen. The prolamins are characterised by their solubility in alcohol of from 70 to 90 per cent. They are nearly or wholly insoluble in water, but their salts with acids or alkalis dissolve freely therein. They yield much glutaminic acid, proline and ammonia, small amounts of arginine and histidine and little or no lysine.

Alcohol-soluble proteins were among the first recognised in seeds, having been described as early as 1805 by Einhof as occurring in the seeds of rye [1805, 2] and barley [1806, 1]. Taddei [1819, 1] found that a part of the gluten of wheat was soluble in alcohol, and Gorham [1821] reported a similar protein, which he called zein, in the seeds of maize. Kreusler [1869] found in the oat-kernel an alcohol-soluble protein, and Johns and Brewster [1916] showed that the seeds of sorghum, *Andropogon sorghum*, also contain a considerable quantity of such protein. Rosenheim and Kajiura [1908] showed that the seeds of rice contain no protein soluble in alcohol. Prolamins have thus been found in the seeds of all the cereals that have been examined with the exception of rice, but have never been found in the seeds of any other family of plants.

The prolamin of wheat, *Triticum vulgare*, was named gliadin by Taddei [1819, 1]. Ritthausen [1872, 2] concluded that the alcohol-soluble protein of wheat consisted of three distinct proteins, gliadin, mucedin and gluten-fibrin, but subsequent investigations have not supported this view [cf. Osborne, 1907; Groh and Friedel, 1914]. The prolamin of rye, *Secale cereale*, is also known as gliadin, for no positive difference, except possibly in specific rotation, has yet been established between it and the gliadin of wheat [Osborne, 1895, 1; Osborne and Clapp, 1908, 3]. The prolamin of maize was named zein by Gorham and maize fibrin by Ritthausen. Zein has been the subject of special study by Chittenden and Osborne [1891-92], by Osborne [1897, 2], and by Osborne and Clapp [1908, 2]. Hordein, which is the prolamin of barley, resembles gliadin in solubility [Osborne, 1895, 2] but differs distinctly in the proportion of the amino-acids which it yields on hydrolysis [Osborne and Clapp, 1907, 5; Kleinschmitt, 1907, 1, 2; Johns and Finks, 1919].

The principal prolamins are therefore:—

Gliadin found in the seeds of	Wheat, *Triticum vulgare* [Osborne and Voorhees, 1893].
	Rye, *Secale cereale* [Osborne, 1895, 1].
Hordein found in the seeds of	Barley, *Hordeum vulgare* [Osborne, 1895, 2].
Zein found in the seeds of	Maize, *Zea mays* [Chittenden and Osborne, 1891-92; Osborne, 1897, 2].
Kafirin found in the seeds of	Sorghum, *Andropogon sorghum* [Jones and Johns, 1918].

(*e*) *Albuminoids.*

No representatives have yet been found in plants of the remaining groups of simple proteins, namely, the albuminoids, histones and protamines. Many of the reserve proteins of seeds show relations to the albuminoids, as has been already pointed out, but the differences in their behaviour toward solvents is so great that none of them can be considered to belong to this group.

(*f*) *Histones.*

The large amount of basic amino-acids which many of the seed proteins yield is similar to that which is considered to be characteristic of the histones; also the reactions of edestan (cf. p. 62) are similar to those of the histones. Whether any real relation exists between the histones and those seed proteins which yield a high percentage of basic amino-acids can be established only by further study. It is possible that the proteins commonly included among histones do not, in fact, differ so widely from other simple proteins as has been generally supposed.

(*g*) *Protamines.*

No substances in any way similar to the protamines have ever been found in plants, and there is no reason to expect to find them among the reserve proteins of seeds. It is possible that such substances occur in pollen grains, but neither the early investigations by Fourcroy [1802], John [1814], or Braconnot [1829], nor the later ones by v. Planta [1885] of hazel pollen, by Kammann [1912] of rye pollen, by Heyl [1919] of ragweed pollen, or by Anderson and Kulp [1922] of corn pollen, have given evidence of their presence. These investigators, however, made no special efforts to discover protamine in pollen and its presence is by no means excluded by their work.

II. Conjugated Proteins.

(*a*) *Nucleoproteins.*

The nucleoproteins deserve attention because they have been considered to be among the most important constituents in the cells of animals and plants. They were first described as existing in vegetable

cells by Hoppe-Seyler [1879], who obtained a preparation from yeast which was very similar to those then recently obtained by Miescher from animal sources. Kossel [1879, 1880] next investigated this substance and found that the phosphorus content of different preparations varied widely, but that most of them contained about 3·5 per cent. of phosphorus, which fact he regarded as evidence of a more stable combination containing this proportion of phosphorus.

Altmann[1] [1889] discovered nucleins to be compounds containing both nucleic acid and protein. It is difficult to determine just what views are now held concerning the character of the union of the nucleic acid and protein, but, from what appears in the literature of this subject, many writers evidently consider nuclein to be something other than a protein nucleate. The available evidence in regard to the nature of this union is very scanty and practically all that is definite relates to the formation of protein nucleates. It is not impossible that other forms of union may exist, but the conditions under which nucleoproteins and nucleins have been obtained make it extremely difficult, if not impossible, to prove the existence of an organic combination between the nucleic acid and protein. The methods thus far employed in isolating these substances depend on processes which would yield a salt of protein and nucleic acid if these two substances were present in the solution. No hydrolytic splitting of the nuclein thus obtained appears to be necessary to set its component parts free, unless hydrolysis is effected with extraordinory ease and with great rapidity. Furthermore, the experiments of Milroy[2] and Löbisch[3] show that artificial mixtures of phosphorus-free protein and free nucleic acid yield products which have the properties usually considered to be characteristic of the nucleins, although from the conditions of their production these artificial compounds could have been nothing other than protein nucleates.

Such preparations of nucleoproteins as have been obtained from vegetable sources can, in the writer's opinion, be considered only as protein nucleates; and they in no sense represent actual constituents of the vegetable cells, although it is not at all impossible that similar products *may* exist there.

[1] Altmann, R. *Ueber Nucleinsäuren.* Archiv. für Anat. u. Physiol., physiol. Abth., 1889, 524-536.

[2] Milroy, T. H. *Ueber die Eiweiss-Verbindungen der Nucleinsäure und Thyminsäure und ihre Beziehung zu den Nucleinen und Paranucleinen.* Zeit. physiol. Chem., 1896, **22**, 307-326.

[3] Löbisch, Wilhelm. *Ueber Nukleinsäure - Eiwessverbindungen unter besonderer Berücksichtigung der Nukleinsäure der Milchdrüse und ihrer angeblichen Beziehung zur Kaseinbildung.* Beitr. chem. Physiol. Path., 1906, **8**, 191-209.

The only extensive study of the character of the "nucleoproteins" obtained from plants which has been made since the true character of the nucleic acids has been established and the basic properties of the protein recognised, was that of Osborne and Campbell [1900], who obtained from the wheat embryo a large number of products which consisted of protein combined with very different proportions of nucleic acid. The aqueous extract of wheat embryo contains much nucleic acid which was first isolated by Osborne and Harris.[1]

The freshly prepared aqueous extract of the embryo-meal is neutral to litmus, alkaline to lacmoid and decidedly acid to phenolphthalein. If heated at once in a water bath to 98° no coagulation occurs unless a very little acid is previously added. On standing at the room temperature for a few hours, protected with thymol, the extract gradually becomes more acid, and, if then heated, a large coagulum forms between 50° and 55°.

In consequence of this continuous development of acid in the extracts, the conditions which determine the proportion in which bases and acids can combine are constantly changing, and, as the proteins are polyacid bases and nucleic acids are polybasic acids, many different salts may be formed under the various existing conditions. We must therefore expect to find the combinations of protein and nucleic acid which separate from the extracts of the embryo-meal under varying conditions to contain different proportions of these two substances; in fact, just such products as Osborne and Campbell [1900] obtained, for details of the preparation of which the original paper must be consulted.

From extracts of the wheat embryo the nucleic acid separates in combination with two distinctly different proteins. In each case the protein exerts a controlling influence on the general properties of the compound formed, those compounds containing the albumin, leucosin, having in the main the properties of albumin, while those containing the globulin have the properties of this group of simple proteins. In this respect the protein nucleates behave much like the hydrochlorides or other salts of the simple proteins.

So far as the writer's experience has gone, no evidence shows that any of the preparations of so-called nucleo-proteins from plants are anything else than protein nucleates formed during the process of extraction and precipitation.

It is clear that definite statements based on a study of isolated products have no value in respect to the actual occurrence of nucleo-

[1] Osborne, T. B., and Harris, I. F., *Die Nucleinsäure des Weizenembryos.* Zeit. physiol. Chem., 1902, **36**, 85-133.

proteins in plants. Concerning the evidence of the existence of such compounds which is furnished by microscopic observations of stained tissues, nothing will be said here, for this lies outside of the scope of this monograph as well as of the personal experience of the writer.

From such knowledge as we now possess it is evident that, at the most, only small quantities of nucleoprotein exist in the entire seed, and that this, if present, will be found chiefly in the tissues of the embryo in which the nuclei of the cells are far more abundant than in the tissues of the endosperm. Nucleoproteins have been described as constituents of fungi and bacteria, but no critical study of any of these has yet been made [cf. Nageli and Loew, 1878; Hoppe-Seyler, 1879; Kossel, 1879, 1880, 1881; Klinkenberg, 1882; Stutzer, 1882; Vandevelde, 1884; Liebermann, 1890; Gottstein, 1893; Malfati, 1891-92; Lasché, 1895; Galeotti, 1898; Wróblewski, 1898; Ruppel, 1898; Ascoli, 1899; Iwanoff, 1902; Tiberti, 1902].

(*b*) *Glycoproteins.*

Little that is definite can be said concerning the occurrence of glycoproteins in plants. It is certain that many of the known seed proteins do not belong to this group, for they give no Molisch reaction and therefore contain no carbohydrate. Krawkow [1897] obtained an osazone from "pea albumin," but in the absence of conclusive evidence that the preparation of this "albumin" was free from admixed carbohydrate, little importance attaches to this observation. Ishii [1894] described a substance obtained from the tubers of yams which had physical properties and an ultimate composition similar to the mucins of animal origin. As he makes no statements concerning the presence of carbohydrate in his preparations, its relations to the true mucins are yet to be demonstrated. Wróblewski [1898] has stated that mucin is one of the constituents of yeast, but gives no experimental evidence of this. Several observations are on record indicating that many bacteria produce a mucin-like substance in the culture medium in which they grow, but such substances are hardly to be considered as vegetable proteins.

(*c*) *Phosphoproteins.*

It seems to be believed by many writers on vegetable proteins that a large number of the proteins of seeds contain phosphorus and consequently should be assigned to the group of phosphoproteins [cf. Wiman, 1896-97]. The fact that the reserve food protein of the egg-yolk consists largely of such a phosphorised globulin-like protein,

and that the protein of milk consists chiefly of the phosphorised casein belonging to this group, has apparently led many to assume, by analogy, that a large part of the reserve food protein of seeds consists of similar substances [cf. Czapek, 1905, p. 59].

Hoppe-Seyler [1866-71, 1] suggested that in the nuts of *Bertholletia* and the seeds of *Pisum sativum* proteins similar to the vitellin of the yolk of hen's eggs may occur, but he based this suggestion solely on the fact that he had extracted a lecithin-like substance from crude preparations obtained from these seeds. He says nothing of the presence of phosphorus in the protein which remained after extracting with warm alcohol, and consequently gives no evidence which shows these to be vitellin-like proteins.

In discussing the relations of edestin to acids it was shown in the first edition of this monograph that the preparations of crude edestin which were obtained by a single precipitation from the extract of the seeds, contained a small amount of phosphorus, which, however, disappeared completely after reprecipitation by dialysis or by dilution. The *crude* preparations of most seed proteins, like those of edestin, contain traces of phosphorus, but, when purified, by repeated precipitation, they are obtained phosphorus-free. The vitellin from the yolk of hen's eggs or casein from cow's milk under these conditions retain their phosphorus completely and in this respect differ in a marked degree from all known vegetable proteins. No good evidence has therefore been obtained to show that phosphoproteins occur in plants, and if these occur at all it is only in very small quantity.

(*d*) *Hæmoglobins.*

Kylin [1910] has suggested that a close relation may exist between the crystalline coloured protein, phycoerythrin and hæmoglobin, although he offered no chemical evidence to support this view. Since he states that the coloured component of phycoerythrin is liberated by acid hydrolysis and can then be extracted from the solution by amyl alcohol the possibility must not be overlooked that this complex protein may contain some derivative of flavone, in this respect resembling the type of complex proteins which the investigations of Osborne, Wakeman and Leavenworth [1921] have indicated may occur abundantly in the alfalfa plant. This suggestion does not seem unreasonable since phycoerythrin is obtained from the tissues of the entire marine plant, *Ceramium rubrum*, and by analogy its proteins might be expected to resemble those of green land plants more nearly than those from other vegetable sources.

(e) *Lecithoproteins.*

Lecithoproteins have not been isolated from plants, and satisfactory evidence of their existence has not yet been brought forward. Schulze and Likiernik [1891] and Schulze and Winterstein [1903] assume the presence of lecithalbumin in seeds from the fact that a part of the lecithin always remains undissolved when the powdered seeds are extracted with ether.

Hoppe-Seyler [1866-71, 1], as stated on p. 32, obtained a lecithin-like substance from crude preparations of the proteins of the Brazil-nut and pea, but his brief statement is not sufficient to warrant the conclusion that this lecithin was anything other than a contamination of the preparations which he examined.

III. Derived Proteins.

1. PRIMARY PROTEIN DERIVATIVES.

The members of the various groups of derived proteins are all represented by corresponding products obtained from vegetable proteins.

2. SECONDARY PROTEIN DERIVATIVES.

(a) *Proteoses.*

The first observation which indicated the presence of proteoses in seeds was made by Vines [1879, 1880, 1] who obtained a proteose-like product from lupine-seeds which he called "hemialbumose." He also noted the presence of a similar substance in the seeds of vetch, hemp and flax. Schulze and Barbieri [1881, 2] soon afterwards examined a number of different parts of many kinds of plants, and concluded from the results which they obtained that plant juices and extracts often contain "peptone" (proteose ?) in small quantity. Even during germination they found only a small quantity of "peptone," and stated that a storing up of this substance does not occur. They confirmed an earlier observation of Kern [1880], made on lucerne and vetch hay, that plants contain ferments which hydrolyse protein during extraction. Products having the solubility relations characteristic of proteoses have been frequently found in the extracts of seeds after the other proteins had been removed. Whether these proteoses were original constituents of the seeds or resulted from the action of proteolytic enzymes is still an open question, for it is extremely difficult to conduct the extraction of the seed and separation of the other proteins in such a way as certainly to exclude the formation of

proteoses. That changes occur in seed extracts is shown by many facts already recorded. Osborne [1892, 2] found that the extract of the flax-seed yielded a nearly constant quantity of diffusible non-protein nitrogen during several days' dialysis. The fact that the amount of nitrogen diffusing during the first dialysis period was only about one-half of that diffusing during the subsequent equally long periods is evidence that this nitrogen did not exist as such in the seed but was formed from some other substance, presumably protein. He has also found more non-protein nitrogen in the dialysate from extracts of the seeds of *Phaseolus* made at 20° than in extracts made with solvents heated to 70°.

That the proteins in many extracts undergo changes in the process of purification is shown by the constant loss of material which is so frequently met with. A striking instance of this was encountered by Osborne and Harris [1905, 2], who subjected 600 grammes of crude conglutin from the yellow lupine to fractionation with ammonium sulphate and finally separated the different fractions by dialysis.. Although the mechanical losses during these operations were small, only 314 grammes of conglutin were recovered. According to Mack [1903, 1904] the seeds of the yellow lupine contain an enzyme which at neutral or acid reaction attacks the protein, and it is probable that the large loss of conglutin was due to the action of this enzyme.

As the formation of proteose would almost certainly accompany the formation of diffusable products which do not give protein reactions, the proteoses found in the extracts cannot be considered to be original constituents of the seeds until more convincing evidence is obtained than any as yet given.

On the other hand, such preparations of proteoses from seeds as were used by Wells and Osborne [1915] were found to be highly anaphylactogenic, in marked contrast to proteoses produced by enzymatic hydrolysis. This observation is difficult to reconcile with the view that the proteoses found in the extracts of seeds are the product of the action of the enzymes on the other protein constituents of the seed.

(*b*) *Peptones.*

The facts which lead to the uncertainties respecting the pre-existence of proteoses in seeds apply also to the peptones. The most elaborate investigation of this subject has been made by Mack [1904], who obtained peptone from lupine-seeds under conditions which he thought entirely excluded its formation by enzyme action. He worked with

very large quantities of seeds and employed Siegfried's method of isolating and purifying peptones by means of ferric-ammonium alum. The several products obtained had a nearly constant composition and when hydrolysed with hydrochloric acid yielded lysine, arginine and glutaminic acid.

By artificially digesting vegetable proteins with pepsin or trypsin, proteoses and peptones have been obtained by Chittenden [1894], Chittenden and Hartwell [1890, 1891], Chittenden and Mendel [1894], Chittenden and Smith [1890], and many others.

(*c*) *Peptides.*

Only two well-characterised peptides have thus far been obtained from seed proteins. The first of these was a dipeptide of proline and phenylalanine, which Osborne and Clapp [1907, 2] isolated from the decomposition products of gliadin which had been hydrolysed by boiling with 25 per cent. sulphuric acid for many hours. This peptide was obtained in beautiful mother-of-pearl crystals of definite form, and yielded a copper salt, the crystals of which were so large and well formed that the measurement of their angles served to definitely characterise the substance. By hydrolysis in a sealed tube with strong hydrochloric acid it yielded proline and phenylalanine in molecular proportions. The identity of this peptide with a synthetic l-propyl-l-phenylalanine has been established by Fischer and Luniak.[1]

Fischer and Abderhalden [1907] obtained l-leucyl-d-glutaminic acid from gliadin by partial hydrolysis at the room temperature with 70 per cent. sulphuric acid for sixteen hours and then for three days in an incubator. This peptide agreed in properties with the synthetic product.

[1] Fischer, E., and A. Luniak. *Synthese von Polypeptiden : XXXII. Derivate des l-Prolins und des Phenyl-alanins.* Ber., 1909, **42**, 4752-4759.

CHAPTER V.

THE RELATION OF PROTEINS TO ACIDS AND BASES.

By L. J. Henderson, Ph.D., Sc.D.

In their relations with acids and bases, proteins behave as if there were present in the molecule a considerable number of acid radicals of various degrees of strength, and a considerable number of similar basic radicals. In solutions which are sufficiently acid these basic radicals must be completely combined with acid, and the acid radicals must be completely free. On the other hand, in sufficiently alkaline solutions the acid radicles must be completely combined with bases, and the basic radicals must be completely free.

Accordingly, it is apparent that there must be a reaction, or a zone, between the state of complete combination with acid and that of complete combination with base, in which there is no combination either with acid or with base, or else in which such combination is a minimum. In particular, there must be a point at which the ionisation of the protein through its acid radicals and that through its basic radicals are equal. As a first approximation this may be considered the isoelectric point, for under these circumstances the protein will move neither to the anode nor to the cathode.

At this point and in its neighbourhood the solubility is likely to be a minimum, for in the case of proteins [Cohn, 1922] it is probably the undissociated molecule which chiefly determines solubility, the ions being relatively soluble substances.

The isoelectric point or zone may fall at an acid, neutral, or alkaline reaction, according to the relative number and strength of the acid and basic groups of the molecule. Thus, if the acid groups be numerous and strong, and the basic groups less numerous and weak, it is evident that the isoelectric state must occur at an acid reaction. Moreover, in case both acid and basic radicals are all weak, there will be a wide zone of reaction throughout which the protein will be very slightly combined with either acid or base, and within which, as a first approximation, it

may be regarded as free from such combination. At the exact isoelectric point this hypothesis may be a very close approximation to the truth. On the other hand, in case certain acid and basic radicals are both of somewhat greater strength, the zone within which there is little or no combination with acid and base may become narrow, or may vanish entirely, so that even at the isoelectric point the protein may be present, in some degree, both as a salt of acid and as a salt of base. Under these circumstances, the isoelectric point will be characterised by the presence both of free protein and of salts of the protein with both acids and bases, but these salts must be ionised to the same extent.

There is much evidence that the relations of proteins to acids and bases may be interpreted in terms of the above hypothesis. We may therefore now turn to quantitative considerations. These lead theoretically to a discussion of acid-base equilibrium in the presence of large numbers of acid and basic radicals; experimentally they lead to the investigation of titration curves.

In the solution of a weak monobasic acid, which is partly neutralised with base, the mass law implies the equation

$$[\overset{+}{\mathrm{H}}] = k \cdot \frac{\text{concentration of free acid}}{\text{concentration of salt}}$$

where k, the apparent ionisation constant, is slightly greater than the true ionisation constant of the acid. This is an approximation which has proved to possess a rather high degree of accuracy.[1] Now, let c represent the total concentration of free acid plus combined acid, and x the concentration of combined acid, that is to say, of salt.

Then

$$[\overset{+}{\mathrm{H}}] = k \cdot \frac{c - x}{x}$$

or

$$x = \frac{c}{1 + \frac{[\overset{+}{\mathrm{H}}]}{k}}.$$

This law also holds in many cases for each acid in solutions containing more than one acid. Let the total concentration of each of several acids be equal (as they must be if they are radicals of a protein molecule), and, as above, designate their concentration by c; let

[1] Henderson, L. J. *Das Gleichgewicht zwischen Basen und Säuren im tierischen Organismus.* Ergebn. Physiol., 1909, 8, 254-325.

$x_1, x_2, x_3, \ldots$ represent the concentration of base combined with them, and $k_1, k_2, k_3, \ldots$ their respective apparent ionisation constants.[1]

Then the relations hold

$$x_1 = \frac{c}{1 + \frac{[H]}{k_1}},$$

$$x_2 = \frac{c}{1 + \frac{[H]}{k_2}},$$

$$x_3 = \frac{c}{1 + \frac{[H]}{k_3}}, \text{ etc.}$$

Evidently the total concentration of base combined with all the acids is given by the sum of all the x's.

$$\Sigma x = x_1 + x_2 + x_3 + \ldots$$

$$\text{or } \Sigma x = \frac{c}{1 + \frac{[H]}{k_1}} + \frac{c}{1 + \frac{[H]}{k_2}} + \frac{c}{1 + \frac{[H]}{k_3}} + \ldots$$

Let $c = 1$ and assume that there are just three acid radicals in question and that $k_1 = 1 \times 10^{-8}$, $k_2 = 1 \times 10^{-9}$ and $k_3 = 1 \times 10^{-10}$. Then approximate computation yields the following table :—

TABLE I.

THEORETICAL CAPACITY TO BIND BASE.

$[H]^+$	10^{-5}N	10^{-6}N	10^{-7}N	10^{-8}N	10^{-9}N	10^{-10}N	10^{-11}N	10^{-12}N	10^{-13}N	10^{-14}N
x_1	—	·001	·01	·09	·50	·91	·99	·999	1·00	1·00	1·00
x_2	—	—	·001	·01	·09	·50	·91	·99	·999	1·00	1·00
x_3	—	—	—	·001	·01	·09	·50	·91	·99	·999	1·00
Σx	0	0	·01	·10	·60	1·50	2·40	2·90	2·99	3·00	3·00

The case of weak bases may be treated in an exactly analogous manner. Let $y_1, y_2, y_3, \ldots$ represent acid combined with several weak bases to form salts, $k'_1, k'_2, k'_3, \ldots$ the corresponding ionisation constants of the bases.

[1] For a full discussion of such considerations compare Henderson, Ergebn. Physiol., 1909, **8**, 254-325; Sorensen, ibid., 1912, **12**, 495; and Michaelis, *Die Wasserstoffionenkonzentration*, 2nd edition. Berlin, 1922.

Then

$$y_1 = \frac{c}{1 + \frac{[\bar{OH}]}{k'_1}},$$

$$y_2 = \frac{c}{1 + \frac{[\bar{OH}]}{k'_2}},$$

$$y_3 = \frac{c}{1 + \frac{[\bar{OH}]}{k'_3}}, \text{ etc.}$$

and

$$\Sigma y = y_1 + y_2 + y_3 + \ldots$$

or

$$\Sigma y = \frac{c}{1 + \frac{[\bar{OH}]}{k'_1}} + \frac{c}{1 + \frac{[\bar{OH}]}{k'_2}} + \frac{c}{1 + \frac{[\bar{OH}]}{k'_3}} + \ldots$$

As before, let $c = 1$. Assume the following values :—

$$k'_1 = 1 \times 10^{-9}, k'_2 = k'_3 = 1 \times 10^{-10}.$$

Then taking account of the fact that, approximately,

$$[\dot{H}] = \frac{1 \times 10^{-14}}{[\bar{OH}]},$$

computation yields the following table :—

TABLE II.

THEORETICAL CAPACITY TO BIND ACID.

$[\dot{H}]$	10^{-9}N	10^{-8}N	10^{-7}N	10^{-6}N	10^{-5}N	10^{-4}N	10^{-3}N	10^{-2}N	10^{-1}N	10^{0}N
$[\bar{OH}]$	10^{-5}N	10^{-6}N	10^{-7}N	10^{-8}N	10^{-9}N	10^{-10}N	10^{-11}N	10^{-12}N	10^{-13}N	10^{-14}N
y_1	—	·001	·01	·09	·50	·91	·99	·999	1·00	1·00
y_2	—	—	·001	·01	·09	·50	·91	·99	·999	1·00
y_3	—	—	·001	·01	·09	·50	·91	·99	·999	1·00
Σy	0	0	·01	·11	·68	1·91	2·81	2·98	3·00	3·00

These two tables may now be brought together by considering the values of Σx and Σy, the total concentration of base and of acid neutralised at different hydrogen ion concentrations by the acid and basic radicals respectively :—

TABLE III.

pH	0	1	2	3	4	5	6	7
+ [H]	10^{0}N	10^{-1}N	10^{-2}N	10^{-3}N	10^{-4}N	10^{-5}N	10^{-6}N	10^{-7}N
Σx	—	—	—	—	—	·00	·01	·10
Σy	3·00	3·00	2·98	2·81	1·91	·68	·11	·01
$\Sigma x - \Sigma y$	− 3·00	− 3·00	− 2·98	− 2·81	− 1·91	− ·68	− ·10	·09
$\Sigma x + \Sigma y$	3·00	3·00	2·98	2·81	1·91	·68	·12	·11

pH	8	9	10	11	12	13	14
+ [H]	10^{-8}N	10^{-9}N	10^{-10}N	10^{-11}N	10^{-12}N	10^{-13}N	10^{-14}N
Σx	·60	1·50	2·40	2·90	2·99	3·00	3·00
Σy	·00	—	—	—	—	—	—
$\Sigma x - \Sigma y$	·60	1·50	2·40	2·90	2·99	3·00	3·00
$\Sigma x + \Sigma y$	·60	1·50	2·40	2·90	2·99	3·00	3·00

Obviously $\Sigma x - \Sigma y$ represents net values of base (positive values of $\Sigma x - \Sigma y$) or of acid (negative values of $\Sigma x - \Sigma y$) in combination at the designated hydrogen ion concentrations. These results permit the construction of a theoretical titration curve (Fig. 1). On this

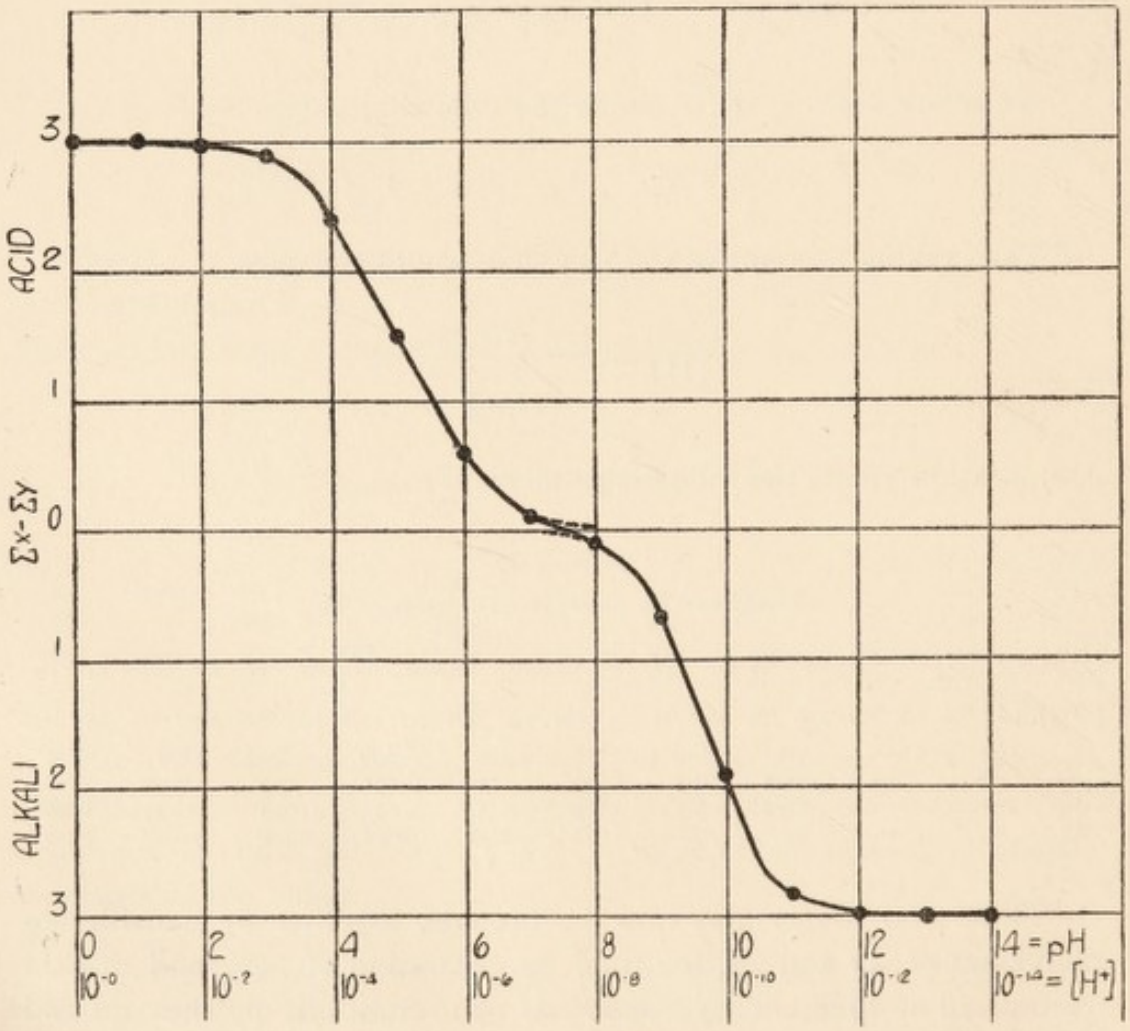


FIG. 1.—Theoretical titration curve.

curve the dotted lines complete the values of the two separate curves of Σx and Σy.

It will be seen that the point at which the combination with acid plus the combination with base ($\Sigma x + \Sigma y$) is a minimum corresponds at least very closely with the point at which the net amount of salt formation ($\Sigma x - \Sigma y$) is 0. This point must also be at least very nearly identical with the so-called isoelectric point, because the salts must ionise to nearly the same amount. Therefore, the sum of the positive and negative electrical charges of the molecule will, other things being equal, be equal.

Extending to either side of this point there is a zone, say from $[\overset{+}{H}] = 1 \times 10^{-7}$N to $[\overset{+}{H}] = 1 \times 10^{-6}$N, through which the amount of salt formation (salts of acid radicals plus salts of basic radicals or $\Sigma x + \Sigma y$) is of nearly the same magnitude as at the isoelectric point.

The position of the isoelectric point in this hypothetical case is evidently dependent upon the fact that an acid radical has been assumed to be present which is stronger than any of the hypothetical basic radicals. It is also evident that in this case the strongest acid radical and the strongest basic radical are alone responsible for determining the position of the isoelectric point. Thus in a simple amphoteric substance, for which $k_A = 1 \times 10^{-8}$ and $k_B = 1 \times 10^{-9}$, we find from the formula[1]:—

$$I = \sqrt{\frac{k_A}{k_B}} \cdot k_W = \sqrt{\frac{10^{-8}}{10^{-9}}} \cdot 10^{-14} = \sqrt{10^{-13}},$$

or, in words, the value of pH for the isoelectric point is 6·5.

Further study of the tables shows that the amount of salt formation at the isoelectric point may be quite different under other circumstances. Thus, if the strongest acid radical and the strongest basic radical were left out from the above case, there would be little change in the isoelectric point, but at that point salt formation would be almost entirely lacking, and the zone of very slight salt formation would be much wider.

On the other hand, the addition of another acid radical of apparent ionisation constant $k = 1 \times 10^{-7}$, and another basic radical of apparent ionisation constant $k' = 1 \times 10^{-8}$ would also leave the isoelectric point nearly unchanged but would lead to a condition in which there must always exist, even at the isoelectric point, a considerable

[1] Michaelis, L. *Die Wasserstoffionenkonzentration*, 2nd edition. Berlin, Springer, 1922, p. 57.

amount of salt formation. The following table and Fig. 2 illustrate this case :—

TABLE IV.

	0	1	2	3	4	5	6	7
$+$ [H]	10^{0}N	10^{-1}N	10^{-2}N	10^{-3}N	10^{-4}N	10^{-5}N	10^{-6}N	10^{-7}N
Σx	—	—	—	—	·00	·01	·10	·60
Σy	4·00	4·00	3·98	3·80	2·90	1·59	·61	·10
$\Sigma x - \Sigma y$	− 4·00	− 4·00	− 3·98	− 3·80	− 2·90	− 1·58	− ·51	·50
$\Sigma x + \Sigma y$	4·00	4·00	3·98	3·80	2·90	1·60	·71	·70

	8	9	10	11	12	13	14
$+$ [H]	10^{-8}N	10^{-9}N	10^{-10}N	10^{-11}N	10^{-12}N	10^{-13}N	10^{-14}N
Σx	1·51	2·49	3·40	3·90	3·99	4·00	4·00
Σy	·01	·00	—	—	—	—	—
$\Sigma x - \Sigma y$	1·50	2·49	3·40	3·90	3·99	4·00	4·00
$\Sigma x + \Sigma y$	1·52	2·49	3·40	3·90	3·99	4·00	4·00

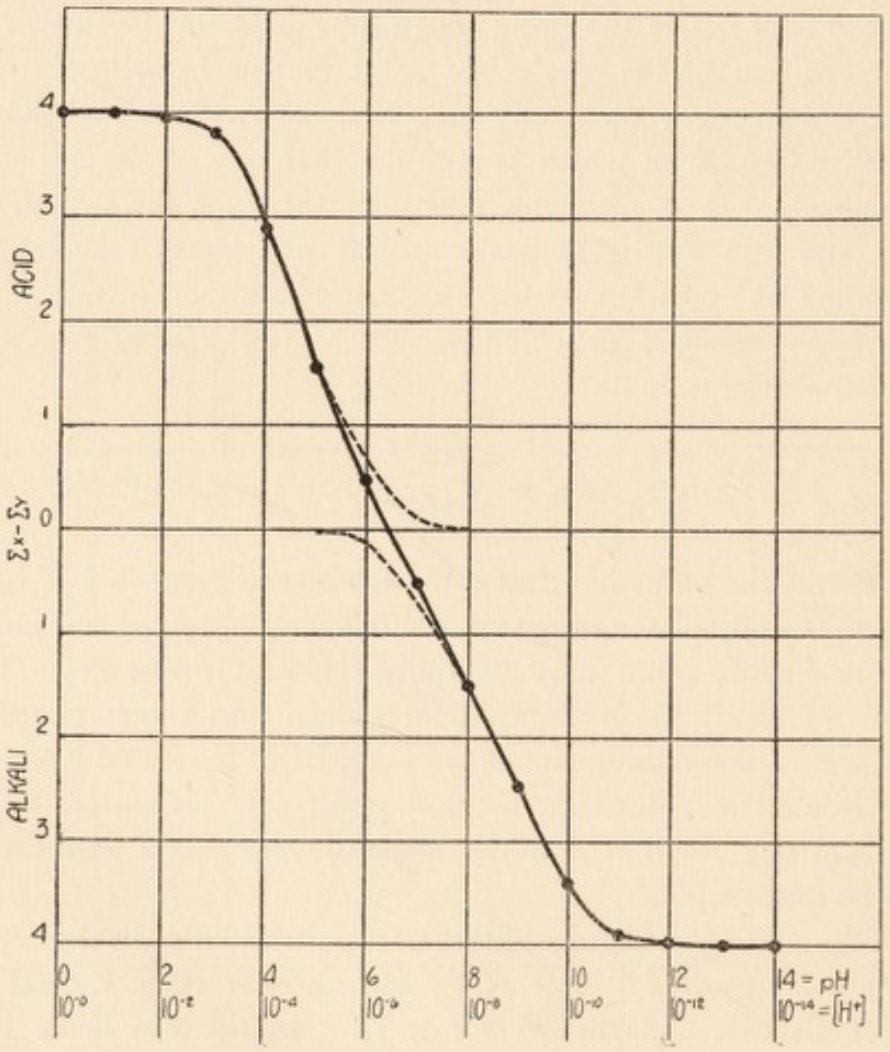


FIG. 2.

No doubt under such conditions the acid and basic radicals of the protein will react with each other in important amounts. No doubt also there is a limit to the strength of acid and basic radicals which

can be present simultaneously in the same molecule. But in the case of oxyhæmoglobin we know a substance whose isoelectric point is very close to neutrality, which contains at least one acid radical so strong that its ionisation constant is not far from 1×10^{-7}.[1] Hence it must also contain at least one moderately strong basic radical.

It is useful to compare the curves of Fig. 1 and Fig. 2, not forgetting, however, that the second curve is longer because of the larger number of acid and basic radicals involved. Apart from this difference, which is a matter of scale, it is easy to see that the second curve is smoother. This difference is a simple result of the theory of buffers.

In general, it may be said that the presence of an acid or basic radical of a given ionisation constant reveals itself by steepness of the curve at the hydrogen or hydroxyl ion concentration corresponding in numerical value to the constant. Thus the sinuosities of a titration curve are direct indications of the ionisation constants of the radicals which are being titrated, and a smooth curve is proof of a regular distribution of the values of these constants throughout the range of reaction under observation. Needless to say titration curves may be constructed theoretically which possess, within certain limits, almost any desired form, and even those that have been determined experimentally vary widely. Their most significant properties are the point where $\Sigma x - \Sigma y = 0$, the slope in this neighbourhood, and then the slope in other portions of the curve. It should be observed that symmetry is not to be expected, though it may be common.

Two important questions now arise: How far does the behaviour of proteins, especially when titrated with acids and bases, correspond with the above theoretical considerations? To what extent are we justified in drawing the conclusion that such correspondence as may be observed between theory and experiment is an indication that the theory is well founded? It must be confessed that these are difficult questions.

Trustworthy experimental evidence that proteins combine chemically with both acids and bases was in hand long before the development of modern theories of ionisation.[2] To this may be added the evidence from Fischer's researches on the constitution of the protein molecule, which is, to say the least, consistent with the theory that

[1] Henderson, L. J. *The Equilibrium between Oxygen and Carbonic Acid in Blood.* J. Biol. Chem., 1920, **41**, 401-430.

[2] Sjöqvist, J. *Physiologisch-chemische Beobachtungen über Salzsäure.* Skandin. Arch. Physiol., 1895, **5**, 277-376. (Good bibliography of older literature.)

occasionally free acid and basic radicals must occur. During later years a large amount of evidence has accumulated which fully establishes the capacity of proteins to form salts with both acids and bases. There is, in the first place, the information derived from such observations as those of Osborne on edestin [1901, 2; 1902, 2].

Thus, pure edestin, from hemp-seed, when free from combined acid or alkali, is entirely insoluble in water, but in the presence of a very little acid, and in the complete absence of salts, it promptly dissolves to a clear solution. From such a solution the edestin is readily precipitated by the addition of a small quantity of a neutral salt, e.g. sodium chloride, and the fact that the edestin has precipitated in combination with the acid is conclusively shown by the behaviour of this precipitate. When such a precipitate is washed thoroughly with dilute alcohol, until all the sodium chloride is removed, and is then dissolved in water, a definite quantity of potassium hydroxide is required to render the solution neutral to phenolphthalein. When thus neutralised, the edestin is completely precipitated, and can be removed from the solution by filtration. If this solution is then evaporated to dryness it leaves a residue of potassium chloride containing nearly all of the alkali originally employed for neutralisation.

Further accurate analyses proved that, under constant conditions, the amount of acid combined with edestin was always nearly constant. Moreover, the amount of acid required just to dissolve pure neutral edestin is approximately equal to the amount in combination with the soluble edestin of ordinary edestin preparations. This is just double the amount of acid combined with the insoluble portion of the protein in such preparations. From these observations of Osborne's the conclusion is inevitable that acid is being neutralised by the protein in accordance with ordinary chemical laws and there seems to be no reason to doubt that definite salts containing acid and protein in true stoichiometrical ratios are being formed. Equally convincing evidence of salt formation may be found in the literature of animal proteins. Among other researches those of L. L. Van Slyke and Bosworth,[1] are especially interesting; they deal with the behaviour of casein.

In the case of tuberin an unpublished investigation of E. J. Cohn's makes the latter conclusion perhaps even more certain. In this experiment pure tuberin suspensions were treated with small but widely varying quantities of sodium hydrate solutions. After equilibrium had been obtained, the solutions were filtered and their

[1] Van Slyke, L. L., and A. W. Bosworth. *Preparation and Properties of Unsaturated or Acid Caseinates and Paracaseinates.* J. Biol. Chem., 1913, **14**, 211-225.

nitrogen content determined. The data, somewhat irregular because of the difficulty of establishing equilibrium, are given in the following table and on the accompanying diagram (see next page).

TABLE V.

SOLUBILITY OF TUBERIN.

Mg. N in 10 c.c. Filtrate.	C.c. $\frac{N}{100}$ NaOH added per 10 c.c. Solution.
0·80, 0·78, 0·80	0·05
1·28, 1·30, 1·59	0·10
1·65, 1·55, 1·54	0·15
2·73, 2·46, 2·70	0·20
3·47, 3·60	0·30

It is evident that, regardless of the amount of base (protein being present in excess), and therefore, within a certain zone, regardless of the true reaction of the solutions, there is a very satisfactory constancy in the ratio of dissolved protein to base. Allowance should be made for the solution of a little free tuberin. This can only be interpreted, so far as I am aware, as salt formation in the strictest sense of the word. It may be added that the same investigator has obtained strictly analogous results of a high degree of accuracy with casein.

Nevertheless, it may be doubted if the preparation of *perfect* protein salts is usually attainable, for there is reason to suspect that the success recorded above depends upon the careful use of a two-phase system and upon other favourable conditions. Inspection of Tables I. and II. of this chapter will convince the reader that frequently, and perhaps nearly always, the neutralisation of an acid or basic radical of a protein molecule can hardly be completed before that of another begins.

The difficulty may be illustrated by considering the neutralisation of phosphoric acid. Here there is a narrow range of reaction within which the first hydrogen is not quite neutralised, though the neutralisation of the second has already begun. In other words, large amounts of NaH_2PO_4 co-exist with small amounts of both H_3PO_4 and Na_2HPO_4. There is also another narrow range within which large amounts of Na_2HPO_4 co-exist with small amounts of NaH_2PO_4 and of Na_3PO_4.

The case would be different if the three ionisation constants of phosphoric acid were more nearly of the same order of magnitude. Then all four substances H_3PO_4, NaH_2PO_4, Na_2HPO_4 and Na_3PO_4, with their ionisation products, might exist together in solution. In the presence of a much larger number of radicals thus overlapping a condition of almost inextricable confusion is to be expected. From such a situation only the most skilful use of all available tricks of manipula-

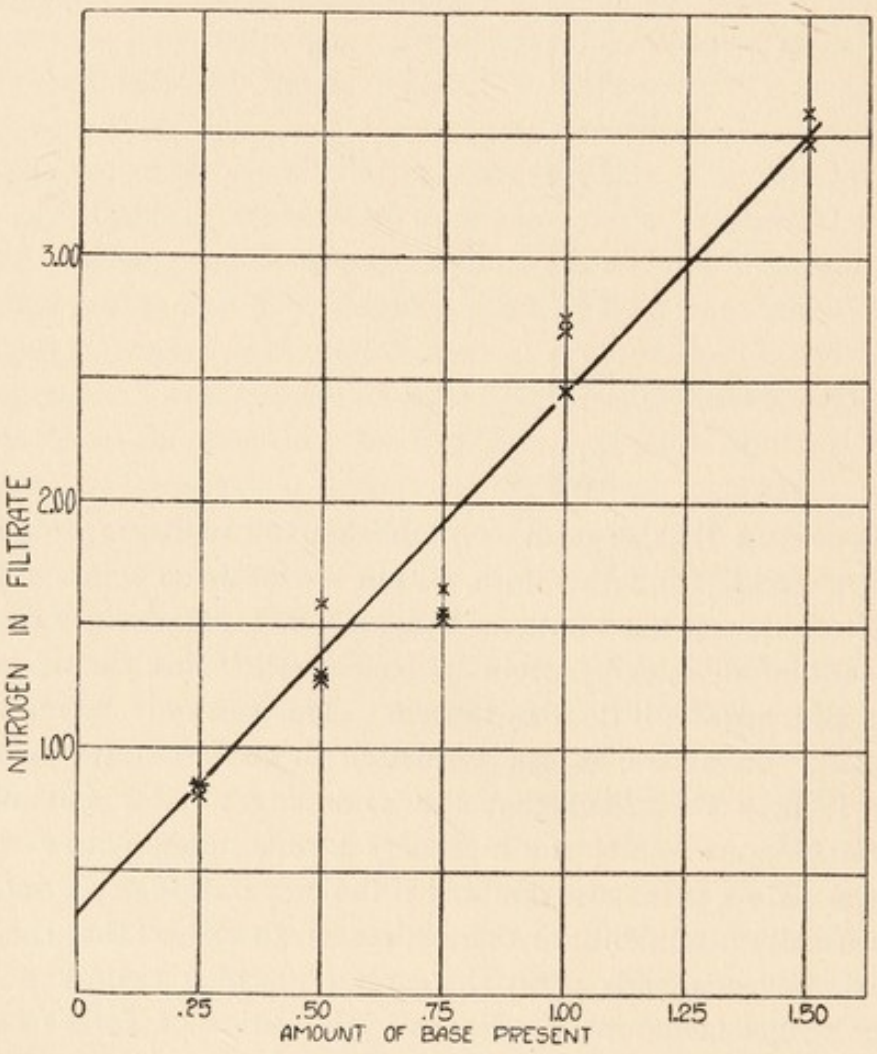


FIG. 3.

tion is likely even occasionally to separate a *perfect* salt, i.e. a compound in which every acid and basic radical is either completely neutralised or else completely free. However, the evidence in support of this somewhat pessimistic view is as yet inconclusive. Furthermore, as will be presently explained, evidence is steadily accumulating that a large number of vegetable proteins are quite free from measurable salt formation near their isoelectric points. They may, therefore, be prepared as pure substances in that state.

If a further justification of the hypothesis that the relations of proteins to acids and bases are purely of a chemical nature be demanded, it may be found in the extensive researches of Sörensen and his associates.[1]

We may, therefore, conclude that the titration curves of proteins have the significance that was provisionally attributed to them at the beginning of this chapter. One reservation should, however, be noted. It is quite possible that in some instances acid and basic radicals of the protein molecule may be liable to some form of tautomeric modification, which is itself dependent upon the reaction of the solution. And it must also be remembered that the protein molecule is liable to irreversible changes in highly alkaline and also in highly acid solutions.

In some respects the chief difficulty in dealing with the titration curves of the proteins depends upon the size of the molecule and the consequent large number of different acid and basic radicals involved. The case is not unlike that of the ultimate analysis of the same substances. In both instances the presence of large numbers of similar radicals leads to a kind of statistical uniformity which tends to obscure individual differences.

Nevertheless, as E. J. Cohn [1922] has pointed out, it is convenient to distinguish between those proteins whose titration curves are steep and those whose curves are flat, at the point where combination with acid equals combination with base, because this distinction corresponds, at least in numerous instances, with the distinction between albumins and globulins or between these and less soluble substances. The probable explanation of this fact has been given above. A flat curve in this region is proof that there can be very little combination with either acid or base throughout a considerable zone, and almost none at all at the isoelectric point. Here the protein exists largely as undissociated molecules. It seems very probable that these may be rather insoluble substances.

The hypothetical protein represented by Table III. and Fig. 1 above may perhaps correspond to an albumin; but the vegetable proteins which have been studied, are, in the free state, insoluble bodies which may better be represented in an excessively simplified form by the second case considered above, in which the strongest acid group and the strongest basic group were eliminated. The consideration of this case has been postponed until this stage in the discussion in order

[1] Sörensen, S. P. L. *Studies on Proteins.* Compt. rend. des travaux du Lab. de Carlsberg, 1915-1917, **12**, 1-372.

to afford a closer comparison with existing experimental data. A computation like that given above yields for this simple case the data of the following table and the curve of Fig. 4 :—

TABLE VI.

	0	1	2	3	4	5	6	7
$[H^+]$	$10^{0}N$	$10^{-1}N$	$10^{-2}N$	$10^{-3}N$	$10^{-4}N$	$10^{-5}N$	$10^{-6}N$	$10^{-7}N$
Σx	—	—	—	—	—	·000	·001	·01
Σy	2·00	2·00	1·98	1·82	1·00	·18	·02	·002
$\Sigma x - \Sigma y$	− 2·00	− 2·00	− 1·98	− 1·82	− 1·00	− ·18	− ·02	·01
$\Sigma x + \Sigma y$	2·00	2·00	1·98	1·82	1·00	·18	·02	·01

	8	9	10	11	12	13	14
$[H^+]$	$10^{-8}N$	$10^{-9}N$	$10^{-10}N$	$10^{-11}N$	$10^{-12}N$	$10^{-13}N$	$10^{-14}N$
Σx	·10	·59	1·41	1·90	1·99	2·00	2·00
Σy	·000	—	—	—	—	—	—
$\Sigma x - \Sigma y$	·10	·59	1·41	1·90	1·99	2·00	2·00
$\Sigma x + \Sigma y$	·10	·59	1·41	1·90	1·99	2·00	2·00

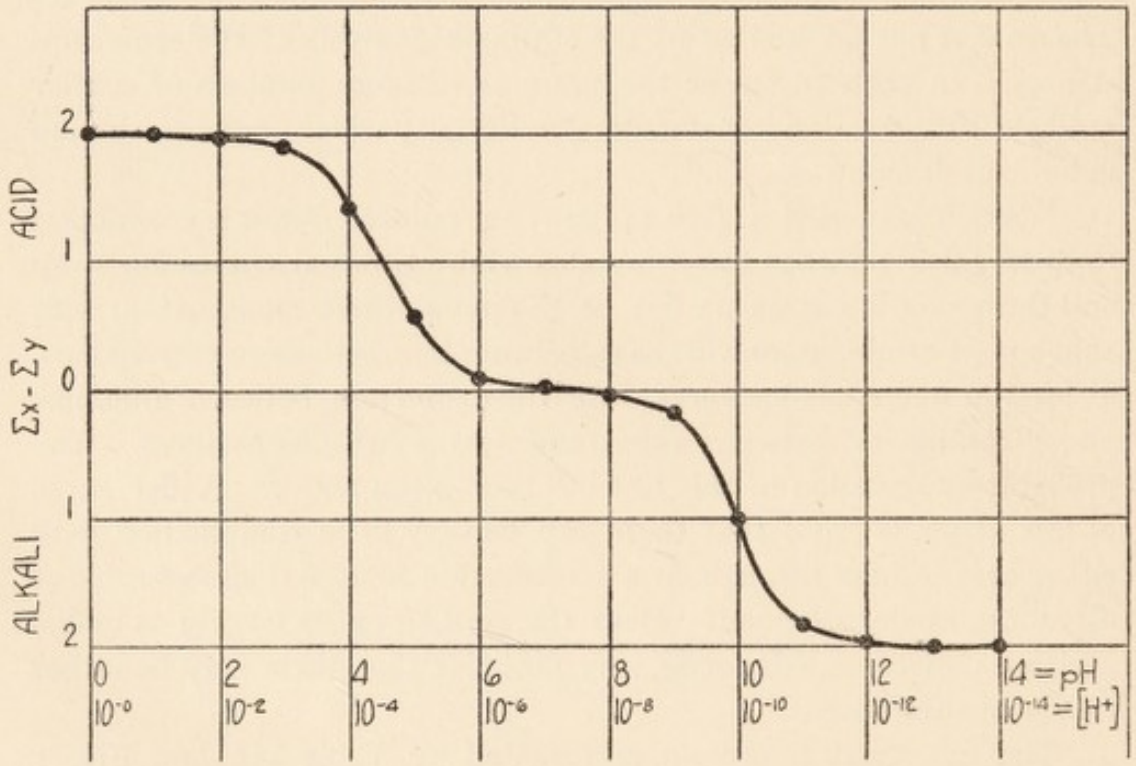


FIG. 4.

There exist more or less complete titration curves for the following vegetable proteins : glutenin [Henderson and Cohn, 1918], tuberin,[1] and edestin [Hitchcock, 1922]. The titration curve of gluten has also been studied by Henderson, Cohn, Cathcart, Wachmann and Fenn [1919]. These curves are given in Fig. 5.

[1] Cohn, E. J. Data to be published in J. Gen. Physiol.

It should be noted that experimental results show the amount of acid or alkali required to bring a protein solution (or suspension) to a given hydrogen ion concentration. Therefore, in all cases where the alkalinity or acidity is high, in case the actual combination between protein and acid or alkali is sought, it is necessary to make a correction for the amount of free acid or alkali present in the solution. This may readily be done by repeating the experiment in the absence of

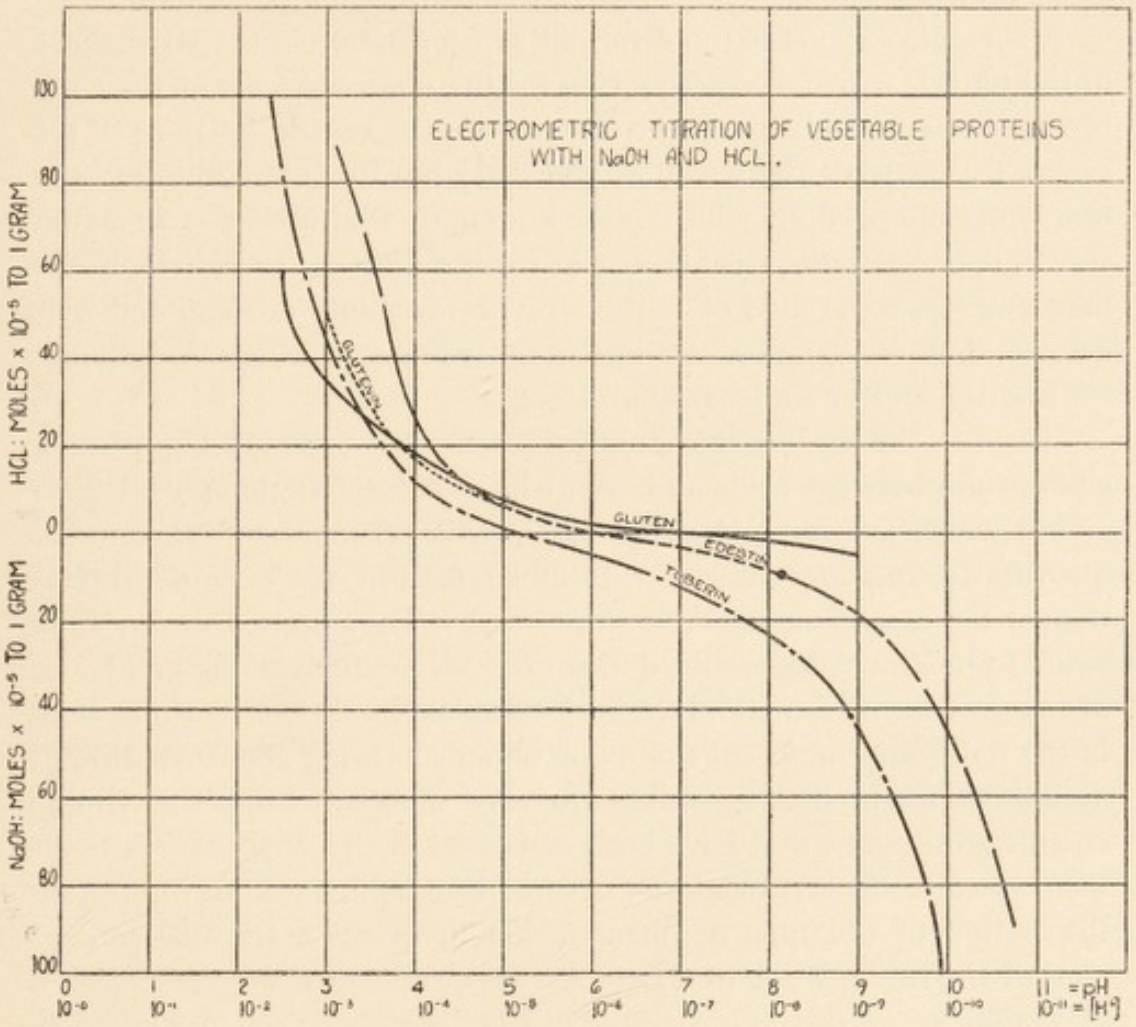


FIG. 5.

the protein. This correction has not been applied in Fig. 5, because considerations bearing on this point are not raised in the present discussion.[1] It is for this reason that the curves as drawn do not tend to become horizontal at their extremities.

On Hitchcock's curve for edestin a point is indicated which may be calculated from Osborne's early observations [1901, 2; 1902, 2]

[1] For a discussion of this question consult E. L. Tague: *A Study of the Determination of Amino-Acids by Means of the Hydrogen Electrode.* J. Amer. Chem. Soc., 1920, **42**, 173-184; D. J. Lloyd, *On the Swelling of Gelatin in Hydrochloric Acid and Caustic Soda.* Biochem. J., 1920, **14**, 147-170; and in the case of edestin, Hitchcock.

in which phenolphthalein was employed. The agreement is very satisfactory.

In the present state of knowledge, it is hardly expedient to press the analysis of the meaning of the titration curves any farther. Throughout the more acid and alkaline ranges little attempt has yet been made to interpret the observations beyond very general conclusions, and what has already been said will perhaps suffice for the middle portions of the curves near the isolectric point.

Everything seems to indicate that salt formation in the strict sense of the term is capable of accounting for the observed facts of the combination of acid and base with protein. In the central portions of the curves this is particularly well established; but there is nothing in the more extreme portions which points in another direction. As stated above, it is, however, possible that in these ranges the protein molecule may undergo some kind of tautomeric or reversible change, and it is certain that irreversible changes occur when the reaction becomes sufficiently acid or sufficiently alkaline.

Even in the case of the simplest electrolytes, the laws governing equilibrium between acids and bases have presented theoretical difficulties which are not yet overcome. Perhaps, in some respect, the quantitative relations are easier to understand in the case of proteins than in the case of such simple substances. They do not, at any rate, seem to present insuperable difficulties. Nevertheless the great size of the protein molecule and the very complex conditions existing both in the fluids and in the cells of living organisms bring about conditions, and therefore problems, which are very different from the simple chemical questions that have been discussed in this chapter. A treatment of such subjects would be out of place here. For an interesting illustration of the type of phenomenon in question the reader may consult the recent work of Loeb, *Proteins and the Theory of Colloidal Behaviour.*[1]

In sum, the theory that normal salt formation is the primary phenomenon, in all cases where the proteins are found in the presence of acids or bases, may be regarded as a well founded working hypothesis.

[1] Loeb, J. *Proteins and the Theory of Colloidal Behaviour*, pp. xii and 292. New York, McGraw-Hill, 1922.

CHAPTER VI.

SOLUBILITY OF VEGETABLE PROTEINS.

A. *Solubility in Water.*

THE aqueous extracts of all the seeds which have been thus far examined contain protein of several types which have commonly been described as globulins, albumins and proteoses. In view of the now generally recognised capacity of proteins to form salts with acids, the salts of which may be soluble in water while the protein at its isoelectric point may be insoluble, it has become impossible to classify properly the proteins found in the slightly acid aqueous extracts of seeds until each has been studied at its isoelectric point. As such studies have been made in very few instances general statements respecting the nature of the water-soluble protein obtained from seeds cannot be made.

It is generally assumed, and seems to be highly probable, that most seeds contain a small proportion of albumins, but few seeds are known in which such occur in any considerable quantity. In most cases a complicated mixture of protein salts occurs, a part of which, like a globulin, can be precipitated by dialysis, while a part remains in solution which, like an albumin, can be coagulated by heating, while a further part, still remaining in solution, has the solubility and some of the other characteristics of the proteoses that result from the hydrolysis of native proteins of either animal or vegetable origin.

B. *Solubility in Saline Solutions.*

Proteins having the properties of globulins can be extracted by saline solutions from all the seeds thus far examined, and in a majority of them these constitute the greater part of the reserve protein.

The concentration of the neutral salt solution required to extract these globulins varies widely, not only with the nature of the salt, but also with that of the protein. Some of the seed globulins are but slightly soluble at room temperature in sodium chloride solutions containing less than 2 per cent. of this salt, while others are readily

soluble in solutions containing only a few tenths of 1 per cent. The degree of solubility of many seed globulins depends much on the temperature of the solution and increases rapidly at about 30°.

Advantage has been taken of this fact to obtain several of the seed globulins in crystalline form, notably that from the hemp-seed [Ritthausen, 1881, 1], the squash-seed [Grübler, 1881], and the oat-kernel [Osborne, 1892, 1].

It is often difficult to determine whether some vegetable proteins are globulins or not because their solubility depends so much on the acids and salts which accompany them in the tissues of the plant. As it is convenient, however, to employ some general designation for those proteins which behave similarly under the conditions usually prevailing during their isolation from the plant cells, those proteins which can be precipitated by dialysing or by diluting the extracts containing them are called globulins in this monograph.

When the aqueous extract of most, perhaps of all seeds, is saturated with ammonium sulphate, and the precipitate, which contains the protein, is dissolved in water, the resulting solution yields on dialysis a precipitate soluble to a greater or less extent in dilute saline solutions, but insoluble in water. Some seeds contain a relatively large proportion of protein which cannot thus be precipitated by dialysis until a certain very small proportion of acid is added, wherewith protein salts are formed insoluble in water, but soluble in dilute saline solutions. A larger proportion of acid yields protein salts which may be soluble in water, but insoluble in saline solutions.

After removing the globulin precipitable by dialysis, a small proportion remains which can be coagulated by heating the solution at a slightly acid reaction. Such protein has usually been regarded as albumin, i.e. protein soluble in water and coagulable by heat.

Some, if not all, of the seed globulins are salts of the protein, the anion present depending chiefly on that of the salt used for the extraction. In the case of edestin from the hempseed, its salts with various anions, like the free edestin itself, are readily soluble in saline solutions though little if at all soluble in water. The solubility in salt solutions is much affected by the amount of acid present; in general the greater the quantity of acid the stronger the saline solution required to dissolve the globulin.

Globulins have been found in relatively greater proportion in oil-seeds than in th se rich in starch. In many of the former, most of the reserve protein is readily soluble in a solution containing 10 per cent. of sodium chloride.

None of the vegetable globulins have been critically studied in respect to their solubility in solutions of different salts except edestin from the hemp-seed which Osborne and Harris [1905, 3] investigated.

As edestin is entirely insoluble in water, the solvent effects of the addition of various quantities of different salts were determined in the following manner. Portions of 2 grammes each of pure crystallised edestin were suspended in sufficient water to make a final volume of 20 c.c. with the different quantities of molar solutions of the several salts which were afterwards added, the edestin being in each case in excess of the amount dissolved. After agitating for some time, the solutions were filtered, nitrogen was determined in 10 c.c. of each, and the amount of edestin dissolved was calculated from the nitrogen in the whole solution. It was thus found that the solubility of edestin is closely proportional to the concentration of the salt solution. In equimolar solutions of sodium, potassium or caesium chlorides its solubility is nearly the same. In those of magnesium, calcium, strontium or barium chloride its solubility is twice as great as it is in equimolar solutions of the chlorides of the monovalent bases, with the exception of lithium chloride, in corresponding solutions of which it does not dissolve as abundantly as in those of the chlorides of the other monovalent bases. Dilute solutions of the sulphates of potassium, sodium, lithium and magnesium have a solvent power similar to that of corresponding solutions of the chlorides of the divalent bases.

Bromides and iodides do not behave like chlorides, for sodium and potassium iodides have a solvent power twice as great as that of the corresponding chlorides, agreeing in this respect with the chlorides of the divalent bases. The bromides are less energetic solvents than the iodides, but more energetic than the chlorides. Barium and calcium bromides are equal to one another in solvent power, which is less than that of sodium or potassium iodide and greater than that of sodium or potassium bromide, the two latter being somewhat less powerful solvents than the corresponding chlorides. Lithium bromide is a much better solvent than lithium chloride, but less energetic than either sodium or potassium bromide.

Salts of strong bases with weak acids which are dissociated in solution with an alkaline reaction have a solvent power approximately proportional to their hydrolytic dissociation. Sodium sulphite and sodium thiosulphate are alike in their solvent power, both being much better solvents than the sulphates. Potassium chromate dissolves edestin more readily than does either the sulphite or the thiosulphate, and is but little less powerful in its solvent effect than sodium carbonate.

Salts of weak bases with strong acids, which are hydrolytically dissociated with an acid reaction, have less solvent power than those of strong bases with strong acids, corresponding in this respect to their hydrolytic dissociation. Thus manganese chloride, manganese sulphate, and ferrous sulphate dissolve edestin to about the same extent as does sodium chloride, having, therefore, about one-half the solvent power of salts of strong divalent bases with strong divalent acids, the two manganese salts being somewhat better solvents than ferrous sulphate.

At 20° the acetates of potassium, sodium or ammonium have no solvent action on edestin, while those of manganese, barium, strontium, calcium, and magnesium dissolve it freely, the degree of solubility in solutions of each of these acetates being in the order named. On the other hand, at 30° sodium acetate dissolves edestin readily. In solutions of acetates of lead, copper, and silver, which are commonly supposed to be precipitants of proteins, edestin, in the complete absence of other salts, dissolves almost as freely as it does in solutions of pure acetic acid. Each of these metallic acetates has equal solvent power and evidence was obtained which showed that the metallic ion of the acetate was united in organic combination with the protein. The solvent effect of lead, copper, and silver acetates is manifestly due to a different reaction with the protein than that which takes place when the protein is brought into solution by means of any of the other salts previously mentioned. Solutions of edestin, produced with each of these other salts, are precipitated by dilution, but those made with solutions of lead, copper, or silver acetate are not, behaving in this respect like those made with free acid.

The results obtained with sodium sulphate, which in sufficient concentration precipitates edestin, are of interest, for, while solutions of this salt up to a certain degree of concentration dissolve edestin in the same proportion as do solutions of a corresponding molecular concentration of potassium, lithium, or magnesium sulphates, the solvent power of more concentrated solutions diminishes with increasing concentration, until by a molar solution of this salt practically no edestin is dissolved. The curve showing the solubility of edestin in solutions of different concentrations of potassium sulphate is the same as that of corresponding solutions of sodium sulphate up to the point of saturation of the solution with potassium sulphate. The solubility of potassium sulphate is so small, however, that molar solutions cannot be made, but if sodium sulphate is added to the saturated solution of potassium sulphate in such quantity that the solution contains as many

molecules of the two sulphates as would the corresponding solution of sodium sulphate alone, the solubility of edestin in the solution of these mixed sulphates is the same as that in a solution containing only sodium sulphate.

Attention is called to these observations in the hope that someone may extend these investigations to other proteins with a view to developing better methods for extracting proteins from seeds than those now employed which depend almost entirely on the use of sodium chloride. The knowledge of the buffer effect of various salts on the hydrogen ion concentration of the extracts, as well as of the importance of the isoelectric point of the individual proteins which has been developed since the experiments above cited were made, should enable future investigators to greatly improve our present unsatisfactory methods of isolating proteins.

C. *Solubility in Acids and Alkalis.*

It was formerly supposed that many proteins were strongly acid in their nature and formed relatively stable salts with bases, as milk casein is now generally considered to do. We, therefore, find many seed proteins described in the older literature as caseins, and among these the so-called legumin from peas and beans was long regarded as a protein of strongly acid character.

Legumin can be extracted from various leguminous seeds by neutral sodium chloride solution, and solutions of the preparations obtained by dialysis, like those of edestin, are distinctly acid. This acidity is probably not caused by the protein itself, but by the combined acid of a legumin salt. Such preparations usually require about 2 c.c. of decinormal potassium hydroxide solution per gramme to make them neutral to phenolphthalein, a quantity slightly greater than that required by similar preparations of edestin which have been proved to be acid salts of this protein. It is therefore improbable that legumin is a protein of a strongly acid type which is soluble in water only when combined with a strong base. In view of these facts there is little doubt that legumin, in the free state, is soluble in water, but when combined with acids, forms salts having properties characteristic of the globulins.

Nearly all the seeds which have been carefully studied contain more or less protein which cannot be extracted by neutral solvents. The greater part of this residual protein is, however, soluble in dilute aqueous alkalis from solutions in which it can be precipitated by the careful addition of acids. The seeds of the cereals contain a relatively

large proportion of such proteins which have been provisionally assigned to the group of glutelins. Very little that is definite has been learned respecting these proteins and until they have been further studied by modern methods it is hopeless to discuss them further.

D. *Solubility in Alcohol.*

The seeds of cereals, with the exception of rice, contain much prolamin soluble in alcohol of from 70 to 90 per cent. In this respect these proteins show a marked contrast in their solubility to all the other proteins of animal or vegetable origin. They dissolve in alcohol of suitable strength in all proportions, so that their solutions, under proper conditions, can be concentrated to thick syrups, from which the protein separates on further evaporation in the form of a transparent film. The addition of alcohol to the concentrated alcoholic solution precipitates these proteins when the concentration of the alcohol reaches a certain degree, which depends on the nature of the dissolved protein, for none of them is at all soluble in alcohol free from water. On the other hand, these proteins are precipitated from their alcoholic solution when the concentration in alcohol is suitably reduced by the addition of water. The limits of concentration in either direction have not yet been determined with accuracy, but, in general, solutions containing less than 50 per cent. or more than 90 per cent. of alcohol dissolve very little of these proteins with the exception of zein of maize, which dissolves in all proportions in alcohol of 92 to 93 per cent.

Other alcohols than ethyl alcohol dissolve gliadin and zein and possibly also the other alcohol-soluble proteins, although as yet experiments with these have not been made. Zein is readily soluble in methyl alcohol and in commercial propyl alcohol. Kjeldahl [1892] has shown that gliadin is soluble in phenol and Mathewson [1906] that it is soluble in dilute methyl and propyl alcohol, in paracresol, and in benzyl alcohol, and that from its solution in phenol it is precipitated by adding ether, acetone, pyridine, benzene or chloroform. Mathewson also found that methyl, ethyl, propyl and amyl alcohols all produce precipitates in the phenol solution, the amount required being less the greater the molecular weight of the alcohol, but precipitates are not produced by adding several volumes of aniline, phenylhydrazine or nitrobenzene, although gliadin is not soluble in these latter substances. When dissolved in phenol the solution can be heated to 140° without producing any notable effect on the specific rotation of the dissolved gliadin.

CHAPTER VII.

PRECIPITATION OF VEGETABLE PROTEINS.

A. *Precipitation by Neutral Salts.*

SOME of the seed proteins are precipitated by saturating their solution with sodium chloride or with magnesium sulphate, all of them are precipitated by saturating with ammonium sulphate or with sodium sulphate at 33°. Since precipitation between definite limits of concentration of ammonium sulphate is a characteristic property of the individual globulins and albumins, this salt affords one of the best means yet employed for separating two or more proteins from one another when present together in extracts of seeds. Thus by adding ammonium sulphate gradually to the extract a precipitate forms which rapidly increases in amount on the further addition of this salt. By filtering out this precipitate and testing the filtrate by adding a little more of the sulphate, it is usually found that no further precipitate forms until the concentration in ammonium sulphate is considerably increased. The second precipitate which then forms is a different protein from the first. These precipitates can then be reprecipitated between the limits of concentration thus established and each protein be obtained in a state of relative purity.

B. *Precipitation by Dilution or by Dialysis.*

Precipitation by dilution or by dialysis is frequently employed in isolating many of the seed proteins. From what has been said before it is evident that the degree of precipitation obtained by this means depends largely on the reaction of the solution. Most of the precipitates are protein salts, and usually separate from solutions only at a suitable hydrogen ion concentration. It is for this reason that many diluted solutions of proteins yield a precipitate only after carbonic acid has been passed through them, for the small proportion of hydrogen ions thus liberated in the solution is sufficient to lead to the formation of their insoluble salts. That such salts are formed by the action of carbonic acid has been shown by Osborne [1901, 3] by

the following experiment with edestin, which in the free state is more soluble in a very dilute solution of sodium chloride than are its salts. Such a solution when diluted with water until it shows a slight turbidity and then saturated with carbonic acid yields a crystalline precipitate. This precipitate when washed free from sodium chloride requires a considerable quantity of decinormal potassium hydrate solution to make it neutral to phenolphthalein. After the edestin is removed by filtration the solution in which the neutralisation is effected contains over 90 per cent. of the added potassium in the form of chloride, which shows that the precipitate produced by carbonic acid is edestin chloride, formed in consequence of the slightly acid reaction imparted to the solution by the carbonic acid. Solutions of legumin which are neutral to litmus, as well as similar solutions of many other seed proteins, when dialysed yield no precipitates, but slightly acid solutions yield such abundantly.

C. *Precipitation by Acids.*

Precipitation by acids may be due either to the acid uniting with a base, previously combined with the protein to form a soluble compound, or by the formation of a salt of the protein insoluble in water. The formation of a precipitate on neutralisation, therefore, depends on the nature of the protein. Edestin dissolved in dilute potassium or sodium hydrate is precipitated by adding enough acid to combine with all of the alkali that is present, for the free edestin is insoluble in water. A solution of legumin is not thus precipitated, for the free legumin is soluble, but a very little more acid precipitates an insoluble salt of legumin. A further addition of acid beyond the amount required to form a precipitate dissolves the protein, the quantity necessary depending upon the proportion of mineral salts contained in the solution, for the soluble acid salts of most seed proteins are insoluble in the presence of small amounts of inorganic salts. Thus edestin dissolved in the least possible quantity of hydrochloric acid necessary for its solution is precipitated by traces of most mineral salts, but a slight excess of acid requires the addition of more salt for precipitation. The precipitation of most seed proteins by acids, therefore, depends largely on the presence of mineral salts in their solutions. An excess of acid appears to act in much the same way, although a larger molecular concentration of acid than of a neutral salt is needed to effect precipitation.

CHAPTER VIII.

DENATURING OF VEGETABLE PROTEINS.

A. *Denaturing by Acids.*

PROTEINS are subject to changes whereby their molecules are slightly altered and their solubility is affected. Our knowledge of the nature of these changes and the various products which result from the denaturing influence of the agents which bring them about, is very limited. It is commonly stated that the action of acids on proteins produces "acid albumin," which is described as insoluble in water but readily soluble in the slightest excess of either acid or alkali. Products having such solubility appear to be formed by the action of acid on nearly all kinds of proteins, and the "acid albumin" which results resembles in its constitution the protein from which it originated, for the changes involved in its formation are slight and do not lead to any profound decomposition of the molecule. Little attention has been paid to the possibility of the formation of intermediate products between the native protein and this "acid albumin," and much confusion still exists in the literature in connection with the action of acids on proteins. Falk[1] has suggested that in the presence of acids a tautomeric change may occur at the peptide union and has pointed out that those conditions which are known to effect such changes in compounds whose constitution is established are the same as those which affect the activity of enzymes. It is not at all improbable that such changes may be involved in the early stages of the denaturing of proteins, though direct evidence of this has not yet been obtained.

Experiments made by Osborne [1902, 1; 1901, 1] showed that small quantities of acids effect profound changes in the solubility of edestin without altering its ultimate composition sufficiently to be detected by analysis. As the results of these experiments appear to have a general application and to shed considerable light on the denaturing effect of acids on proteins they will here be described in detail.

[1] Falk, K. G. *A Chemical Study of Enzyme Action.* Science, 1918, **47**, 423-429.

Crystallised edestin, on solution in the least possible quantity of hydrochloric acid, yields a solution which is precipitated by the addition of a little sodium chloride. This precipitate, treated with a strong solution of sodium chloride, never wholly redissolves. If the part that does dissolve is recrystallised and the experiment repeated, a part of the preparation originally soluble in a neutral salt solution again remains undissolved, and this occurs each time the treatment is repeated. This insoluble derivative, which cannot be made soluble again in a neutral salt solution by any known means, is not "acid albumin," for it is not soluble in a slight excess of potassium hydroxide solution. Similar products result from nearly all the seed proteins, and their formation may be considered to be a general property of these substances. As no suitable name had ever been suggested for such insoluble products, the writer has proposed that they be called in general *proteans*, that that obtained from edestin be called *edestan*, and that similar derivatives from other proteins be given corresponding names.

Preparations of nearly all proteins made by the customary methods contain more or less substance of such altered solubility, and there is little doubt, in view of what we have learned in regard to the formation of these products from edestin, that they are the result of the action of a slight quantity of acid present in the solutions from which they were originally obtained. The following experiments, made with edestin, give the evidence on which this belief is founded. Unfortunately these experiments were made at a time when the modern methods for measuring the hydrogen ion concentration of the solutions had not been developed so that this important factor was not accurately defined. However, as the results of the experiments were positive, they are here given in the hope that they may stimulate a further study of this change in the protein molecule which in the past has seriously interfered with the preparation and purification of many of the vegetable proteins.

Several 1 gramme portions of neutral edestin, which contained 2·16 per cent. of edestan, were suspended in 10 c.c. of pure water and kept at different temperatures for six hours with frequent shaking. An equal volume of 20 per cent. sodium chloride solution was added and the solution at once made neutral to phenolphthalein. The solution was then filtered and nitrogen determined in the thoroughly washed residue collected on the paper, from which the quantity of edestan was calculated.

PERCENTAGE OF EDESTAN FORMED BY CONTACT WITH WATER.

Treated at once with 10 per cent. NaCl at 20°.	Treated with sodium chloride solution after six hours.			
	Water + CO_2 at 20°.	Pure Water at 20°.	Pure Water at 30°.	Pure Water at 50°.
2·16	6·75	4·32	7·11	29·00

These figures show that even at 20° the quantity of edestan which is formed by water alone is twice as great as in the original preparation and that the quantity is decidedly greater if the water contains carbonic acid. At 50° about four times as much edestan is formed as at 30° and nearly eight times as much as at 20°, showing the velocity of the reaction to be doubled by each increase of 10° in the temperature. In view of the larger amount of edestan produced by water containing carbonic acid we should expect to find that the proportion of this insoluble product would be increased by the addition of small quantities of strong acid to the solution. This has been found to be the case, and the following figures give the result of experiments which have shown this :—

PERCENTAGE OF EDESTAN FORMED BY ACIDS AT 20°.

9 c.c. $\frac{N}{100}$ HCl.		14 c.c. $\frac{N}{100}$ HCl.		18 c.c. $\frac{N}{100}$ HNO_3.	19 c.c. $\frac{N}{100}$ HNO_3.	20 c.c. $\frac{N}{100}$ HNO_3.
3 hours	20 hours	3 hours	20 hours	24 hours.	24 hours.	24 hours.
9·01	12·15	29·80	33·55	68·38	75·20	79·02

These figures, compared with those of the preceding table, show that edestin yields much more edestan in contact with acids than in contact with water. In the experiment in which 9 c.c. of centinormal hydrochloric acid were used the proportion of edestan that formed was very much less than in the experiment containing 14 c.c. of the same acid. In the first experiment the amount of acid used was less than that with which the edestin can combine to form a water-soluble compound, and the solution therefore contained only so much free acid as was produced by hydrolytic dissociation of the edestin salts. In the experiment with 14 c.c. of acid a small amount of acid in excess of that required to form a soluble compound was present, and the effect of this larger quantity of free acid produced by the greater dissociation of the weaker acid compounds of edestin is made manifest in the greatly increased quantity of edestan which was produced.

That the amount of edestan which is formed depends on the degree of ionisation of the acid which produces it is shown by the following experiments, in which portions of neutral edestin, each weighing 1 gramme, were suspended in 6 c.c. of water and 14 c.c. of centinormal hydrochloric, phosphoric and acetic acids were respectively added. The quantity of edestan that had formed after frequently agitating during two hours is given in the following table:—

PERCENTAGE OF EDESTAN FORMED BY EQUIVALENT QUANTITIES OF DIFFERENT ACIDS UNDER THE SAME CONDITIONS.

14 c.c. $\frac{HCl}{100}$,	14 c.c. $\frac{H_3PO_4}{100}$,	14 c.c. $\frac{H_4C_2O_2}{100}$.
19·29	16·02	5·65

The solution of phosphoric acid contained 0·98 gramme of H_3PO_4 per litre and was made on the assumption that this acid would behave toward edestin as a monobasic acid. The quantity of edestan formed by acetic acid as compared with that formed by hydrochloric acid is doubtless due to the lesser ionisation of the former acid.

The edestan contained in preparations of edestin, made by the usual methods, has the same properties as the edestan which results from the action of acids on the unchanged edestin, for if preparations of edestin, made by the commonly employed methods, are treated with water, and the solution of the soluble part is precipitated by the addition of a little sodium chloride, the precipitate produced, when treated with a large proportion of sodium chloride, leaves an insoluble residue which in all respects appears to be the same as that obtained by the action of acids on the unchanged edestin.

Edestan is a voluminous white powder which swells somewhat in water, and forms a colourless transparent jelly with very dilute hydrochloric acid. In the dry state it is little, if at all, soluble in strong ammonia, but the gelatinous mass formed by treating the substance with very dilute hydrochloric acid is slightly soluble therein and yields a solution which gives a precipitate with ammonium chloride. Consequently when hydrochloric acid is added to its ammoniacal solution a precipitate forms, even when much of the ammonia is still unneutralised. The ammoniacal solution is not precipitated by sodium chloride.

Edestan exists in preparations of edestin chloride in combination with acid. The amount of acid required to form a compound sparingly soluble in water appears to be definite, as the following experiments show. A quantity of edestan obtained from a preparation of edestin chloride was suspended in water and dialysed until the

dialysate was free from chloride. The dialyser then contained an opalescent fluid and a voluminous precipitate. The acidity of the substance dissolved in this solution was found in two experiments to correspond to 21·5 and 23·4 c.c. of a centinormal solution per gramme. Other experiments gave similar results, from which it appeared that the acidity of the edestan chloride was almost exactly three times that of the edestin chloride soluble therein. From these results we may conclude that the basic property of this altered product is greater than that of the original protein, and as this substance originates so readily and in such large proportions in the presence of a small amount of free acid, it is evident that experiments which have been made to determine the acid-combining power of proteins, in which an excess of acid has been employed, may not necessarily show the acid-combining power of the unchanged proteins.

The reactions of edestan are similar to those which are considered to be characteristic of the histone group, but there is manifestly no connection between this substance and the true histones. The globin obtained from hæmoglobin by the action of dilute acids is generally designated a histone on the ground of the similarity of the reactions of the two substances. It is possible, if not probable, that globin more nearly resembles edestan than the true histones and that it is a similar product of alteration of the protein constituent of the hæmoglobin which has been produced by the action of acids, in the same way as edestan is produced from edestin. Products similar to edestan are formed apparently from some of the animal proteins with great ease, as, for example, from the myosins of muscle tissue which rapidly become insoluble in neutral salt solution during the development of acid which occurs in these tissues soon after death.

Whether other products than edestan are formed by the action of acids on proteins before true "acid albumin" results has not yet been determined; it is not improbable that such may be the case.

B. *Denaturing by Alkalis.*

It has long been known that proteins undergo a change when dissolved in solutions containing a moderate quantity of caustic alkali. A product of this change which is known as "alkali albumin" or "alkali albuminate" resembles in solubility the acid albumin which results from proteins through the action of dilute acids. No satisfactory study has ever been made of these two substances and little, therefore, is known in respect to their relations to one another.

Such scattered observations as are on record indicate that the vegetable proteins are less easily affected by alkalis than are the animal proteins. Ritthausen's experience in extracting seed proteins with dilute caustic alkali solutions showed that the precipitates produced by neutralisation still retained, to a large extent, their solubility in neutral saline solutions, from which it was clear that much of the protein thus extracted had not been converted into "alkali albumin."

Chittenden and Osborne [1891-92] found that zein was particularly resistant to the action of alkali, for even after digesting with 2 per cent. potassium hydroxide solution at 40° for twenty-four hours, the zein still retained its original solubility in alcohol and gave no evidence of the formation of any "alkali albumin." In this experiment the possibility, however, is not excluded of the formation from zein of an "alkali albumin" soluble in alcohol, for it may be that the zein had suffered a change quite analogous to that which results in the formation of "alkali albumin" without producing a substance whose solubility was like that of the "alkali albumin" obtained from other proteins.

Experience indicates that seed proteins are less easily altered by small quantities of alkali than they are by acids, a fact which is contrary to the generally accepted view in regard to the action of alkalis and acids on proteins in general.

Since proteins readily lose ammonia through the hydrolytic action of alkalis on the amide group which nearly all proteins supposedly contain, it is probable that the so-called denaturing of proteins by alkalis is the result of hydrolysis. Further discussion of this question will be found in Chapter IX. on the hydrolysis of vegetable proteins.

C. *Denaturing by Alcohol.*

Alcohol produces a marked denaturing effect on many of the animal proteins, and the ease with which such changes are effected differs with the different proteins. Thus, ovalbumin is quickly converted into a product insoluble in water, but serumalbumin resists this change for a longer time. Many of the proteins of seeds appear to be but little affected by a long treatment with alcohol, and the evidence that any change whatever is caused by alcohol is of such an uncertain character that definite statements in regard to the action of alcohol cannot at present be made. Zein shows an apparently unique behaviour toward alcohol, for when dissolved in strong alcohol the original solution gradually becomes gelatinous and finally is

converted into a firm jelly which is a combination of the zein with alcohol similar to that formed by gelatin with water when its hot solutions are cooled. The formation of this combination depends on the concentration of the solution in zein and apparently also on other conditions, the nature of which is not known. Whether an actual denaturing of the protein here occurs is uncertain, but this appears to be the case, for it has not yet been found possible, by any of the numerous means that have been employed, to restore the zein to its original solubility in alcohol after this change has once taken place. Such a change has not been observed with any of the other alcohol-soluble proteins.

D. *Denaturing by Metallic Salts.*

It is generally recognised that the addition of salts of the heavy metals to solutions of a protein result in the denaturing of the protein. It is probable, from experiments which have been made with edestin, that this denaturing is largely if not wholly due to the fact that such metallic salts are hydrolytically dissociated with a strong acid reaction and that, in the presence of the acid thus set free, the protein is rapidly denatured. Solutions of ferric chloride behave toward edestin in almost exactly the same manner as pure hydrochloric acid, the edestin being denatured with the formation of a product soluble in dilute acid but not precipitated by an excess of ferric chloride. It is probable that the acid set free by hydrolytic dissociation of the metallic salts is the chief cause of the difficulty encountered in attempting to prepare definite salts of the protein with metals.

E. *Denaturing by Heat.*

It appears to be commonly believed that all proteins having the properties of globulin are completely coagulated by heating their slightly acid solutions and that this property is also shared by nearly all of the other native proteins. In this respect the seed proteins differ in a marked degree from the animal proteins, for most of them are very incompletely coagulated by heating their solutions, even to boiling, and many of them are not coagulated at all under these conditions. In this connection should be considered the part which acid plays in the production of a heat coagulum, for it is well known that alkaline solutions of proteins cannot be coagulated by heat and that neutral solutions are usually coagulated with difficulty. It is customary in attempting to separate a protein from its solution by heat to add a very small quantity of acetic acid. From what we have

said in relation to the denaturing effect of acid on protein it is evident that acid cannot be added to the solutions of many of the seed proteins without of itself causing a change in the solubility of the protein, and it becomes difficult in such cases to distinguish between the denaturing effect of the acid and that of the heat. It has, therefore, been the practice in determining the effect of heat on vegetable proteins to heat them in solution without the addition of any more acid than that which has combined with them during the process of isolation. Osborne and Campbell[1] have shown that crystallised ovalbumin, which has an acid reaction toward phenolphthalein similar to that of the preparations of the seed proteins, is completely coagulated when its solutions are sufficiently heated. If, however, enough alkali is first added to make the solution faintly alkaline to phenolphthalein, no coagulum forms on heating, although the albumin has suffered a chemical change which is made evident by the precipitate which forms on cooling the solution and then adding a quantity of acid corresponding to that originally present in the crystalline substance.

The behaviour of edestin solutions toward heat is similar to that of a large number of other seed globulins, and in this connection the experiments of Chittenden and Mendel [1894] are of interest, for they show that the acid combined with the crystallised edestin is insufficient to effect its complete coagulation, as the part of the edestin which is not coagulated remains unchanged even after long heating in a boiling solution. Chittenden and Mendel employed a sodium chloride solution of edestin which had been obtained by extracting hemp seed with a solution of this salt at 60°, a process which yields preparations consisting chiefly of the more acid hydrochloride of this protein, which is to some extent hydrolysed when dissolved. On heating this solution to boiling, a part of the edestin was coagulated. On removing the coagulum and dialysing the filtrate the edestin was precipitated unchanged, as shown by its complete crystallisation. When a very little acetic acid was cautiously added to the filtrate from the coagulum and this again heated to boiling, a second coagulum resulted at 95°—the same temperature as that at which the first coagulum began to form. The coagulation in this case, as in the first, was still incomplete, and the filtrate from the coagulum required a further addition of acid in order to give a coagulum on again heating to boiling. From this we might conclude that, in the complete absence of acid, edestin would not be affected by simply heating its solution, were it not for the fact

[1] Osborne, T. B., and G. F. Campbell, *The Protein Constituents of Egg-White.* J. Amer. Chem. Soc., 1900, **22**, 422-450.

that the writer has found that edestin which contains no combined acid behaves in the same way as the edestin chloride.

Some seed proteins, as, for instance, leucosin obtained from wheat, are more easily coagulated than the seed globulins, but whether these can be completely separated from their solution by heating to a few degrees above the temperature at which a flocculent coagulum is formed, is still an open question. The aqueous extract of wheat flour becomes turbid on heating to 48° to 50° and a flocculent coagulum is formed at 52°. Whether the leucosin is thus completely coagulated is difficult to decide, for after heating the solution for some time at 65°, at which temperature it remains clear, a second but smaller coagulum begins to separate at 73°, which gradually increases in amount as the temperature is raised to 82°. No more coagulum is formed even on boiling. Whether this second small coagulum, formed at the higher temperature, is a residue of leucosin which remains uncoagulated at the lower temperature or a distinctly different protein having a higher coagulation point, remains to be determined.

Most seed extracts behave in a similar manner on heating, and in view of the incomplete coagulation of edestin and other seed proteins it is a question whether or not the coagula obtained at the several temperatures are really formed from different protein substances. The temperature at which a coagulum separates in such solutions depends much upon the rate of heating, so that when the temperature is raised rapidly the first coagulum is obtained at a much higher degree than when raised slowly. The presence of sodium chloride in the aqueous extract of wheat flour has little effect on the temperature at which the coagulum separates. In respect to its behaviour toward heat leucosin resembles the animal proteins more closely than do the globulins, which constitute the greater part of the reserve protein of most seeds. We have already stated the reasons for believing leucosin to be chiefly contained in the embryo of the seed, and it is probable that the physiologically active seed proteins, in this respect as well as in others, more closely resemble the physiologically active animal proteins than do the true reserve proteins of the seed.

CHAPTER IX.

PRODUCTS OF HYDROLYSIS OF VEGETABLE PROTEINS.

A. *Hydrolysis by Acids.*

THE amino-acids which have been obtained from vegetable proteins are the same as those yielded by animal proteins. Ammonia is also a constant product of their hydrolytic decomposition. It is thus plain that no fundamental chemical difference exists between the vegetable and the animal proteins. Such differences as have been detected consist chiefly in the proportion in which some of the amino-acids are yielded by many of the proteins from plants as compared with most of those of animal origin. In general the plant proteins yield more glutaminic acid and ammonia than do the animal and many of them also yield less lysine. Proline has been obtained in relatively large amount from a number of the plant proteins, and arginine is yielded in larger proportion by some of them than by most animal proteins except the protamins. The vegetable proteins soluble in alcohol, on the other hand, yield these basic amino-acids in remarkably small proportion, zein [Osborne and Leavenworth, 1913] being the only one which has yet been found to yield no lysine.

It has long been recognised that many vegetable proteins contain more nitrogen than the animal proteins, and it has been shown [Osborne, Leavenworth and Brautlecht, 1908] that this difference is chiefly due to a relatively larger proportion of arginine in the former. A few, however, of the seed proteins which are rich in nitrogen yield but little arginine. In these the high nitrogen content is due to a large proportion of amide nitrogen.

It is probable that only a part of the sulphur of vegetable, as well as animal, proteins belongs to cystine because when these are decomposed by heating with strong alkali a smaller proportion of the sulphur is converted into sulphide than is the case when cystine itself is similarly treated. No evidence has yet been obtained to indicate the nature of this still unidentified sulphur-containing substance. The proportion of sulphur, and therefore presumably of the cystine-yielding

complex in vegetable proteins, differs greatly. Thus vicilin, from some of the leguminous seeds, contains only 0·1 per cent. of sulphur, while antiarin [Kotake and Knoop, 1911], from the latex of *Antiaris toxicaria*, contains over 7·0 per cent.

Troensegaard [1921, 1923] has advanced the view that the protein molecule is for the most part made up of heterocyclic rings which can be easily split by acids, alkalis or enzymes because a large part of the oxygen of the protein is present as hydroxyl. Experience has shown that such hydroxyl causes easy splitting of heterocyclic rings and he assumes that by means of acids or alkalis the splitting of the ring may lead to the formation of amino-acids. Since Troensegaard's experiments were largely made with gliadin attention is here called to them, though in the author's opinion further evidence in support of this theory is required before it can be accepted as a better conception of the structure of the protein molecule than that of peptide union between the amino-acids which has been founded on the work of Emil Fischer.

The experiments of Chittenden [1894] and his associates have shown that the proteoses and peptones formed from vegetable proteins by the action of pepsin hydrochloric acid are similar to those obtained from animal proteins.

Underhill [1903] found that both the natural and artificial vegetable proteoses have the same physiological effect when injected into the circulation of animals as have those of animal origin, namely, a lowering of the blood pressure, rendering the blood uncoagulable, accelerating the flow of lymph, deep narcosis, and other toxic symptoms.

Knaffl-Lenz [1913] tested the action of peptone obtained from carefully purified preparations of several vegetable proteins when injected into dogs and cats and found that the effect produced corresponded closely with the intensity of the tryptophan reaction given by the proteins. Thus zein peptone even in relatively large amount had no peptone action and did not protect the animal in the slightest degree against a subsequent injection of Witte's peptone. A sufficient quantity of the gliadin peptone caused typical reactions. Smaller quantities had no effect on the coagulability of the blood, but produced an evident peptone immunity. Of the other vegetable peptones, cucurbitin, the crystalline globulin from the squash-seed, which showed the strongest tryptophan reaction, gave the peptone reactions as strongly as did Witte's peptone. Legumin and edestin peptones were less potent and vicilin peptone still less so.

Fischer and Abderhalden [1903] obtained by tryptic digestion of edestin a resistant product which they believed to contain all of the proline and phenylalanine and from the products of acid hydrolysis of gliadin a dipeptide of leucine and glutaminic acid [1907]. Osborne and Clapp [1907, 2] similarly isolated a dipeptide of proline and phenylalanine from this latter protein after prolonged hydrolysis with sulphuric acid.

Whether the vegetable proteins are in general more difficult to hydrolyse completely than are the animal has not yet been definitely determined. That combinations difficult to hydrolyse may occur is shown by the presence of the above-mentioned dipeptide of proline and phenylalanine, which required several hours' heating in a closed tube with concentrated hydrochloric acid before it was completely hydrolysed. The writer once found that when gliadin was boiled with 25 per cent. sulphuric acid for twelve hours a considerable quantity of an insoluble product resembling humin formed, which when further hydrolysed by boiling with strong hydrochloric acid yielded several amino-acids, among which glutaminic acid and cystine were conspicuous.

B. *Hydrolysis by Alkalis.*

In comparison with acids, alkalis have been little used to effect hydrolytic decomposition of proteins, as some of the amino-acids, notably arginine, cystine, and tryptophan are thus destroyed. Osborne, Leavenworth and Brautlecht [1908] found that continued boiling with a strong solution of sodium hydroxide yields a quantity of ammonia corresponding to the sum of the amide nitrogen and one-half of the nitrogen of the arginine. With these exceptions the products of alkaline hydrolysis are, so far as is now known, the same as those produced by acids. In regard to the proportion of ammonia eliminated by alkaline hydrolysis, and the forms of union of nitrogen in the molecule of some vegetable proteins, the reader is referred to p. 71.

C. *Colour Reactions.*

From what has been said of the products of protein decomposition it is evident that the colour reactions of the vegetable proteins are practically the same as those of the animal proteins. None of these reactions, therefore, deserves consideration except those showing the presence of carbohydrates. In the case of vegetable proteins, associated so intimately with a variety of carbohydrates, Molisch's reaction is of especial interest, for this indicates the presence of even minute quantities of any of the members of this group. Preparations of protein

which do not give this reaction may be regarded as entirely free from any carbohydrate or from any complex which can yield a carbohydrate or furfural, as, for instance, glucosides or nucleic acid. The fact that a large number of the vegetable proteins give absolutely no trace of colour with Molisch's test, shows that none of these belong among the glycoproteins. Whether protein preparations which give Molisch's reaction contain a carbohydrate group as a constituent of their molecules, or as a constituent of some group organically united with the protein molecule, or as simply a contamination, cannot yet be definitely decided. Attempts to isolate glucosamine or any other carbohydrate from such proteins have, up to the present time, failed, and we have no good ground to believe that any of the seed proteins actually contain a carbohydrate group notwithstanding statements to the contrary in the older literature. Still, we have no conclusive evidence that some of them do not contain such a group, for it is extremely difficult to isolate carbohydrate from a mixture of protein decomposition products, and the fact that this has not yet been accomplished is by no means conclusive evidence of the absence of such substances. Molisch's reaction is not given by most of those proteins whose physical properties favour their purification. Preparations of cucurbitin, edestin, excelsin, juglansin, corylin, amandin, the flax-seed globulin, and legumin from the pea or vetch, have been obtained which give no trace whatever of this reaction. On the other hand, preparations of other proteins, especially those from the leguminous seeds, frequently give very strong reactions, but it has been noted that when a number of different preparations of one or the other of these proteins are tested under uniform conditions the intensity of the reaction varies greatly. In such cases it is highly probable that this reaction is caused by a small amount of some contaminating substance which is difficult to separate from the protein.

D. *Nitrogen in Vegetable Proteins.*

Partition of Nitrogen in Seed Proteins.

The different forms in which nitrogen occurs in seed proteins have been extensively studied by Osborne and Harris [1903, 1]. Following Hausmann's [1900] method, slightly modified, they obtained the results given in the table, p. 72. Several proteins of animal origin were likewise analysed and are given for comparison.

The most striking feature shown by this table is the wide range in the amount of basic nitrogen obtained from the different proteins, namely, from one-third to one-thirtieth of the total nitrogen of the

protein, while the proportion of ammonia differs from one-fourth to one-sixteenth of the total nitrogen. The non-basic nitrogen, on the other hand, is more constant even than the total nitrogen and forms from about one-half to three-fourths of the latter.

PARTITION OF NITROGEN IN DIFFERENT PROTEINS.

Protein.	Source.	In per cent. of protein. N as ammonia.	Basic N.	Non-basic N.	N in MgO, pp.	Total N.	In per cent. of nitrogen. N as ammonia.	Basic N.	Non-basic N.
Globulin,	cocoanut	1·36	6·06	10·92	0·14	18·48	7·3	32·8	59·0
,,	squash-seed	1·28	5·97	11·04	0·22	18·51	7·4	32·3	59·6
Edestin,	hemp-seed	1·88	5·91	10·78	0·12	18·69	10·1	31·6	57·7
Excelsin,	Brazil-nut	1·48	5·76	10·89	0·17	18·30	8·0	31·5	57·8
Corylin,	hazel-nut	2·20	5·75	10·89	0·16	19·00	11·6	30·3	59·5
Globulin,	cotton-seed	1·92	5·71	11·01		18·64	10·3	30·6	59·1
,,	castor-bean	1·96	5·64	11·03	0·12	18·75	10·5	30·1	58·7
Juglansin,	walnut	1·78	5·41	11·50	0·15	18·84	9·4	28·7	61·2
Conglutin,	lupine α	2·12	5·20	10·40	0·18	17·90	11·8	29·1	58·1
	lupine β	2·65	5·13	10·29	0·14	18·21	14·0	28·2	56·6
Legumin,	pea [1]	1·68	5·11	11·10	0·15	18·04	9·3	28·3	61·5
,,	lentil	1·69	5·16	11·10	0·11	18·06	9·3	28·5	61·3
,,	horse-bean	1·62	4·92	11·41	0·11	18·06	9·0	27·2	63·2
,,	vetch	1·75	5·17	10·92	0·18	18·02	9·7	28·7	60·7
Globulin,	flax-seed	2·00	4·77	11·49	0·22	18·48	10·8	25·8	62·0
Vicilin,	pea	1·70	4·92	10·22	0·21	17·05	10·0	28·8	60·0
,,	lentil	1·75	4·59	10·77	0·13	17·24	10·1	26·6	62·5
,,	horse-bean	1·93	4·53	10·35	0·23	17·04	11·3	26·6	60·7
Vitellin,	egg-yolk [1]	1·29	4·36	10·04	0·59	16·28	7·9	26·7	61·6
Vignin,	cow-pea	1·91	4·28	10·81	0·25	17·25	11·1	24·8	62·6
Globulin,	sun-flower	2·57	4·27	11·50	0·24	18·58	13·8	23·0	61·9
Conalbumin,	egg-white [1]	1·00	3·76	10·93	0·42	16·11	6·2	23·3	67·8
Amandin,	almond	3·05	4·15	11·63	0·17	19·00	16·0	21·8	61·2
Phaseolin,	kidney-bean	1·69	3·62	10·56	0·33	16·20	10·4	22·3	65·2
,,	adzuki-bean	1·74	4·18	10·01	0·27	16·20	10·7	25·8	61·7
Glycinin,	soy-bean	2·11	3·95	11·27	0·12	17·45	12·1	22·6	64·6
Legumelin,	lentil	1·08	3·59	10·97	0·42	16·06	6·7	22·3	68·3
,,	horse-bean	0·96	3·42	11·10	0·44	15·92	6·0	21·4	69·5
,,	adzuki-bean	1·03	3·84	10·93	0·30	16·10	6·4	23·9	67·9
,,	soy-bean [1]	1·18	3·08	11·44	0·39	16·09	7·3	19·1	71·1
Leucosin,	wheat	1·16	3·50	11·84	0·43	16·93	6·8	20·6	69·6
Casein,	cow's milk	1·61	3·49	10·31	0·21	15·62	10·3	22·4	66·0
Ovalbumin,	egg-white [1]	1·34	3·20	10·68	0·29	15·51	8·6	20·6	69·0
Glutenin,	wheat	3·30	2·05	11·95	0·19	17·49	18·8	11·7	68·3
Gliadin,	,,	4·33	1·09	12·17	0·07	17·66	24·5	6·2	68·9
,,	rye	4·08	0·91	12·56	0·11	17·66	23·1	5·2	70·0
Hordein,	barley	4·01	0·77	12·20	0·23	17·21	23·3	4·5	70·9
Zein,	maize	2·97	0·49	12·51	0·16	16·13	18·4	3·0	77·5

[1] Revised figures, given in view of later unpublished determinations.

Comparison of the Nitrogen Precipitated by Phosphotungstic Acid with that in the Basic Amino-Acids.

We are able to form some judgment in regard to the accuracy of the figures given for the nitrogen precipitated by phosphotungstic acid,

since the only strongly basic amino-acids yielded by proteins are arginine, histidine and lysine, and these are, therefore, precipitated from a dilute solution by phosphotungstic acid. The nitrogen thus precipitated should, consequently, be equal to the nitrogen contained in these three basic amino-acids, if no other basic substances are present among their decomposition products. Cystine is also precipitated to some extent, but as this amino-acid usually occurs among the products of hydrolysis of proteins in very small proportion, the effect of cystine nitrogen is practically negligible. The existence of such an agreement is shown from the following table :—

	Per cent. of the protein.						Basic N. in p.c. of that precipitated.
	Histidine.	Arginine.	Lysine.	Basic N Calculated.	Basic N Precipitated.	Difference.	
Globulin, squash-seed	2·42	14·44	1·99	5·69	5·99	− 0·30	95·00
Excelsin, Para-nut	2·50	14·29	1·64	5·57	5·76	− 0·19	96·70
Edestin, hemp-seed	2·19	14·17	1·65	5·48	5·91	− 0·43	92·70
Globulin, cotton-seed	3·46	13·51	2·06	5·69	5·71	− 0·02	99·65
Globulin, castor-bean	2·74	13·19	1·54	5·29	5·64	− 0·35	93·80
Amandin, almond	1·87	12·16	0·72	4·56	4·15	+ 0·41	109·90
Legumin, pea	1·69	11·73	4·98	5·20	5·11	+ 0·09	101·50
Legumin, vetch	2·94	11·06	3·70	5·07	5·17	− 0·10	98·07
Conglutin-α, yellow lupine	2·51	10·93	2·74	4·77	5·16	− 0·39	92·50
Vicilin, pea	2·17	8·91	5·40	4·50	4·92	− 0·42	91·46
Glycinin, soy-bean	2·10	7·69	3·39	3·59	3·95	− 0·36	90·90
Vitellin, hen's egg-yolk	1·90	7·46	4·81	3·82	4·36	− 0·54	87·62
Vignin, cow-pea	3·08	7·20	4·31	3·98	4·28	− 0·30	92·90
Glutelin, maize	3·00	7·06	2·93	3·63	3·52	+ 0·11	103·10
Ovalbumin, hen's egg	1·71	4·91	3·76	2·76	3·20	− 0·44	86·25
Leucosin, wheat	2·83	5·94	2·75	3·25	3·50	− 0·25	92·87
Conalbumin, hen's egg	2·17	5·07	6·43	3·45	4·16	− 0·71	83·00
Legumelin, pea	2·27	5·45	3·03	2·95	3·45	− 0·50	85·50
Legumelin, soy-bean	2·04	5·35	4·91	3·21	3·08	+ 0·13	104·22
Phaseolin, kidney-bean	2·62	4·87	4·58	3·15	3·62	− 0·47	87·00
Glutenin, wheat	1·76	4·72	1·92	2·37	2·05	+ 0·32	115·60
Casein, cow's milk	2·46	3·39	5·95	2·99	3·49	− 0·50	85·70
Gliadin, wheat	0·58	3·16	0·63	1·49	1·09	+ 0·40	136·00
Gliadin, rye	0·39	2·22	0·00 ?	0·83	0·90	− 0·07	90·20
Hordein, barley	1·28	2·16	0·00 ?	1·05	0·77	+ 0·28	136·40
Zein, maize	0·82	1·55	0·00	0·72	0·49	+ 0·16	146·90

Sixteen of the twenty-six proteins given in this table contain an amount of bases in which the nitrogen does not fall below 90 nor above 110 per cent. of that precipitated by phosphotungstic acid. Three of the others show differences which are relatively great though absolutely small; but as they contain very little base these differences are unquestionably caused by unavoidable errors of analysis; for the

phosphotungstates of the bases are somewhat soluble, and, therefore, when the amount of base is small the nitrogen precipitated by this acid is less than that actually contained in them. On the other hand, the bulky precipitate of the phosphotungstates, which is obtained when the amount of base is large, carries with it some of the mono-amino-acids, thereby compensating the error caused by solubility. The six remaining proteins yield a quantity of bases containing an amount of nitrogen less than that precipitated by phosphotungstic acid by a little more than 10 per cent. of the latter. Two of these, namely, phaseolin and legumelin from the pea, yield more bases when hydrolysed for twenty-four hours than when hydrolysed for twelve hours, hence this difference may possibly be due to an incomplete hydrolysis even after this longer time of boiling.

It is evident from the figures given in the table that in most cases the agreement between the nitrogen in arginine, histidine and lysine and that precipitated by phosphotungstic acid is so close that Hausmann's method can be employed for controlling the results of determinations of the bases by the method of Kossel, Kutscher and Patten [1900, 1903]; for where a wide difference between the nitrogen obtained by these two methods is found the accuracy of the direct determination of the bases should be established by careful repetition.

The accuracy of the determinations of the individual bases, that is, the completeness with which they can be separated from one another, as well as from other substances, is shown by the evident purity of the products obtained in the course of analysis. The arginine copper nitrate double salt separates completely from its solution on slow evaporation, leaving no trace of any other substance in the final liquid. The histidine solutions readily yield pure histidine dichloride, and the character of the crystallisation of the lysine picrate, in which form this substance is weighed, is such as to leave no doubt of its purity, which can, moreover, be further established by analysis of the product in the form in which it is actually weighed. The agreement between duplicate determinations of the several bases made on one and the same protein is further evidence of their accuracy.

In regard to the constancy of the determination of the ammonia nitrogen the following results serve to illustrate how closely repeated determinations by different analysts using different preparations may be expected to agree. (Cf. Osborne, Leavenworth, and Brautlecht [1908].)

NITROGEN AS AMMONIA IN PER CENT. OF THE PROTEIN.

Gliadin	4·40,[2] 4·44,[2] 4·35, 4·30, 4·33, 4·33, 4·30.[1]
Hordein	4·10,[2] 4·01.[1]
Zein	2·99,[2] 2·97.[1]
Edestin	1·83,[2] 1·86, 1·88,[1] 1·93,[1] 1·86, 1·81, 1·80, 1·87.
Globulin, squash-seed	1·35,[2] 1·28.[1]
Glycinin	2·14,[2] 2·11.[1]
Vignin	1·89, 1·86, 1·91,[1] 1·82.
Globulin, cotton-seed	1·94, 1·96, 1·92.[1]
Legumin, pea	1·68, 1·68.[1]
Vicilin, pea	1·64, 1·78, 1·60.[1]
Vitellin, hen's egg yoke	1·24, 1·29, 1·28,[1] 1·24.[1]
Conalbumin, hen's egg	1·13, 1·21.[1]

[1] Figures given in preceding table.
[2] Distilled *in vacuo* at 40°.

Is the Nitrogen Yielded as Ammonia Amide Nitrogen?

As the amino-acids which result from protein hydrolysis yield scarcely more than traces of ammonia by long boiling with strong hydrochloric acid, practically all of the ammonia must arise from some other form of binding of the nitrogen in the protein molecule. That this nitrogen may be in amide union seems probable from the following experiments in which five portions of gliadin, each weighing 1 gramme, were dissolved in 50 c.c. of 20 per cent. hydrochloric acid, and the solutions boiled for the times indicated in the following table:—

Boiled for 30 min., nitrogen as NH_3 = 4·30 per cent.
Boiled for 1 hour, nitrogen as NH_3 = 4·35 per cent.
Boiled for 2 hours, nitrogen as NH_3 = 4·33 per cent.
Boiled for 3 hours, nitrogen as NH_3 = 4·33 per cent.
Boiled for 4 hours, nitrogen as NH_3 = 4·33 per cent.
Boiled for 6 hours, nitrogen as NH_3 = 4·40 per cent.

When treated with strong hydrochloric acid at 20° for two hours, only 0·22 per cent. of nitrogen as ammonia was obtained, while after seventeen hours at the same temperature 1·67 per cent. was found. Under similar conditions after seventeen hours at 20° asparagine yielded 1·4 per cent. of nitrogen as ammonia, and one-half of its nitrogen after boiling for thirty minutes.

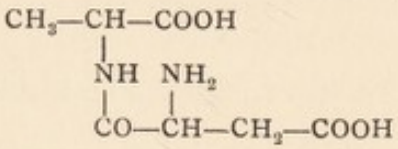
These results were later confirmed by Thierfelder and von Cramm [1919] who further showed that synthetic amides of dipeptides yielded ammonia in the same way as did gliadin under similar conditions of hydrolysis.

Further evidence that the ammonia results from the hydrolysis of amide groups, $CONH_2$, with the formation of a corresponding amount of carboxyl groups, COOH, was obtained by Osborne and Nolan [1920] who found that the acidity of the products of hydrolysis of gliadin was increased in proportion to the ammonia set free. There is little reason

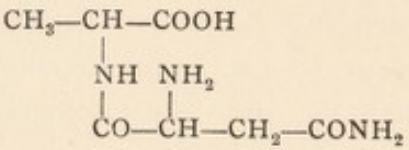
to doubt that the nitrogen yielded by proteins as ammonia is actually in amide combination with one of the carboxyl groups of the dibasic amino-acids.

Ratio of Ammonia to Glutaminic and Aspartic Acids.

The work of Emil Fischer has made it almost if not quite certain that the amino-acids are united in the protein molecule in peptide union; that is by the union of the NH_2 group of one amino-acid with the carboxyl group of another; thus



which represents a peptide of alanine and aspartic acid. The dibasic acids would therefore afford carboxyl groups with which nitrogen might unite in amide union, as shown by the following formula, which represents the amide of the peptide just mentioned :—



A relation may consequently exist between the quantity of amide nitrogen which the different proteins yield and their content in glutaminic and aspartic acid. Osborne and Gilbert [1906] showed that a large proportion of glutaminic acid is in many cases accompanied by a similar large proportion of amide nitrogen. Osborne, Leavenworth and Brautlecht [1908] showed that the amount of ammonia yielded by hydrolysis with acids agreed so closely with that required for amide union with the sum of the glutaminic and aspartic acids found in a large number of proteins of both vegetable and animal origin as to make it highly probable that practically all of the ammonia originated from such a combination and that one of the carboxyl groups of each molecule of the dibasic acids was thus united with a NH_2 group. Later determinations made by Foreman [1] of glutaminic acid in casein have shown that this protein yields a larger proportion of this amino-acid than had been previously obtained and the still later discovery of Dakin [2] that casein also yields a notable quantity of oxyglutaminic acid has made it improbable that this relationship to the

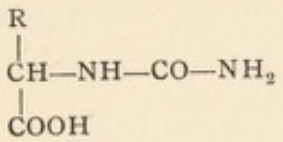
[1] Foreman, F. W. *A New Method for Preparing Esters of Amino-Acids: Composition of Caseinogen.* Biochem. J., 1919, **13**, 378-397.

[2] Dakin, H. D. *On Amino-Acids.* Biochem. J., 1918, **12**, 290-317.

dibasic acids is as strictly quantitative as previously supposed. Nevertheless we have reason to believe that, apart from insignificant quantities which result from secondary decomposition, the ammonia is present in amide union in the protein molecule. The amount of glutaminic and aspartic acids yielded by all proteins thus far examined is sufficient to afford the carboxyl groups needed for such a combination without taking into consideration any oxyglutaminic acid which these may also yield. In the case of such vegetable proteins as gliadin or hordein, which yield nearly 50 per cent. of glutaminic and aspartic acids, it is difficult to imagine that a relatively large proportion of carboxyl groups are not united with nitrogen as in amides.

Bergell and Feigl[1] have suggested that the dibasic acids may be present as a diamide R—CO—NH—CO—R which they have shown to be stable in acid solutions but to yield ammonia on boiling with alkalis. Since the excess of ammonia yielded by distilling proteins with alkali over that yielded by boiling with acids is so nearly equal to one-half of their arginine nitrogen we have as yet no reason to believe that any of them contain a diamide grouping.

Andersen and Roed-Müller[2] have suggested the possibility that a part of the nitrogen may exist in the uramino binding



They base this theory on an observation by Lippich[3] that proteins yield more carbon dioxide on alkaline hydrolysis than on acid hydrolysis; the difference being greater than can be accounted for by the amount of arginine contained in them. They show that there is no evidence contrary to this theory of uramino acid bindings in a protein but admit that the only satisfactory proof that such actually exists would be the isolation of substances containing such linkages. This has not yet been accomplished.

Nitrogen Converted into Ammonia by Alkaline Hydrolysis.

Among the known decomposition products of proteins cystine and arginine are notably unstable in alkaline solutions. According

[1] Bergell, P., and Feigl, J. *Ueber neue Verbindungen von Aminosäuren und Ammoniak*, II. Mitteilung. Zeit. Physiol. Chem., 1908, **54**, 258-287.

[2] Andersen, A. C., and Roed-Müller, R. *Zur Kenntnis der Eiweisskörper: II. Über die Bindung des Ammoniaks in den Eiweisskörpern.* Biochem. Zeit., 1915, **70**, 442-463.

[3] Lippich, F. *Über analytische Anwendungen der Uramidosäurereaktion.* Zeit. physiol. Chem., 1914, **90**, 124-144.

to Onslow[1] tryptophan is comparatively stable in hot baryta solution when in the presence of other products of protein hydrolysis but is easily decomposed when alone. In boiling 10 per cent. solutions of sodium hydroxide to which some copper sulphate was added Herzfeld [1913, 1] states that tryptophan is largely decomposed.

Experiments by Osborne, Leavenworth and Brautlecht [1908] show how some vegetable proteins behave when subjected to alkaline hydrolysis. One gramme, air dry, of each of the proteins given in the table below was distilled with 300 c.c. of decinormal sodium hydroxide solution, and when 200 c.c. had distilled over, the distillate was titrated. The residual solution was then made up to 300 c.c. with decinormal sodium hydroxide solution and the distillation repeated. The solution was then again made up to 300 c.c. with water and again distilled, the process being repeated until practically no more ammonia came over. The results obtained are given in the following table:—

Distillation.	Gliadin, Wheat, 0·9198 gm.	Legumin, pea, 0·8979 gm.		Vicilin, pea, 0·9264 gm.		Excelsin, Para-nut, 0·9024 gm.
	Mgr. N.	Mgr. N.		Mgr. N.		Mgr. N.
1	27·2	15·4	17·6	17·8	15·4	11·4
2	12·0	4·4	5·8	3·6	5·2	4·5
3	1·6	2·2	2·6	2·2	3·0	3·5
4	2·6	1·8	1·6	1·6	1·2	2·4
5	0·4	1·8	1·6	1·2	1·6	1·4
6	—	1·0	1·0	1·0	0·8	1·8
7	—	1·3	1·2	1·0	1·0	1·6
8	—	1·0	0·8	0·8	1·0	1·6
9	—	0·6	0·8	0·4	0·6	1·6
10	—	0·8	0·4	0·4	0·4	1·6
11	—	1·4	1·0	1·2	0·8	1·6
12	—	0·8	0·6	0·2	0·4	0·8
13	—	0·6	0·8	—	—	0·4
14	—	0·2	0·4	—	—	0·9
15	—	—	—	—	—	0·0
Total	43·8	33·3	36·2	31·4	31·4	35·1
Per cent. of dry and ash-free protein	4·76	3·71	4·04	3·39	3·39	3·72
Amide N	4·30	1·69	—	1·70	—	1·48
½ Arginine N	0·51	1·88	—	1·42	—	2·25
Sum	4·81	3·57	—	3·12	—	3·73

Gliadin, which contains much amide nitrogen and but little arginine nitrogen, and excelsin, which contains little amide nitrogen and

[1] Onslow, H. *On the Stability of Tryptophan in Baryta Hydrolysis.* Biochem. J., 1921, **15**, 383-391.

very much arginine nitrogen, both gave results in close agreement with the calculation, while legumin and vicilin yielded slightly more nitrogen by alkaline hydrolysis than that calculated.

Most of the nitrogen came over in the first two distillations, corresponding to the ease with which amide nitrogen is converted into ammonia by caustic alkalis. The nitrogen subsequently coming off as ammonia was evolved slowly in much the same way as from arginine, although a little more quickly. The close agreement between the results thus obtained and those calculated shows that these proteins contain little nitrogen which can be thus converted into ammonia except the amide and one-half the arginine nitrogen.

E. *The Rate of Hydrolysis of Proteins.*

The only comprehensive study of the rate of hydrolysis of plant proteins by means of enzymes is that of Frankel [1916], while the rate of hydrolysis of wheat gliadin by means of acids and alkalis has been studied by Vickery [1922].

Frankel subjected pure preparations of edestin, amandin, conglutin, legumelin, phaseolin and the globulins from cotton seed, soy-bean, and squash seed, to the successive action of pepsin, trypsin and of erepsin. Phaseolin, conglutin and edestin were also treated directly with alkaline trypsin, followed by erepsin, and with acid pepsin followed by alkaline erepsin. The results of his studies are shown by Figs. 6 and 7, in which the extent of the hydrolysis is expressed as the ratio of the amount of amino nitrogen found at successive periods to the amount found when a sample of the protein was hydrolysed with 20 per cent. hydrochloric acid for twenty-four hours. Pepsin hydrolysed from 9 to 11 per cent. of the peptide bonds during the first three hours. The rate of hydrolysis subsequently became much slower, reaching 15 to 20 per cent. in 100 hours; during subsequent periods of 300 hours the maximum attained was only 21 to 24 per cent.

Trypsin likewise effected a very rapid hydrolysis during the first few hours but the rate diminished rapidly when approximately 60 per cent. of the bonds were broken. Prolonged digestion with this enzyme acting upon a substrate previously digested with pepsin only effected hydrolysis of approximately 70 per cent. of the peptide bonds. Trypsin acting directly upon alkaline solutions of native proteins hydrolysed from 30 to 40 per cent. of the peptide bonds within twelve hours (Fig. 8 and 9) but only effected hydrolysis of from 50 to 60 per

cent. during the longest periods studied even when fresh enzyme solution was added to the digest.

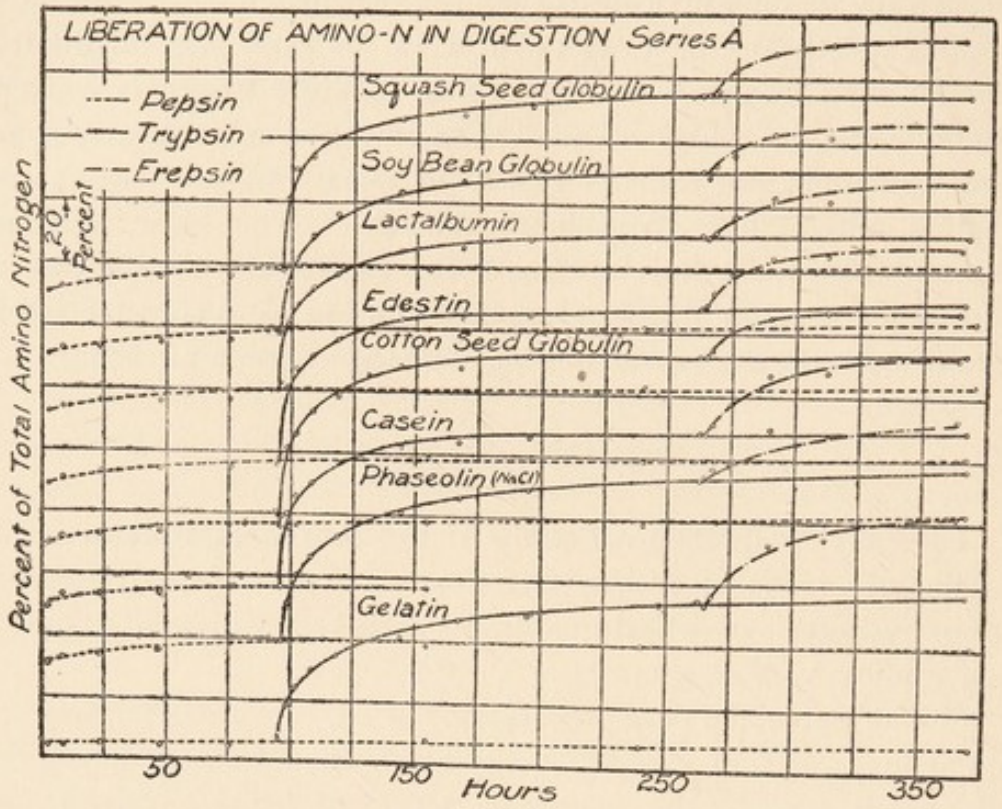


FIG. 6.

(Reproduced by kind permission from J. Biol. Chem., 1916, **26**, 45.)

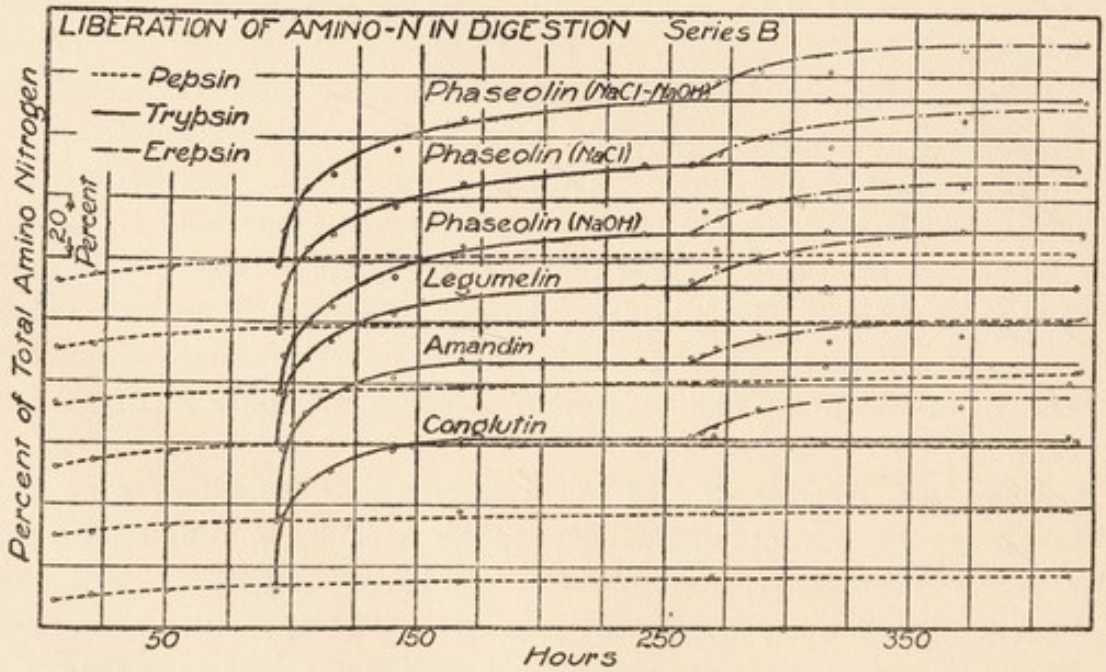


FIG. 7.

(Reproduced by kind permission from J. Biol. Chem., 1916, **26**, 50.)

Erepsin acting on substrates previously digested with pepsin and trypsin brought the hydrolysis up to 85 to 90 per cent. of completion. The rate of hydrolysis under these conditions was relatively slow

compared with the very rapid initial rate when ereptic digestion followed a previous digestion with pepsin and the total hydrolysis

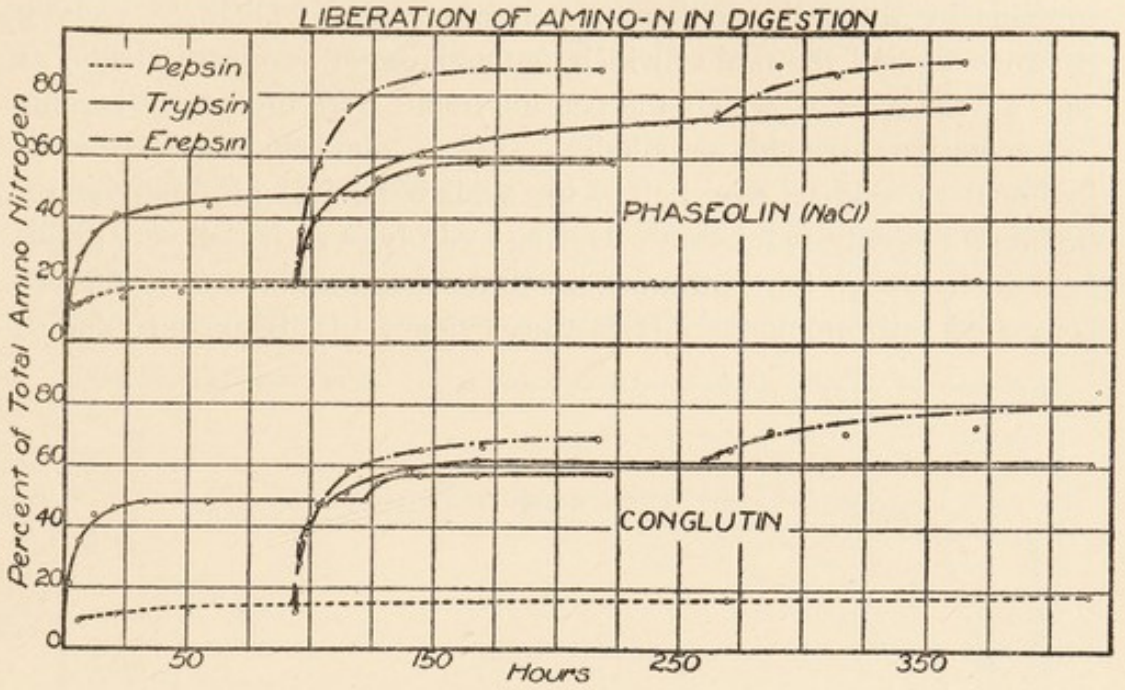


FIG. 8.

(Reproduced by kind permission from J. Biol. Chem., 1916, **26**, 56.)

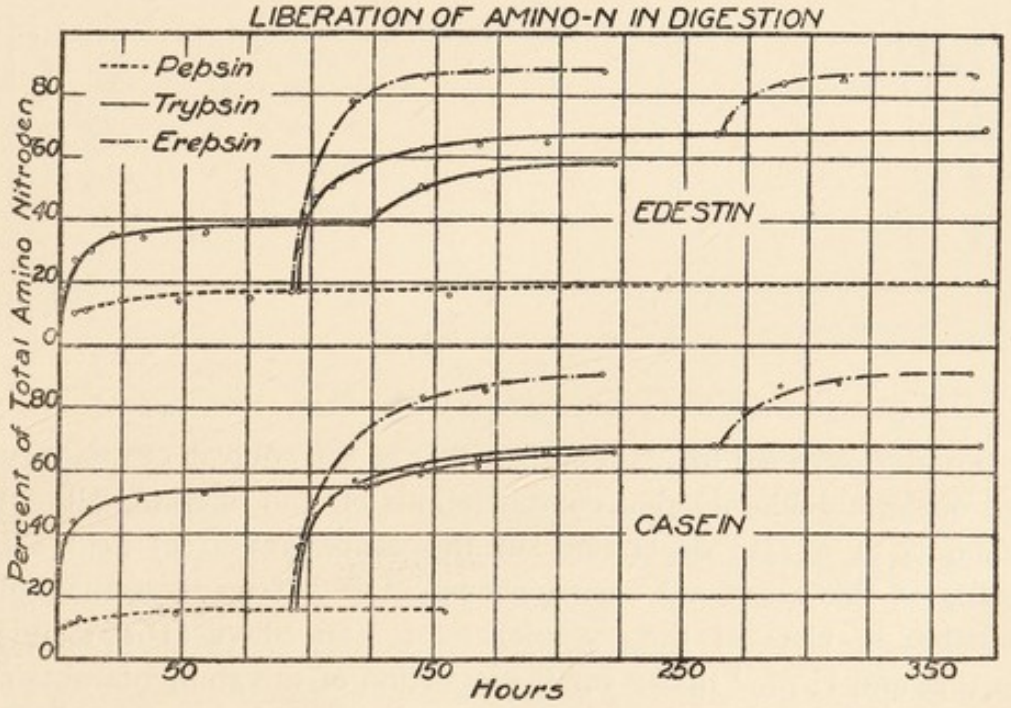


FIG. 9.

(Reproduced by kind permission from J. Biol. Chem., 1916, **26**, 57.)

thus effected reached nearly if not quite the same level as that obtained when the three enzymes acted successively (Figs. 8 and 9).

Although ammonia has long been recognised as a product of the hydrolysis of proteins and probably originates for the most part from

acid amide groupings in the protein molecule, practically no study has been made of the rate at which the ammonia is liberated from the proteins by the action either of enzymes, acids or alkalis. Vickery has investigated the rate at which ammonia is set free from gliadin as well as the rate at which the peptide bonds were broken by various concentrations of acids or alkalis. It was found that even 0·027 N hydrochloric acid set over half of the available amide nitrogen free on boiling for twenty-four hours. During hydrolysis with such very dilute acid the hydrogen ion concentration diminished as amide nitrogen was converted into ammonia. It is thus evident that the acidity due to

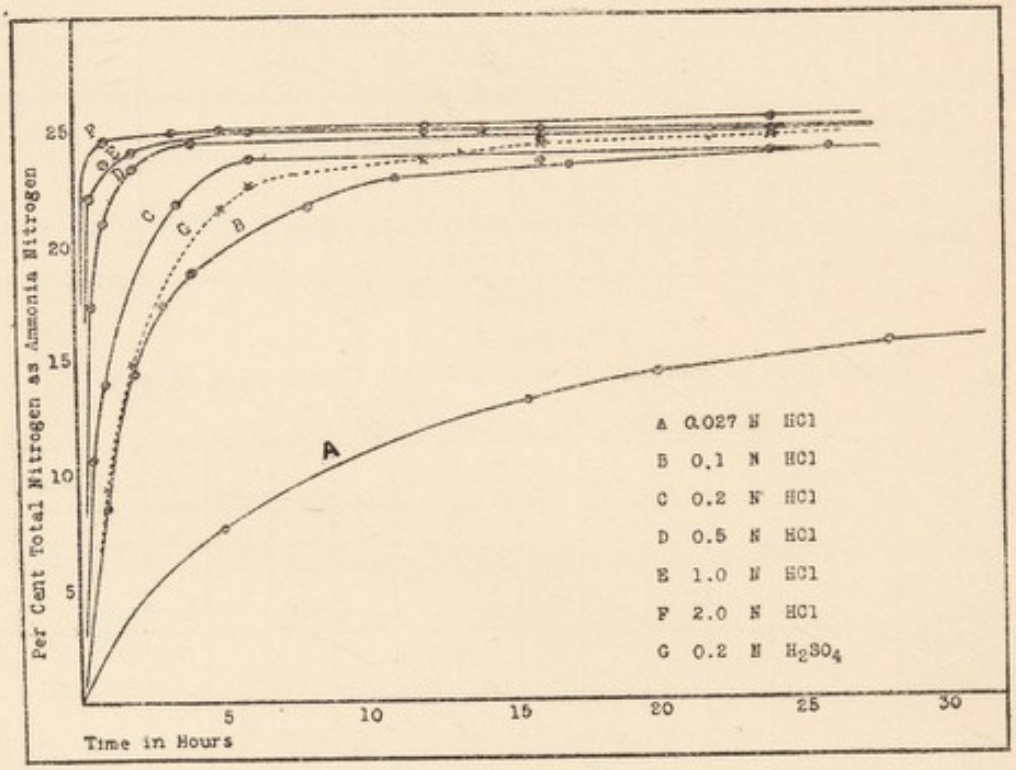


FIG. 10.

(Reproduced by kind permission from J. Biol. Chem., 1922, 53, 508.)

carboxyl groups set free is less than that of the equivalent quantity of hydrochloric acid. Higher concentrations of acid liberated all of the ammonia at a rate depending on the concentration of acid. The results of this work are clearly shown in Fig. 10. It seems highly probable in view of the experience of Van Slyke [1912] and of Gortner and Holm[1] that a small proportion of the ammonia obtained on prolonged acid hydrolysis of proteins has its origin in deamination. Vickery found 25·0 per cent. of the nitrogen of gliadin as ammonia when the protein was boiled forty hours with 0·5 N hydrochloric acid and 25·6 per cent. when boiled for the same time with 20 per cent.

[1] Gortner, R. A., and G. E. Holm. *The Effect of Prolonged Acid Hydrolysis upon the Nitrogen Distribution of Fibrin with Especial Reference to the Ammonia Fraction.* J. Amer. Chem. Soc., 1917, **39**, 2736-2745.

hydrochloric acid. This effect probably furnishes an explanation of the fact that all the curves of Fig. 6 do not reach the same maximum within the limits of the diagram, as well as the discrepancy just noted.

The results obtained when gliadin is boiled with 0·2 N sodium hydroxide [curve A, Fig. 11] show three distinct phases of the reaction. The decomposition of the amide groupings takes place very rapidly and is practically complete within two hours. Ammonia is then evolved at a slower but quite uniform rate for several hours, doubtless representing decomposition of arginine. Finally, a point is reached at which only traces of ammonia are set free. This phase

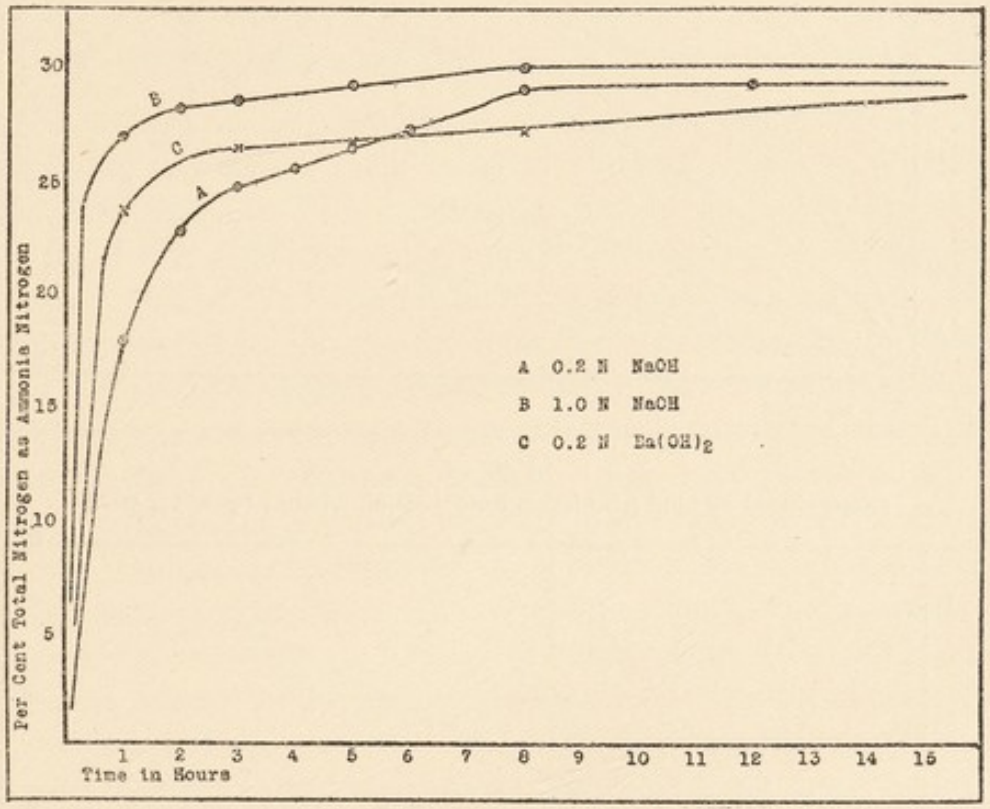


FIG. 11.
(Reproduced by kind permission from J. Biol. Chem., 1922, 53, 509.)

probably represents other secondary decompositions. These three reactions run side by side from the beginning but as their rates are different each becomes in turn evident upon the curve. It is of interest to note that 0·2 N barium hydroxide effects hydrolysis of amide nitrogen more rapidly than does 0·2 N sodium hydroxide but causes less rapid secondary decomposition.

The rate at which the peptide bonds of gliadin are hydrolysed by acids of any concentration presents marked contrasts to those which Frankel observed with enzymes. Only with normal or stronger acids are the rates higher than reported by Frankel for the action of erepsin or trypsin on protein previously treated with pepsin. With the

stronger acids the initial rate of hydrolysis is very rapid but, when about three-quarters of the peptide bonds have been split, the rate is markedly slower and, except with 20 per cent. hydrochloric acid,

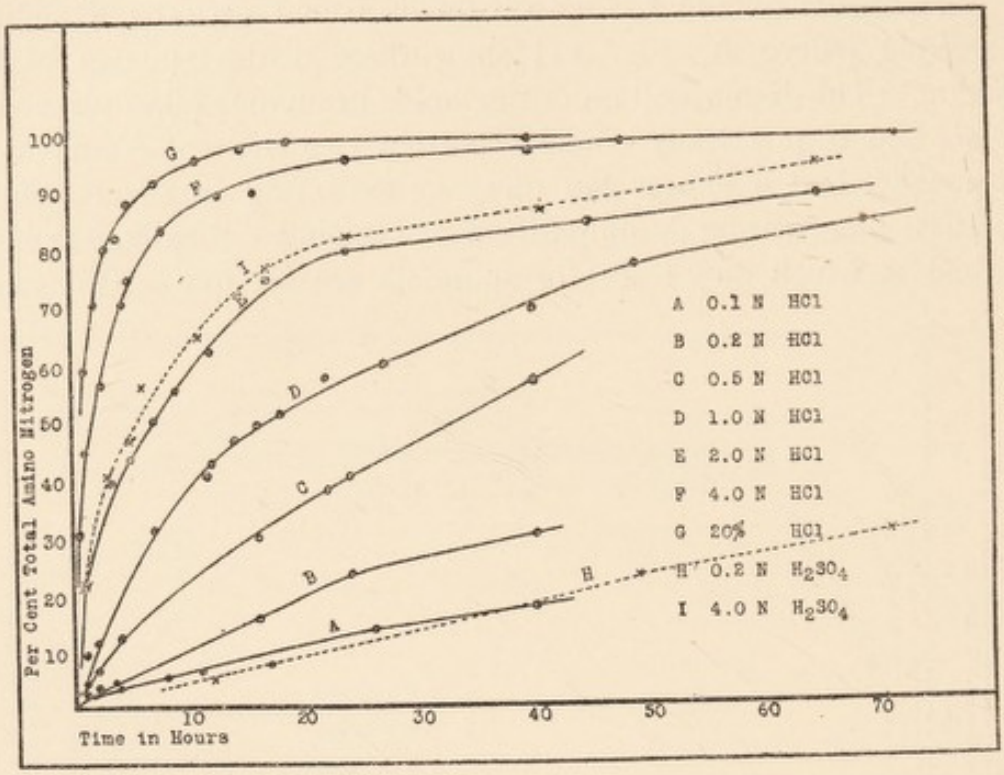


FIG. 12.
(Reproduced by kind permission from J. Biol. Chem., 1922, 53, 509.)

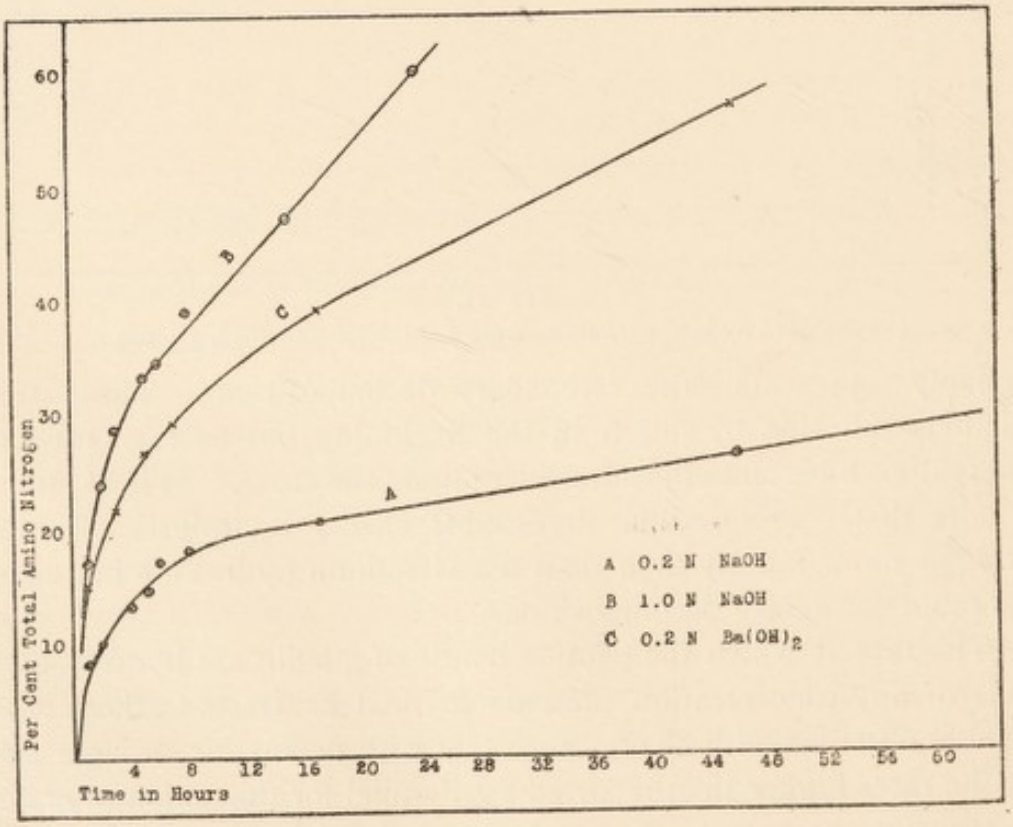


FIG. 13.
(Reproduced by kind permission from J. Biol. Chem., 1922, 53, 510.)

prolonged boiling is necessary to effect complete hydrolysis. With 20 per cent. acid, however, Fig. 12 shows that hydrolysis is practically complete within twenty to twenty-four hours.

The initial rate at which the peptide bonds of gliadin are split by alkalis is more rapid than that caused by acids of equivalent concentration, but subsequently becomes approximately the same. A surprising feature of the curves shown in Fig. 13 is that barium hydroxide hydrolyses the peptide union at a much more rapid rate than does sodium hydroxide of equivalent concentration. No explanation of this observation is at present forthcoming.

F. *The Undetermined Nitrogen of Protein Hydrolysis.*

The nitrogen of the protein, other than the amide and the basic nitrogen, largely exists, so far as is now known, in the form of peptides of *a*-amino-acids. It is not probable that all of the decomposition products of the proteins are yet known, for attempts to determine the amount of each of the known substances has in no case given a result which did not fall far short of the total of the protein. A considerable part of this deficit is doubtless made up of the known substances that are determined, for losses necessarily occur in the processes of isolating and separating them [cf. Osborne and Heyl, 1908, 1 ; Osborne and Jones, 1910, 2]. This loss, however, probably does not account for all of the unknown residue, even assuming the presence of oxyglutaminic acid recently discovered by Dakin. If it is assumed that the amino-acids that are found in a well-conducted analysis of the decomposition products of a protein are united in the molecule with the elimination of water, and that the dibasic acids are united to an amide group which replaces one hydroxyl, and the sum of the percentages of these radicals is subtracted from 100, the percentages of the unknown residue can be approximately estimated. If the nitrogen unaccounted for in the same analysis is calculated as per cent. of this unknown residue, it is found that in the case of gliadin this unknown part contains 13·3, in excelsin 14·0, and in legumin 14·3 per cent. of nitrogen. Among the known mono-amino-acids yielded by proteins this percentage of nitrogen is equalled only by glycocoll, alanine, tryptophan, and serine, even if the calculation is made for the radicals in polypeptide union, that is, after subtracting one molecule of water from their molecular weights. In making this calculation no account is taken of the fact that the amount of the amino-acids isolated in a condition fit for weighing is distinctly less than the quantities actually yielded by hydrolysis. As this loss falls mostly on substances which contain less than 13 per cent. of nitrogen,

the actual proportion of nitrogen in the unknown residue of the protein must be even higher than that indicated by the above calculation. As it is improbable that this unknown residue is wholly made up of undetermined quantities of the four amino-acids above mentioned, it is fair to presume that the proteins contain a considerable amount of some still unknown substance or substances relatively rich in nitrogen [cf. Emil Fischer [1]].

G. Sulphur in Vegetable Proteins.

It has been demonstrated that many proteins yield cystine on hydrolysis, and the probability has become great that much, if not all, of the sulphur of some of them is cystine sulphur. The fact that cystine is a constituent of the protein shows that there must be at least two atoms of sulphur in their molecules.

With the exception of one or two unsatisfactory observations, no proteins have been described which contain no sulphur whatever. Only one carefully studied vegetable protein has yet been obtained which contains so small a proportion of sulphur as to make the existence of sulphur-free protein in any way probable. This protein is vicilin, obtained from several leguminous seeds, some preparations of which have been found to contain as little as 0·1 per cent. of sulphur. The sulphur content of various preparations of vicilin, which were obtained by Osborne and Campbell [1898 1, 2, 3, 4], by fractional precipitation, fell between 0·2 and 0·1 per cent. The accuracy of the determinations in these different fractions was established by repeated closely agreeing determinations, and there can be no doubt that differences in the sulphur content of these fractions actually existed. In view of this variable content in sulphur and of its very small total amount, it is possible that these preparations of vicilin were mixtures of different proportions of sulphur-free and sulphur-containing proteins. In no other case has better evidence of the possible existence of sulphur-free protein yet been obtained.

Numerous attempts have been made to establish a definite ratio between the sulphur which can be split off as sulphide by boiling with alkalis and the total sulphur of the protein. In every case the amount of sulphide sulphur thus obtained was less than the total sulphur, and it was for a long time assumed that this fact showed the presence of two different forms of sulphur in the protein molecule. Experiments with cystine show that not more than two-thirds of the sulphur which this substance contains can be converted into sulphide by boiling with

[1] Fischer, E. *Die Chemie der Proteine und ihre Beziehungen zur Biologie.* Sitzungsber. der königlich preussischen Akademie der Wissenschaften, 1907, 35-56.

alkali, and that, unless special care is taken, the proportion obtained is but little more than one-half. Therefore, unless the proportion of sulphide sulphur is decidedly less than that yielded by cystine, the fact that only a part of the protein sulphur can be converted into sulphide is not necessarily evidence of the existence of two forms of sulphur in the protein molecule.

Determinations of the total sulphur in a number of vegetable proteins have led to constant results, so there is no doubt that these proteins contained definite quantities of sulphur. Determinations, by Schulz' method, of the sulphur of different seed proteins, converted into sulphide by heating with caustic soda, likewise yielded uniform results which are given in the following table, together with similar results obtained with some animal proteins which are introduced for comparison :—

Ratio of Sulphide Sulphur to Total Sulphur.

	Total Sulphur.	Sulphide Sulphur.	Per cent. of total Sulphur as Sulphide.	
Seralbumin	1·930[1]	1·280[1]	66	
Oxyhæmoglobin, dog	0·568[2]	0·335	59	
Serglobulin, horse	1·110[3]	0·630[1]	57	
Gliadin	1·027	0·619	60	
Conglutin, blue lupine I.	0·393	0·262	66	⅔
Conglutin, blue lupine II.	0·359	0·239	66	
Conglutin, yellow lupine I.	0·530	0·344	65	
Conglutin, yellow lupine II.	0·954	0·558	58	
Conglutin, yellow lupine III.	1·378	0·889	64	
Oxyhæmoglobin, horse	0·380[4]	0·190[1]	50	
Vignin	0·426	0·214	50	
Amandin	0·429	0·217	50	
Globin	0·420[1]	0·200[1]	48	½
Glycinin	0·710	0·320	46	
Vicilin	0·200	0·092	46	
Legumin	0·385	0·165	41	
Edestin	0·880	0·346	40	
Zein	0·600	0·212	35	
Ovovitellin	1·028	0·348	34	
Fibrin	1·100[3]	0·380[5]	34	⅓
Excelsin	1·086	0·350	32	
Ovalbumin	1·616	0·491	30	
Phaseolin	0·312	0·072	23	¼
Casein	0·800[6, 7]	0·101	13	⅛

[1] Schulz, F. N. *Die Bindungsweise des Schwefels im Eiweiss.* Zeit. physiol. Chem., 1898, **25**, 16-35.

[2] Jaquet, A. *Beiträge zur Kenntniss des Blutfarbstoffes.* Zeit. physiol. Chem., 1890, **14**, 289-296.

[3] Hammarsten, O. *Ueber das Fibrinogen.* Pflüger's Archiv, 1880, **22**, 431-502.

[4] Zinoffsky, O. *Ueber die Grösse des Hämoglobinmoleciils.* Zeit. physiol. Chem., 1886, **10**, 16-34.

[5] Krüger, A. *Ueber den Schwefel der Eiweissstoffe.* Pflüger's Archiv, 1888, **43**, 244-264.

[6] Chittenden, R. H., and H. M. Painter. *Casein and its Primary Cleavage Products.* Studies from the Laboratory of Physiological Chemistry of Yale University, 1885-1886, **2**, 156-199.

[7] Hammarsten, O. *Zur Frage, ob das Casein ein einheitlicher Stoff sei.* Zeit. physiol. Chem., 1882, **7**, 227-273.

If we assume that the proteins given in the following table contain cystine, and that the amount of sulphide sulphur obtained from them by Schulz' method is equal to only two-thirds of the sulphur actually present in this cystine, we find that the proportion of the total sulphur still unaccounted for is greater than any probable error involved in the analyses by quantities so considerable that we are forced to conclude that many of these proteins contain sulphur in some complex other than cystine.

	Total Sulphur, per cent. of the Protein.	Cystine Sulphur calculated, per cent. of the Protein.	Difference unaccounted for	
			Per cent. of the Protein.	Per cent. of the total Sulphur.
Oxyhæmoglobin, horse	0·380	0·285	0·100	26
Vignin	0·426	0·321	0·105	24
Amandin	0·429	0·326	0·103	24
Globin	0·420	0·300	0·120	29
Glycinin	0·710	0·480	0·230	32
Vicilin	0·200	0·138	0·062	31
Legumin	0·385	0·248	0·137	36
Edestin	0·880	0·519	0·361	41
Zein	0·600	0·318	0·282	47
Vitellin, hen's egg-yolk	1·028	0·522	0·506	49
Fibrin	1·100	0·570	0·530	48
Excelsin	1·086	0·525	0·561	51
Ovalbumin	1·616	0·737	0·879	54
Phaseolin	0·312	0·108	0·204	65
Casein	0·800	0·150	0·650	81

In connection with sulphur in vegetable proteins, attention should be called to the unique protein which Kotake and Knoop [1911] found in the latex of *Antiaris toxicaria*. This was obtained in crystals giving all of the colour reactions of proteins, and contained 7·2 per cent. of sulphur. After hydrolysis cystine equal to 10·6 per cent. of the dry protein was isolated in a state of purity, a quantity far greater than has heretofore been obtained from any known protein.

CHAPTER X.

THE PROTEINS OF GREEN PLANTS.

VARIOUS plants contribute large amounts of protein to the ration of farm animals but practically nothing is known of the chemistry of these proteins, even their proportion not yet being established. It is true that the agricultural chemist states the percentages of protein in green fodders, but his analyses are made by indirect methods founded on assumptions unsupported by satisfactory evidence. A serious gap therefore exists in our current knowledge of the chemistry of nutrition which makes it impossible to apply to the practical problems of feeding on the farm what has been learned of the nutritive value of the proteins of seeds as well as of the protein concentrates.

Our present meagre knowledge of the protein constituents of living plants is chiefly due to the difficulties encountered in separating the contents of the cells from their enveloping walls. Attempts to grind the fresh leaf and extract the contents of the cells with water result in mixtures that cannot be filtered clear, and consequently appear to present no opportunity to obtain the protein in a state fit for chemical examination. Extracting dried leaves with water yields solutions containing only a part of the total nitrogen, and most of this soluble nitrogen does not belong to protein. Such solvents as are usually used for extracting proteins from animal tissues, or from seeds, fail to dissolve much of the residual nitrogen.

In consequence of these facts little chemical evidence has been obtained of the nature of the compounds containing a large proportion of the nitrogen in any plant.

Uno [1902] stated the percentage of protein obtained by coagulating the expressed juices of the leaves, flowers, roots and stems of many species of plants, but, as he gives no evidence of the amount or nature of the protein in this coagulum, his work contributes little to our knowledge of this subject.

The first serious attempt to isolate the protein in quantity from leaves was made almost simultaneously by Osborne and Wakeman

[1920] who used spinach leaves, and by Chibnall and Schryver [1921] who worked with cabbage.

Osborne and Wakeman found that by grinding the fresh leaves in suitable mills and centrifuging at high speed opaque green solutions could be obtained which were free from all suspended particles except those of extremely small size visible only under the higher powers of the microscope. By adding about 20 per cent. of alcohol to these solutions, which contained the greater part of the protoplasm of the cells of the leaves together with the contents of the vacuoles, a voluminous precipitate separated which consisted of protein together with a considerable quantity of other substances previously in colloidal solution.

Chibnall and Schryver found that by treating the ground leaves with water saturated with ether (employed as a cytolytic agent) opalescent colloidal solutions were obtained from which flocculation of the colloid takes place, either on standing at 20°, or more rapidly on warming to 40°. Subsequently Osborne, Wakeman and Leavenworth [1921] showed that, by grinding and pressing fresh young alfalfa plants, it is possible to obtain large quantities of the undiluted juice free from chlorophyll and all suspended particles. This juice is a yellow brown colloidal solution, transparent in thin layers by transmitted light, but opaque and almost black by reflected light. On the addition of about 20 per cent. of alcohol a voluminous precipitate forms which contains nearly all of the protein and other colloids in the juice. This precipitate, which when washed and dried at 107° contains about 11 per cent. of nitrogen and 12 per cent. of ash, is a mixture of protein, calcium salts of both phosphoric and organic acids and also of colouring substances, possibly related to the flavones. When treated in the moist state with 75 per cent. alcohol containing 0·1 per cent. hydrochloric acid, about one-fourth dissolves, none of which is protein, but most of which is calcium, phosphoric acid and organic substances. About four-fifths of the nearly nitrogen-free solids thus dissolved are precipitated by neutralising, nearly 90 per cent. being inorganic.

Dilute aqueous hydrochloric acid likewise dissolves none of the protein, notwithstanding the fact that this is thereby converted into a hydrochloride containing about 3·5 per cent. of hydrochloric acid. Subsequent extraction with boiling absolute alcohol removes a notable quantity of a very dark red-brown colouring matter which is readily soluble in absolute alcohol. When the solution of this colouring matter is poured into distilled water a colloidal solution results which does not settle out over night, but separates at once on adding a small

amount of hydrochloric acid and sodium chloride. The residue after thus extracting with aqueous hydrochloric acid, ether and alcohol contains 0·62 per cent. of ash and the ash-free substance 14·6 per cent. of nitrogen, of which 6·6 per cent. is converted into ammonia by hydrolysing with hydrochloric acid. It also contains 0·83 per cent. of phosphorus and 0·95 per cent. of sulphur. When the above moist residue is suspended in water it settles very slowly and incompletely, but separates immediately as a coarse flocculent precipitate when dilute sodium hydroxide solution is carefully added to the isoelectric point. The clear filtrate from this precipitate contains sodium chloride equal to the sodium hydroxide added, and equivalent to 3·5 per cent. of hydrochloric acid in the solids thus treated. Deducting this combined acid the residue contains nitrogen equal to 15·1 per cent. These solids, consequently, consist chiefly of protein hydrochloride.

When this is suspended in water at the room temperature none is dissolved, as shown by the absence of a biuret reaction in the filtered solution. At temperatures approaching 100°, however, it is converted into a clear yellow jelly which does not pass into a true solution, even after long heating. After cooling, the addition of an excess of hydrochloric acid, or of sodium chloride, converts this jelly into a coarse flocculent precipitate resembling an ordinary protein coagulum, such as is commonly produced by heating. This settles rapidly, leaving the solution clear. A little colouring matter is thus removed, as shown by the strong yellow colour of the filtered solution when made alkaline. An excess of sodium hydroxide above that required to neutralise the combined acid produces a viscid, deep yellow colloidal solution which is very difficult to filter even when highly diluted, and though appearing almost clear is quite opaque in a beam of sunlight. The precipitate produced by acidifying contains the same percentage of nitrogen as that of the substance from which it was derived.

The behaviour of this product towards acids and alkalis is so unlike that of most native proteins as to suggest that it is a combination of protein with some as yet unidentified complex. This view is supported by the fact that, like the corresponding product from the spinach leaf, when it is boiled with 60 per cent. alcohol containing 0·3 per cent. sodium hydroxide the combination is apparently hydrolysed, since after the protein is precipitated with acid its nitrogen content is raised to 16·3 per cent. As this precipitate is then readily soluble in an excess of either acid or alkali and the solution from which it separates contains a notable quantity of colouring matter, it is possible that the protein in the original colloid precipitate is combined with

the coloured complex. It is likewise probable that the protein moiety suffers from the action of the hot alkali and that some of its amide nitrogen is converted into ammonia. This decomposition, however, is not so great as one would expect since the percentage of the total nitrogen converted into ammonia by complete hydrolysis with acids is reduced by this treatment only from 6·6 to 5·9 per cent. The fact that only 10·3 per cent. of the total nitrogen of the original product is found in the filtrate from the precipitate produced by acidifying the hot alkaline alcohol solution is further evidence that the protein moiety of this product is not hydrolysed by the hot alkali to any great extent.

The alfalfa protein thus prepared gives the following results on analysis :—

	Protein. per cent.	Nitrogen. per cent.
Ammonia nitrogen	0·96	5·86
Humin ,,	0·60	3·67
Basic ,,	3·76	22·98
Non-basic ,,	11·04	67·49
Total nitrogen	16·36	100·00

	Protein. per cent.		Protein. per cent.
Tyrosine	3·19		
Histidine	2·56	(containing N = 0·69)	
Arginine	7·11	(,, ,, = 2·29)	
Lysine	3·34	(,, ,, = 0·64)	
Total nitrogen			3·62

The results of this investigation of the alfalfa juice show that the colloid precipitate produced by adding 20 per cent. of alcohol consists chiefly of three groups of substances, each having pronounced colloidal character, namely, calcium phosphates, colouring matters, and protein, the latter probably combined with a coloured complex and consequently belonging to the group of conjugated proteins.

The filtrate from the colloid precipitate contains much nitrogen but very little protein, probably less than 1 per cent. of the solids of the plant. Some of this protein can be coagulated by heating the acidified solution, but more of it has the properties characteristic of proteoses. Neither of these protein fractions have been studied.

After removing the water-soluble constituents of the alfalfa 6·4 per cent. of its solids and 2 per cent. of its nitrogen are dissolved by extracting with strong alcohol. This extract contains no protein but all of the chlorophyll together with other substances, the nature of which has not yet been learned.

The plant residue which remains after the above extractions with

water and alcohol contains more than half of the nitrogen of the plant.

About 14 per cent. of this residual nitrogen can be extracted by dilute aqueous solutions of sodium hydroxide at room temperature, but only a part of this separates on acidifying. This precipitate contains both protein and pentosans. By boiling the plant residue with 60 per cent. alcohol containing 0·3 per cent. sodium hydroxide most of the remaining nitrogen can be extracted, leaving a residue of plant fibre equal to 32 per cent. of the original dry solids of the alfalfa but containing only 5·6 per cent. of its nitrogen.

The cautious addition of acid to the alkaline alcohol extract precipitates protein containing 60 per cent. of the dissolved nitrogen. Some, if not all, of the remaining 40 per cent. of the nitrogen may have been derived from the same protein as that yielding the precipitate, its solubility being due to hydrolytic changes caused by the hot alkali.

This protein remaining in the residue after extraction with water and alcohol behaves much like that found in the colloid which is precipitated by alcohol from the juices of the plant. That it too may be combined with the flavone-like pigment is indicated by the liberation of a relatively large proportion of the latter simultaneously with the protein when the plant residue is heated with alkaline alcohol.

Whether the colloid precipitate produced by adding alcohol to the expressed juice of the alfalfa plant is to be considered as a constituent of the contents of the vacuole and sap or simply as a part of the protoplasm which has been dispersed in these fluids by grinding in the mill, cannot be decided from such data as we now have. Evidence that the latter supposition may prove true is given by experiments recently made by Chibnall [1923] in the author's laboratory. Spinach leaf cells were plasmolysed by means of ether, and pressed out in the hydraulic press. The pressed residues were then allowed to imbibe water and subsequently pressed. This operation does not rupture the leaf cells and yields a brown juice which has a slight Tyndall effect. On adding about 30 per cent. of alcohol a white precipitate separates containing 32 per cent. of ash and only 2·4 per cent. of nitrogen, in contrast with over 10 per cent. found in the colloidal precipitates derived from either spinach or alfalfa leaves by the method previously described. After distilling out the alcohol at a low temperature, the filtrate gives a coagulum on boiling which contains about 14·5 per cent. of nitrogen equal to about 1·5 per cent. of the total leaf nitrogen. Presumably this represents either globulin or albumin. When the

cells of the residue from which the water-soluble constituents have been thus removed are ruptured by grinding with water, the colloidal substances become dispersed in the water and are thus readily removed from the solid residue by pressing. The expressed deep green fluid when passed through a felt of paper pulp is dark brown and when treated with a little acetic acid yields a flocculent precipitate. When this is washed with alcohol and then with ether it forms a nearly white powder which on drying contains 14·9 per cent. of nitrogen and only 1·7 per cent. of ash. The ash-free substance therefore contains 15·2 per cent. of nitrogen.

Although sufficient data are not yet available for a discussion of the relations of the fluid thus obtained from the plasmolysed cells to the fluids obtained by grinding the fresh plant directly, we seem justified in concluding that the much larger quantity of precipitate produced by alcohol under the latter conditions originates from the protoplasmic matter of the cells and that the substances in the filtrate in both cases represents the contents of the vacuoles. It thus appears that Chibnall's method yields the protein of the protoplasmic lining of the cells of the leaf in a much purer condition than when the cells are ruptured by grinding before removing the water-soluble constituents of the vacuole, and therefore offers a better opportunity for studying these two parts of the cell than has heretofore been available.

The results obtained by Chibnall and Schryver in their studies of the cabbage, scarlet runner bean, and spinach plants show that the proteins in the leaves of these plants are similar in their general properties to those of the alfalfa plant.

It is too early to generalise concerning the proteins of green plants, but it would appear from what is now known that relatively very small quantities of albumins, globulins or proteoses are present in the juice, and that most of the protein of the plant occurs as a hitherto unrecognised type which is unlike any of the native proteins thus far described in its behaviour towards acids or alkalis.

CHAPTER XI.

THE NUTRITIVE VALUE OF THE VEGETABLE PROTEINS.

THE recognition of the wide differences in the chemical constitution of the individual proteins obtained from vegetable sources raised the question of their relative value in nutrition, about which little was known at the time the first edition of this monograph was written.

All previous attempts to feed isolated proteins in supposedly adequate artificial mixtures of purified foodstuffs had failed because these were never eaten in sufficient quantity for more than a short time. After Osborne and Mendel [1911, 1] discovered that by using "protein-free milk" foods could be made containing one or another purified protein, on which, for the first time, not only adult rats were maintained but young ones grew at a normal rate for several weeks, it became possible to compare the relative nutritive value of different proteins in a way fairly satisfactory.

These experiments fall into two groups, one dealing with the individual proteins, fed as prepared in the laboratory, the other with mixtures of proteins as they occur in seeds, or in the parts of seeds used for feeding men or animals. These groups should be considered separately.

The experiments with the pure proteins likewise fall into two groups: (*a*) those designed to show whether the protein in question is alone adequate for maintenance or growth; and (*b*) those designed to show how little of it is needed, either to maintain the animal, or to promote its growth at a normal rate.

Experiments of the type of group (*a*) have definitely proved that the nutritive value of a protein, or of a mixture of proteins, is determined by the minimum quantity of any one essential amino-acid which they yield on digestion, but they do not give any information respecting the *relative* value of different proteins which yield some of each of the essential amino-acids. The results must be regarded simply as qualitative, not quantitative.

Group (*b*) involves the *quantitative* relations of the proteins in nutrition. If the relative nutritive value of different proteins is to be

established, it is necessary to know how much of each protein is needed to secure a normal rate of growth. For this purpose experiments of a type entirely different from those just described must be made.

The effect of like quantities of two or more proteins on the rate of growth must be compared. In conducting such experiments difficulties are encountered which have often misled those who failed to appreciate the experimental limitations.

In respect to maintenance experiments designed to show whether or not any individual amino-acid is essential, no decisive experiments have ever been made, except those with zein and tryptophan. Such experiments relate to the physiological requirements of the animal and are of so little importance in respect to the relative nutritive value of the proteins, which is the subject of this chapter, that they will be dismissed without further discussion.

In regard to growth the situation is different, for here we have evidence that notable amounts of essential amino-acids must be available before their presence is revealed by growth. Thus we now know that gliadin yields nearly one per cent. of lysine, yet when young animals are fed on an otherwise adequate diet containing 18 per cent. of this protein and the water-soluble vitamin is supplied daily by tablets containing 30 milligrammes of a yeast fraction (a dose demonstrated to be ample for normal growth and containing less than 3 milligrammes of nitrogen) they do not grow at all. The lysine furnished by the gliadin is no more than is needed for maintenance. Furthermore, when the diets containing 28 per cent. of "protein-free milk," 18 per cent. of zein and 0·5 per cent. of tryptophan are fed no growth whatever can be made until lysine is added, thus proving that whatever quantity of lysine may be yielded by the "protein-free milk" it is no more than enough for maintenance. When young rats are fed on diets containing 28 per cent. of "protein-free milk," 13·5 per cent. of zein and 4·5 per cent. of casein, which yields at least 1·5 per cent. of tryptophan, almost no growth is made until more tryptophan is added. They then grow very rapidly, thus demonstrating that the casein furnished very little more tryptophan than is necessary for maintenance [1914, 1].

The experiments just cited clearly demonstrate that the "protein-free milk," even when fed in such large proportion of the food, does not furnish enough of either tryptophan or lysine to affect the experiments by which a deficiency in these two essential amino-acids has been regarded as demonstrated.

The difficulties encountered in experiments with "protein-free milk" have long been recognised by Osborne and Mendel who for this reason some years ago abandoned the use of this source of water-soluble vitamin in favour of dried brewery yeast or extracts derived therefrom which can be fed in small tablets apart from the food. In this way the amount of nitrogen in the vitamin-bearing addition to the diet is reduced, but the use of yeast is subject to criticisms of the same type as those applying to "protein-free milk," though with less force.

Although the relative nutritive values of individual proteins have not yet been demonstrated beyond criticism, the outcome of some of the experiments has not been seriously affected by the difficulties just discussed, while that of others has given a better basis for comparison than have the results of the old time methods based on nitrogen equilibrium. The available data will therefore be reviewed in the following pages with the understanding that the reader will keep in mind the shortcomings of the methods by which they have been obtained and not ascribe to them an importance which is undeserved.

The proteins, thus far fed, have been obtained from three groups of plants, namely the cereals, legumes and some other species whose seeds are rich in oil.

Nearly one-half of the total proteins of the maize kernel is zein which contains no glycocoll [Osborne and Clapp, 1908, 2], tryptophan [Osborne and Harris, 1903, 6], lysine [Osborne and Leavenworth, 1913], oxyproline or isoleucine [Dakin, unpublished communication]. Osborne and Mendel [1914, 1; 1916, 1] found that rats of all ages rapidly declined and died on diets containing about 15 per cent. of their calories in the form of zein unless tryptophan, equal to about 0·5 per cent., was added to their food. Little, if any, decline in weight then occurred even during 100 days, but no growth took place until a similar quantity of lysine also was added. Inasmuch as growth was then at a nearly normal rate we are justified in assuming that the qualitative deficiencies of zein are made good by tryptophan and lysine, since these two amino-acids were the only variables in the diets.

Unless the unknown constituents of the "protein-free milk" furnished sufficient glycocoll, oxyproline and isoleucine, to enable the young rats to grow at a nearly normal rate, which seems at best very doubtful, we can conclude that these three amino-acids are not essential for growth. If future experiments made with some concentrated preparation of the water-soluble vitamin should confirm the above conclusion the nutritive value of the other amino-acids and the conception

of the amino-acids serving in nutrition as "building stones" would be questionable.

Similar experiments with gliadin, which yields a very small amount of lysine [Osborne and Leavenworth, 1913], and also with hordein from barley, which likewise yields but little lysine [Johns and Finks, 1919], showed that these two proteins furnish enough tryptophan for maintenance but not enough lysine to permit growth. Hogan [1918] has shown the same to be true for kafirin from sorghum, but this protein is also deficient in cystine.

The prolamins of these cereal grains can be supplemented also by combining them with other proteins containing the lacking amino-acids as effectively as by adding the amino-acid itself. It is for this reason that normal growth can be made when the total protein of these seeds is fed in sufficient amount [Osborne and Mendel 1920, 1] since, besides the prolamins, these seeds contain also a nearly equal amount of a protein belonging to the group of glutelins, each yielding the amino-acids in which the accompanying prolamin is deficient. Thus, Osborne and Mendel [1912, 1] have shown that young rats can grow at a normal rate when either maize or wheat glutelin forms the sole protein of the diet with the exception of the small amount present in the "protein-free milk" which served as the source of water-soluble vitamin.

Besides the prolamins and glutelins each of these seeds of the cereals contains a small proportion of other proteins concerning the nutritive value of which, in their isolated state, nothing is yet known.

The proteins obtained from leguminous seeds present especial interest. While nearly all of these, so far as known, are not wholly deficient in any essential amino-acid, their purified preparations fail to promote normal growth, even when fed at high percentage levels.

The first observation of nutritive deficiencies of proteins from leguminous seeds was made by Osborne and Mendel [1911, 1; 1912, 1] who found that when phaseolin, which constitutes the chief protein of the kidney bean, *Phaseolus vulgaris*, was fed together with "protein-free milk" young rats lost weight, but regained this rapidly when the phaseolin was replaced by edestin, or by casein. They later found that when the phaseolin had been cooked with water, or dissolved in alkali and reprecipitated by acid, the young animals, while maintained for many weeks, gained very little in weight. Osborne and Mendel (unpublished) also found that the cooked phaseolin was better utilised by the animal than the raw. Waterman and Johns [1921] later attributed the improvement in nutritive value after heating this protein

with water to the greater digestibility which they observed in vitro. Johns and Finks [1920] found that by adding 0·36 per cent. of cystine to the food containing 18 per cent. of cooked phaseolin the diet was rendered adequate for growth at a normal rate.

Phaseolin contains about 0·3 per cent. of sulphur, the exact amount being still a matter of doubt [cf. Osborne, 1902, 3]. Of this approximately one-fourth, equal to 0·07 per cent., is converted into sulphide by heating with alkalis. Casein contains 0·8 per cent. of sulphur, only one-eighth of which can be converted into sulphide. If the cystine content of a protein can be roughly estimated from the sulphide sulphur, assumed to be equal to two-thirds of the total sulphur of cystine, phaseolin and casein respectively contain about 0·4 and 0·6 per cent. of this amino acid. If the failure of rats to grow on the phaseolin diet is due to a deficiency in cystine, as it certainly appears to be, it is surprising that normal growth should be made when a like quantity of casein is substituted for the phaseolin. There are two possible explanations for this; one that the cystine in phaseolin is combined with other amino-acids in a peptide union unhydrolysable by the enzymes of the digestive tract; the other, that the sulphur of the casein which is not converted into sulphide by heating with alkalis serves as a source of sulphur as does cystine itself. It may be that cystine does not function simply as an amino-acid but rather as a source of available sulphur.

Although the proteins of the adsuki bean, *Phaseolus angularis*, closely resemble those of the kidney bean [Osborne and Campbell, 1897, 5; Osborne and Harris, 1903, 1; Jones, Finks and Gersdorf, 1922], Johns and Finks [1921] found that when 18 per cent. of the uncooked isolated globulin from this seed was fed with "protein-free milk" young rats grew at about two-thirds of the normal rate, and when 0·36 per cent. of cystine was added to the food the rate of growth was normal.

According to Johns and Finks [1918] most of the protein of the Chinese velvet bean, *Stizolobium niveum*, consists of the globulin, stizolobin, which can be precipitated either by dialysing or by heating sodium chloride extracts to boiling. Young rats rapidly lose weight when fed on diets containing 18 per cent. of the preparations obtained by dialysis, but grow normally on those containing preparations made by boiling [Finks and Johns, 1921].

Conglutin from the seeds of the yellow lupin, *Lupinus luteus*, contains about 0·5 per cent. of sulphur, two-thirds of which, like that of cystine, is converted into sulphide by heating with alkali [Osborne,

1902, 3]. Nevertheless, Osborne and Mendel [1912, 1] found that conglutin merely suffices to maintain young animals without growth. Later unpublished experiments made with otherwise adequate diets including the fat-soluble vitamin gave no better results. A diet containing 9 per cent. of conglutin and an equal quantity of phaseolin, both of which had been heated with water, also failed to promote growth.

They also found [1912, 1] that legumin from the garden pea, *Pisum sativum*, sufficed for slow growth when fed at an 18 per cent. level, and that growth was distinctly better after the raw protein had been replaced by cooked. Legumin from vetch seeds, *Vicia sativa*, under similar conditions of feeding, led to slightly better growth than did that from the pea, while later unpublished experiments showed that heating with water did not improve the growth-promoting properties of this protein. Analyses of the faeces of rats fed on this protein showed them to contain only 10 per cent. of the ingested nitrogen, from which it appears that the legumin was as well utilised as is the average food protein.

When vignin from the cow-pea, *Vigna sinensis*, was the sole protein of the diet the animals grew very slowly for many weeks, but when the vignin was heated with water growth was more rapid.

Legumelin, found in small proportion in several leguminous seeds by Osborne and Campbell [1898, 5], is distinctly different in properties and constitution from the other proteins with which it is associated. These differences are reflected in its nutritive properties, if the fact that fairly rapid growth was made on a diet containing "protein-free milk" and 18 per cent. of a preparation obtained from the pea can be accepted as characteristic of legumelin from the seeds of other species.

Unfortunately the experiments with legumin, vignin and legumelin were all made before the need of the fat-soluble vitamin was recognised, but, as normal growth was made under similar conditions when equal quantities of other proteins were fed, it is improbable that the outcome of these experiments was affected thereby.

Sure [1920] showed that rats fed on a low calorie diet containing 18 per cent. of arachin, the chief protein of the pea-nut, *Arachis hypogea*, grew very little during several weeks, even when cystine, tryptophan, the "leucine fraction" of amino-acids and l-proline were added. No better growth was made when the rats were fed on a mixture of arachin and conarachin, in the proportion in which these two globulins are extracted from the pea-nut by salt solution. In these experiments the alcoholic extract of ether-extracted wheat

germs was used as a source of water-soluble vitamin. The mixed globulins extracted from the pea-nut by salt solution, as well as preparations obtained by extracting the entire seed with alkali and precipitating with acid, have likewise proved inadequate for growth when fed in the author's laboratory with "protein-free milk" and butter fat as sources of vitamins. Rapid growth, however, occurred as as soon as diets were fed which contained an equal amount of protein supplied by oil-free pea-nut meal (unpublished). This experience agrees with that of Daniels and Loughlin [1918].

Osborne and Mendel [1912, 1] showed that normal growth is made by young rats fed on diets containing "protein-free milk" and 18 per cent. of edestin, cucurbitin, excelsin or the globulin from the cotton-seed. Johns, Finks and Paul [1919] found the same to be true for the globulin from coconut presscake, and that it was equally efficient when 2 per cent. of yeast was included in the food, instead of 28 per cent. of "protein-free milk."

What has been said respecting the uncertainties pertaining to attempts made to compare two or more isolated proteins applies with even greater force to comparisons respecting the total protein contained in different natural food products. No seed is known which does not contain several different proteins and in every case examined it has been found that seeds containing a protein deficient in one or more amino-acids also contain another protein in which these amino-acids are present. It is therefore obvious that we cannot conclude that because a considerable part of the protein isolated from a given seed fails to meet the nutritional needs of a growing animal that the mixture of protein in the entire seed is inferior. Unfortunately it has not been possible to obtain from any seed a preparation which strictly represents its total proteins in the proportion in which these naturally occur. It is therefore necessary to prepare diets which include the entire seed in question as the sole source of protein. Many experiments have been made with such foods, but these involve the introduction of relatively large amounts of non-protein substances which are not alike for any two vegetable food products. The results, therefore, cannot be accepted as necessarily representing the influence of the protein alone on nutrition. In so far as such experiments are planned to show the food value of the entire seed, or other vegetable product, they may yield useful results and in many cases give an approximate idea of the relative value of the proteins in some of them. They may also indicate the advantages to be gained by combining two or more food products with the purpose of supplementing the

amino-acid deficiencies of the proteins of one with the amino-acid excesses of those of the other.

Numerous experiments have been made to show the relative food value of proteins by feeding many kinds of seeds, singly and in combination with one another as well as with various animal products. In the reports of these it has been assumed that all but a negligible part of the nitrogen of the food belongs to protein, which certainly for most of the vegetable products used is not true.

McCollum[1] attaches great significance to the records of reproduction, and ascribes the lack of fertility and infanticide, which occurs with great frequency among the females, to deficiencies in the protein of the rations on which they had been fed.

In view of the importance attached to these features of such experiments, especially those made with vegetable proteins, it seems advisable to point out that Osborne and Mendel [1919, 2] as well as others[2] have shown that rats can grow to full adult size at the normal rate on artificial diets and yet be infertile, as shown not only by attempts to mate them without success, but also by microscopic examination of the ovaries and testicles. Such results have been obtained with casein, which previously had been considered to be adequate for normal nutrition during growth. While absolutely convincing proof has not yet been obtained that the infertility observed under these conditions of restricted feeding is not due to the protein of the diet, there is much evidence that some other, as yet unrecognised, factor is the cause of this abnormal development.

Since in the reports of experiments made by many investigators data are lacking to show how much protein was eaten to make a given gain in weight, the author is unable to review intelligently an important part of the literature dealing with the nutritive value of the proteins *in situ*.

In this connection the reader is referred to a discussion of the difficulties encountered by Osborne and Mendel [1918] in attempting to compare the nutritive values of concentrates designed to contain the total proteins of barley, oats, rye, or wheat, and [1920, 1] in feeding the entire seeds of each of these cereals as the sole source of protein. The results of the latter experiments indicated that, expressed in grammes of gain per gramme of protein eaten, the proteins of barley

[1] McCollum, E. V. *The Newer Knowledge of Nutrition.* New York, The Macmillan Co., 1922, 449 pp.

[2] Evans, H. M., and K. S. Bishop. *On the Existence of a Hitherto Unrecognised Dietary Factor Essential for Reproduction.* Science, 1922, **56**, 650-651.

and oats are of about equal value in promoting growth and are somewhat superior to those of rye or wheat, and also that the total proteins of each of these seeds is more efficient than has generally been supposed.

Osborne and Mendel [1919, 1] found that when the entire wheat kernel, containing about 10 per cent. of protein [N × 5·7], was fed as the sole source of protein, together with butter-fat and a suitable mixture of inorganic salts, 20 milligrammes of protein each week per gramme of body weight were insufficient for prolonged maintenance of most adult rats, and that in general about 23 milligrammes were required.

To prepare an adequate diet from the entire grain, containing enough protein for the normal growth of the young, it was necessary to mix 92 per cent. of the ground seed, 5 per cent. of butter fat, and 3 per cent. of the salt mixture with water and bake the dough into hard cakes. As this food, containing about 9 per cent. of protein, was much lower in calorific value than the fat-rich mixtures used in the other experiments, the rats ate about 50 per cent. more of it by weight than of the latter, and thus actually consumed as much protein as did rats on the fat-rich diets containing 15 to 17 per cent. of protein. This shows how misleading may be conclusions based on the percentage of protein in the food when nothing is known of the amount eaten.

On this diet vigorous growth was made during many months and several litters of young were produced. Unfortunately the actual amount of protein eaten through the entire period could not be determined because the rats frequently scattered the dried food. Nevertheless the consumption of food was ascertained at frequent intervals, from which it was clear that intake was high and consequently the protein is not of relatively superior quality. More precise data respecting protein from this important source should be obtained if possible.

Osborne and Mendel [1919, 1] attempted to compare the relative values of the protein in the different parts into which the wheat kernel is usually separated in the process of milling. They found that a weekly intake of 23 to 26 milligrammes of the proteins of patent flour per gramme of body weight was needed to maintain adult rats, whereas when one-fifth of the protein was replaced by the protein of extracted beef muscle only 16 milligrammes sufficed. In this connection the experiments made by Sherman [1920] are of interest, because they not only indicate that wheat flour satisfies the maintenance requirements of man in much the same way as it meets those of

the rat, but also that a relatively small proportion of the proteins of milk increase the maintenance value of the flour proteins in the same way as those of muscle tissue did in the experiments just cited.

Since the patent flour contained too little protein to permit the preparation of a suitable diet containing a sufficient amount of the fat-soluble vitamin and inorganic salts, the protein content was increased by adding dried and finely ground gluten obtained from the same lot of flour. When young rats were fed on a food mixture containing 14·8 per cent. of protein derived wholly from patent flour, together with 9 per cent. of butter-fat and 4 per cent. of a suitable salt mixture, growth was very slow and the rate was not appreciably accelerated when a yeast fraction rich in vitamin B was fed, apart from the food.

The embryos of the wheat kernel appear to contain protein of much better quality than does the endosperm, if such a conclusion can be drawn from experiments made with a commercial preparation containing approximately 77 per cent. of its nitrogen from this part of the seed, 14 per cent. from the bran, and 9 per cent. from the endosperm. With protein (N × 6·25) from this source adult rats were well maintained for long periods on a weekly intake of 16 to 20 milligrammes per gramme of body weight.

Young rats grew at a normal rate and reproduced on a diet containing only 7 per cent. of protein from this commercial product, making an average gain of body weight equal to 1·6 grammes per gramme of protein eaten.

Bran contains so much indigestible matter that only one rat ate enough of the food to enable it to grow well. This animal made an average gain of 2·05 grammes of body weight per gramme of crude protein eaten (i.e. N × 6·25), despite the fact that over 20 per cent. of the ingested nitrogen was voided in the fæces.

No comparable experiments have been made with the maize kernel, but Hogan [1916] has shown that rats can make good growth on maize meal supplemented with an adequate salt mixture. Hogan gives no data respecting the proportion of protein in this diet, but since maize meal usually contains less than 10 per cent. of protein, it is evident that this diet was comparatively low in protein. It thus appears that the amino-acid deficiencies of zein, which constitutes nearly one-half of the total protein of this seed, are supplemented by its other proteins so well that when enough of the meal is eaten normal growth can be made by rats. Hogan found, however, that swine did not grow appreciably on this food mixture but made rapid growth when casein

also was added. Whether this was because the diet contained a higher percentage of protein or because the casein supplemented the maize proteins is not clear, but the fact that a suitable proportion of the latter proteins proved adequate for the growth of rats indicates that the failure of the pig to grow was due to too small an intake of protein. This view receives support in the observation made by McCollum, Simmonds, and Pitz [1916], who state that pigs can grow well when fed a mixture of 70 per cent. maize meal and 30 per cent. maize "gluten feed" supplemented by a suitable salt mixture.

Hogan well attributes the apparent discrepancy between the outcome of experiments with rats and pigs on this diet to the great difference in the size of the two species, in consequence of which much more protein per unit of body weight is eaten by the rat, a fact which must always be kept in mind in comparing the nutritive value of proteins when fed to animals of markedly different size.

Johns, Finks and Paul [1920] have also shown that the proteins of maize meal when fed at a sufficiently high level suffice to promote normal growth of rats. On a diet containing

	Per cent.	
Gluten meal[1]	53 =	19·4 per cent. protein
Whole ground maize	18 =	1·5 " "
Salt mixture	4	
Butter-fat	18	
Lard	7	

normal growth was made, the water-soluble vitamin being furnished by the ground seed. In view of the fact that Osborne and Mendel [1914, 2] were unable to secure more than very slow growth when the total protein of the diet, equal to 17 per cent., was furnished by maize "gluten meal" and the water-soluble vitamin by "protein-free milk," the apparent supplementing effect of the relatively small proportion of whole maize meal used in the experiment just described is surprisingly good.

Maize gluten, the proteins of which are derived from the endosperm of the seed, and which is extensively used on the farm as a protein concentrate, was rendered efficient for the growing rat when a relatively small part of the protein in the diet was replaced by other proteins rich in tryptophan and lysine [Osborne and Mendel, 1917, 1].

The proteins of the embryos of the seeds of wheat [Osborne and Campbell, 1900] are different in chemical properties and structure from those of the endosperm, and the same is presumably true for those of the maize kernel.

[1] Gluten meal is a mixture of the protein of the endosperm of the seeds of maize obtained as a by-product in the manufacture of corn starch.

As to the value of the proteins of the embryo of the maize kernel little is known. Two types of commercial germs are available, one from the manufacture of starch, in which process the grain is first digested with water containing sulphurous acid and the embryo mechanically separated and subsequently dried; the other in which the germs are excised from the untreated grain. The former product has always failed as a source of protein when fed in the author's laboratory, and was presumably the type used by McCollum, Simmonds, and Pitz [1916]. The latter when supplemented with butter-fat and a suitable salt mixture gave normal growth when the diets contained nitrogen equal to about 13 per cent. of protein, but data were not obtained from which a quantitative comparison could be made with the proteins of the endosperm or with those of the entire grain (unpublished).

Although the experimental conditions under which comparisons of the nutritive value of the proteins of the cereal grains must be made leave much to be desired in the way of rigid proof, the results of numerous investigations indicate that these proteins are inferior to those of animal origin, and that their nutritive value is markedly improved by relatively small additions of the proteins contained in those animal products which are commonly used for food. Although this improvement may not be wholly due to the protein factor alone, the fact that the animal proteins are richer in the amino-acids which have been proved to be essential for growth than are the proteins of the cereal grains confirms the belief that the proteins were the chief factor concerned in the outcome of the experiments above discussed.

The leguminous seeds which are rich in protein have many times been recommended as cheap and desirable substitutes for the more expensive animal proteins. With the exception of the soy-bean, however, these seeds have never been used extensively as a major constituent of the diet.

As already shown the proteins obtained from these seeds contain all of the essential amino-acids, and, with the exception of cystine, in apparently sufficient amount to meet the nutritional requirements of young rats.

The ground seeds of many species of leguminous plants are eaten by rats as well as other animals in larger quantity when cooked with water than when raw. The evidence available, while by no means conclusive, indicates that cooking not only destroys some obnoxious constituent, but also may alter the configuration of the protein, whereby it is better utilised. Seeds of different species are not alike

in respect to this nutritive defect. Some, like those of the pea, *Pisum sativum*, are eaten readily in the raw state, those of the soy-bean, *Soja hispida*, less readily, but in sufficient quantity to enable the animal to grow at a rate somewhat below the normal, while after cooking normal growth is made on an otherwise identical diet [Osborne and Mendel, 1917, 3]. On the other hand, rations containing meal made from the kidney bean, *Phaseolus vulgaris*, are scarcely eaten at all unless cooked [Johns and Finks, 1920].

On account of the dietary importance of the leguminous seeds several attempts have been made to determine their nutritive value. None of the published records give any idea of the relative nutritive value of the proteins in these seeds, because either no data are given respecting the amount of protein ingested, or no proof is furnished that the amount of protein eaten was not in excess of the minimum required for normal growth. The only experiments in which the above requirements were approximately met are those of Osborne and Mendel [1917, 1], who found that when the diet contained nitrogen equal to 9 per cent. of protein furnished by soy-bean flour, which presumably had been heated during the process of manufacture, the gain of body weight per unit of protein eaten compared favourably with that made on diets containing other proteins of high quality.

McCollum, Simmonds, and Parsons [1921, 1, 2, 3, 4] give the results of extensive experiments with supposedly adequate and comparable diets, containing 9 per cent. of protein from seeds of several cereals, some animal products, and those from some of the commonly used leguminous seeds, fed either separately or in various combinations. Since no data are given respecting the amount of food eaten, it is impossible to draw satisfactory conclusions from their reports as to the actual value of the proteins in the leguminous seeds which they used, or as to their supplementing value for the proteins of the cereal grains.

Concerning the relative value of proteins contained in green forage plants, or the supplementing value of these for the proteins of seeds, almost nothing definite is known. It is a matter of common experience that many kinds of animals derive a large part of their supply of protein from various species of green plants. When attempts are made to obtain experimental evidence of the part played by the protein factor of diets containing green plants almost insuperable difficulties are encountered.

In the first place we do not yet know how to isolate the protein from any fresh plant in a state of purity, and such attempts as have been made in the author's laboratory to feed the crude protein or the

colloids precipitated from the alfalfa plant (see Chapter X.) have failed completely, apparently because, for some unknown reason, young rats will not eat enough of the foods containing such preparations.

In the second place, when the entire plants, or the residues, presumably rich in protein, from which the soluble matters have been extracted, are fed, it is impossible to determine how much protein is consumed, since we do not know the proportion of non-protein nitrogenous substances which they contain. A product from spinach leaves, which contained nitrogen equal to 39 per cent. of protein (N × 6·25), was fed to young rats by Osborne and Mendel (unpublished) with marked success, although the nitrogen in the diet was equivalent to only 10 per cent. of protein. On the other hand, when a similar product from the juice of the alfalfa plant was fed in a diet containing nitrogen equal to 20 per cent. of protein, all of the young rats died within a few days, probably because they would not eat. This refusal to eat cannot be explained in view of the fact that McCollum [1917] obtained good growth and reproduction with rations containing 40 per cent. of the alfalfa leaf together with various cereal grains. While the experiments of these authors show that the alfalfa leaf as a whole supplements the nutritive deficiencies of the cereal grains, they do not indicate the part played by the protein in this complicated mixture of food factors.

Until more is known about the chemical make-up of the green forage plants, and methods are developed whereby their proteins can be isolated and further studied, no better basis will exist for correctly choosing the proper combinations of the products available for farm animals than that which we now have founded on empirical experiments with mixtures arbitrarily selected.

In this connection attention should be directed to the work of Völtz,[1] who showed that lambs made very considerable growth on diets in which about 80 per cent. of the nitrogen was furnished by urea, the rest being derived from rye straw or chaff, hydrolysed by dilute sodium hydroxide at ordinary temperatures. The only plausible explanation of this anomalous observation is that the bacteria in the omasum of these ruminants converted the urea into protein, which subsequently was digested in the stomach and intestine, and so became available in the form of amino-acids for the nutritive requirements of these animals. Under such circumstances the non-protein nitrogenous

[1] Völtz, W. *Der Ersatz des Nahrungseiweisses durch Harnstoff beim wachsenden Wiederkäuer. Der Futterwert des nach dem Beckmannschen Verfahren aufgeschlossenen Strohs und der Spreu.* Biochem. Zeit., 1920, **102**, 151-227.

substances in green foods may, in whole or in part, serve the same purpose as protein in feeding ruminants. In the light of such experiments it is obvious that the knowledge of the nutritive value of proteins gained by feeding rats cannot be applied to ruminants.

On the other hand, those mixtures which experience has shown to be best for feeding young pigs and chickens are such as experiments with rats indicate should be efficient. Although sufficient data are not yet available to justify final conclusions, it is probable from such experiments as have been made that the protein requirements of man are also much the same as those of rats.

CHAPTER XII.

SOME PHYSIOLOGICAL RELATIONS OF VEGETABLE PROTEINS TO THE ANIMAL ORGANISM AND THE BIOLOGICAL RELATIONS OF SEED PROTEINS TO ONE ANOTHER.

A. *Toxalbumins.*

WARDEN and Waddell [1884] obtained from the seeds of *Abrus precatorius* a toxic substance which they named abrin and considered to be a protein. This appears to be the first suggestion that toxic proteins exist in seeds, and it gave rise to the designation of toxalbumins, which is now applied to this group of proteins.

Three years later Dixson [1886-87] by neutralising an extract of the seeds of *Ricinus communis*, obtained a precipitate which was very toxic and consisted largely of protein. Stillmark [1888], who reinvestigated this substance, ascribed its toxic properties to the protein which, at Kobert's suggestion, he called ricin.

Power and Cambier [1890] isolated from the bark of *Robinia pseudacacia* a similar toxin which they considered to be a nucleoprotein. Schmidt's Lehrbuch[1] appears to be the first publication in which this toxalbumin is described under the name of robin.

Siegel [1893] discovered a similar toxic protein in the seeds of *Jatropha curcas*, to which he gave the name of curcin.

Elfstrand [1897] found a toxic substance in extracts of the seeds of *Croton tiglium*, which appeared to be of protein nature, and for this he proposed the name crotin.

Dunbar [1903] ascribed the effect which extracts of rye pollen had on hay-fever patients to a toxic protein contained in pollens.

Wienhaus [1909], at Kobert's suggestion, proposed the name phasin for all of the proteins which are non-toxic, but which, like the above-mentioned toxalbumins, agglutinate suspensions of the red blood corpuscles of animals.

The protein nature of these substances has been the subject of controversy, but it appears to be the opinion of the majority of those

[1] Schmidt. *Lehrbuch der pharmaceutischen Chemie*, 1896, 3rd edition, **2**, 1647.

who have worked with them that the protein itself, and not some non-protein substance associated with the preparations which have been studied, is the toxic or agglutinating agent. This belief is founded on the fact that the chief constituent of the preparations obtained from these seeds is protein ; that the more thoroughly these preparations are freed from contamination with non-protein substances the greater their toxicity ; that the toxin is indiffusible, and that all those processes which tend to denature the protein, or to cause its hydrolytic decomposition, also diminish or destroy the toxicity of the preparations.

Those who deny the protein nature of the toxin have for the most part based their opinion on the assumption that digestion with trypsin, carried to the point where the solution fails to give the reactions characteristic of protein, does not destroy the toxin. The opponents of this view contend that the toxicity of these toxalbumins is so great that the solutions tested were too dilute to give these reactions, although they still contained enough of these extraordinarily toxic proteins to manifest their presence by fatal results when administered to animals.

We apparently have the same question as to the chemical nature of the toxalbumins as we have in respect to that of the enzymes, with which the toxalbumins seem to have much in common. Cf. Osborne [1895, 3], and Sherman and Schlesinger [1911, 1912].

Abrin.

Warden and Waddell obtained their preparation of abrin by extracting the fat-free powder of the ground seeds of *Abrus precatorius* with water and precipitating with alcohol. Martin and Wolfenden [1889] separated a globulin and a proteose from the precipitate thus produced, and considered both to be toxic. By brief heating these were denatured, the globulin at 75° to 80°, the proteose at 80° to 85°, while at the same time the toxicity was destroyed. Hellin [1891] discovered that abrin had the property of agglutinating the red blood corpuscles, and also showed that the effects of poisoning with abrin were like those caused by ricin. Ehrlich [1891] found that animals immunised to abrin were not immune to ricin, thus establishing a difference between these two toxins.

Répin [1895], Heuseval [1900], and Kobert [1913] state that abrin does not lose its toxicity by digestion with trypsin. Hausmann [1902] found that the toxicity of abrin was greatly reduced by the action of pepsin-hydrochloric acid. On the other hand, digestion with trypsin for several weeks left the toxicity of the solution quantitatively unaltered.

The small precipitate produced in this solution by adding ammonium sulphate to 8/10ths saturation, when dissolved in water, although it gave no biuret reaction, agglutinated red blood corpuscles and proved toxic in doses corresponding to 3·5 milligrammes per kilogramme of body weight.

Hausmann's evidence of the non-protein nature of abrin founded on the failure of the biuret reaction cannot be accepted in view of the relatively large doses which he employed, for we now know that the toxicity of the toxalbumins is so great that a lethal dose may be contained in solutions too dilute to give this reaction.

Sommerfeld [1913] described the relations of toxin to agglutinin from *Abrus* and found these to be very similar to those of ricin.

Ricin.

As already stated, Dixson was the first to isolate from seeds of *Ricinus* a highly toxic product which consisted chiefly of protein. Stillmark, at Kobert's suggestion, named this ricin. He obtained his preparations by saturating sodium chloride extracts of the seed with magnesium sulphate, dissolving the precipitate in water and dialysing until salts were removed. He also discovered the remarkable fact that solutions of ricin added to suspensions of red blood corpuscles caused these to agglutinate and settle, leaving the solution clear.

Ehrlich in [1891] found that animals could be immunised against ricin, and this discovery led to numerous investigations in the field of immunology and to a recognition of the close relations of the toxalbumins to the bacterial toxins. (Cf. Schaer [1891], Tichomiroff [1895], Werhovsky [1895], Stepanoff [1896], Ehrlich [1897], Heuseval [1900], Kobert [1900], Roemer [1901], Hausmann [1902], Jacoby [1902, 1, 2], Kraus [1902], Rehns [1902, 1, 2], Braun, and Behrendt [1903], Brieger [1903], Arrhenius [1904], Frænkel [1904], Jodlbauer and v. Tappeiner [1905], Pascucci [1905], Woronzow [1907], Sachs [1905], Cornevin [1897].)

Cushny [1898] undertook an extensive study of various methods for isolating ricin, and obtained preparations of much greater toxicity than those before described. All his attempts to separate a non-protein toxic substance from his solutions failed, and he states that when the preparation contained protein it was toxic, but when the protein was removed it was not. He ascribed the failure of his predecessors to detect protein in solutions which were highly toxic to the fact that the ricin is fatal in such extremely small amounts that such solutions were too dilute to give protein reactions. He found that the

toxin was precipitated by saturating its solution with ammonium sulphate, was coagulated by heating and thereby rendered non-toxic, and that the toxicity of the solutions decreased as protein was removed from them. He further found that saturation with magnesium sulphate removed all of the ricin from its solution, and that the albumose which was not thus precipitated had no toxic or agglutinating properties. Cushny therefore concluded that ricin is either a protein or is so intimately associated or combined with protein that it could not be separated by any of the means then available.

Jacoby [1901] thought he had demonstrated the non-protein nature of ricin because he found that preparations which he subjected to prolonged tryptic digestion, and were still toxic, failed to give the biuret reaction. Jacoby's method for preparing this so-called ricin must certainly have yielded preparations containing a large proportion of the non-toxic globulin of the seed and relatively little ricin. It is, therefore, not surprising that the very dilute solutions which he tested failed to give the biuret reaction although they still contained enough ricin to be toxic.

Landsteiner and Jagic [1904] found when blood corpuscles were agglutinated by ricin that the toxin could be extracted from the precipitate by dilute hydrochloric acid, thus indicating that the toxin and agglutinin are one and the same substance.

Osborne, Mendel, and Harris [1905] separated the different proteins of the seed of *Ricinus* from one another, and showed that the toxic properties were associated only with those proteins which were soluble in water and coagulable by heat. By dialysing the sodium chloride extract of the seed, the greater part of the protein separated as a globulin which was free from toxic properties. The aqueous solution from which this globulin separated, when fractionally precipitated with ammonium sulphate, yielded the toxin in a fraction obtained within comparatively narrow limits of concentration in this salt. The toxicity of these fractions was closely proportional to the amount of coagulable albumin which they contained, and, by further fractionation with ammonium sulphate, preparations resulted which consisted largely of non-toxic albumin. The most toxic fraction, and at the same time the richest in albumin, consisted of a mixture of about 70 per cent. of albumin and 30 per cent. of proteose. The toxicity of this product exceeded that of any preparation of ricin before described, for 0·0005 of a milligramme per kilo of body weight was fatal to rabbits when subcutaneously injected. Very dilute solutions of this preparation agglutinated the red blood corpuscles of

dog, pig, and cat. An examination of the properties, ultimate composition, partition of nitrogen, and colour reactions showed this preparation to have, in all respects, the properties characteristic of pure protein, and no evidence was obtained which indicated that it contained more than the most insignificant quantity of any other substance. They concluded that the toxic property belongs to the albumin, and that consequently true toxalbumins occur in seeds.

Woronzow [1909] later reported experiments from which he drew the conclusion that ricin was a globulin rather than an albumin, as asserted by Osborne, Mendel, and Harris. While Woronzow's observations agree with those of the latter authors, his conclusion that ricin is not an albumin does not seem justified in view of the fact that saturation with magnesium sulphate is characteristic of many vegetable albumins, and cannot be regarded as evidence of the presence of a globulin. The fact, observed by Woronzow, that water extracts less toxin from the seed than sodium chloride solutions, does not necessarily show that ricin is not an albumin, since this may be a constituent of the "protein grains," which are not dissolved by water, but are readily dissolved by sodium chloride solutions. If these grains are thus dissolved it may well be that albumin is liberated from them.

The evidence thus far recorded, as to the action of trypsin on ricin, strongly favours the view that these toxalbumins are not destroyed by this enzyme, but an examination of the details of the experiments which have been tried leaves one much in doubt as to the weight which should be attached to them. The earlier experiments which were mostly made with crude preparations of ricin, and which have since been shown to contain but little of this toxin, were made at a time when the enormous toxicity of this substance was not appreciated.

It appears, however, from all the experiments which have been reported that ricin is not easily altered by proteolytic enzymes. That there should exist in nature a form of protein not easily hydrolysed by the enzymes of the digestive tract would not be surprising. The fact that the toxalbumins produce such powerful physiological effects upon animals indicates that they differ in some pronounced manner from other known proteins. All the other facts which we know in regard to these toxalbumins speak strongly in favour of their protein nature, and in view of the experiments of Osborne, Mendel, and Harris it is difficult to believe that the toxicity is not a property of the protein substance itself.

Lau [1901] showed that besides the red blood corpuscles ricin

agglutinates suspensions of many other lipoid-rich cells of animal origin, and, on the ground of the experiments which he made with such cells, concluded that the toxin and agglutinin are identical.

Reid [1913] washed cells of dog's brain with physiological salt solution, agglutinated them by adding a solution of ricin, extracted the ricin from the precipitate by very dilute hydrochloric acid, and found that the clear extract promptly agglutinated a suspension of cat's blood corpuscles. This showed that not only the toxin but also the agglutinin were united with the brain cells, thus indicating that the two are one and the same substance. Reid, further, found that the cells of many other organs of the body are agglutinated by ricin, and states that a differentiation of ricin into a toxin and an agglutinin, of which the latter plays no intravitam role, is not justified.

Contrary to Müller's [1899] conclusions, Reid found that the agglutinating power of ricin is not affected by digestion with pepsin. As to the validity of the evidence which he offers in support of his views, the reader is referred to the data given in his paper.

Curcin.

Stillmark [1889] obtained from extracts of the seeds of *Jatropha curcas* a preparation having toxic as well as agglutinating properties similar to those of ricin. Siegel [1893] was unable to remove the protein from preparations made from extracts of this seed without destroying its physiological activity.

Felke [1913] acidified extracts of *Jatropha* seeds with dilute acetic acid, and added enough saturated sodium chloride solution to make the salt content 8 per cent. The precipitate, which contained most of the protein and nearly all of the toxin, was completely soluble in water. When a quantity of this solution equivalent to 20-30 milligrammes of the precipitate was injected into rabbits death ensued. Extracts of the seed heated to 60° lost their toxicity, as they also did after adding a little sulphuric acid. The toxic effects of curcin are similar to those of ricin, but its solution does not agglutinate the corpuscles of many kinds of blood nor does it agglutinate suspensions of brain cells from man or cat. Felke considered curcin to be a toxalbumin.

Crotin.

The toxalbumin which occurs in the seeds of *Croton tiglium* has been the subject of relatively few investigations. Stillmark [1888] applied the same methods which he had used for preparing ricin to

the seeds of *Croton tiglium* and obtained products which, when injected into cats or rabbits, produced effects similar to those caused by ricin, and found that it also agglutinated red blood corpuscles at a dilution of 1 to 40,000. He concluded that croton seeds contained a substance similar to, if not identical with, ricin.

Elfstrand [1898] made an extensive investigation of this toxin and obtained from the oil-free croton seeds two proteins, a globulin and an albumin, which were toxic and likewise agglutinated red blood corpuscles. The fact that solutions of the albumin lose their toxicity at the same temperature as that at which they are coagulated led him to suspect that the toxicity of the globulin might be due to an incomplete separation from the toxic albumin. From the results of his experiments Elfstrand considered it probable that this toxalbumin, which he named crotin, is an enzyme.

Kobert [1913] states that the lethal dose of crotin for warm blooded animals is much less than that of ricin, and that the action of crotin and ricin on various kinds of blood is not alike. Thus the red corpuscles of the guinea pig, man, horse, rabbit, hedgehog, snake, hen, or cat are agglutinated by ricin but not by crotin, those of the five latter, however, are hæmolysed. On the other hand, the red corpuscles of the pig, ox, sheep, or frog are energetically agglutinated, as are all these by ricin. Of the protein nature of crotin there appears to be satisfactory evidence, and it may be included among the toxalbumins on the basis of Elfstrand's investigation.

Robin.

The name robin designates the protein substance produced by Lau [1901], according to the method of Power and Cambier [1890], who discovered this toxic substance in extracts of the bark of *Robinia pseudacacia*. This toxin produces physiological effects very similar to those caused by abrin, ricin, or crotin. Power considered robin to be a nucleoprotein, soluble in water with an acid reaction, and precipitable by alcohol, acids, or excess of salts. On heating its solutions the greater part is coagulated between 70° and 80°, and on boiling its toxicity is completely destroyed. Preparations of robin give all the reactions characteristic of proteins. Power estimated the content of robin at 1·1 to 1·7 per cent. of the bark, and states that it hydrolyses some glucosides and also coagulates milk. He considered it to be an enzyme.

Ehrlich [1897] found that robin produced an antirobin serum when injected into animals.

According to Kobert [1913] solutions of robin agglutinate the red corpuscles of many kinds of blood, some of them at great dilution. Robin is distinguished from ricin by its failure to agglutinate the red corpuscles of dog's blood.

B. *Phasins.*

Wienhaus [1909], at Kobert's suggestion, proposed the name phasin for the substances obtained from extracts of seeds which are non-toxic, but agglutinate suspensions of red blood corpuscles. This designation was derived from *Phaseolus*, from the seeds of which the first phasin was obtained. Whether the agglutinating factor is identical with the toxin or not was the subject of much discussion until non-toxic agglutinins were later obtained from many species of seeds. Since then it has been generally assumed that these two factors are not identical. In this connection attention should be called to the experiments of White and Avery [1913], who found that a very carefully purified preparation of crystallised edestin, which the author made expressly for their work, had strong agglutinating properties. These investigators dissolved the edestin in the least possible quantity of N/10 sodium hydroxide solution, and then diluted to the required concentration with a N/1000 solution. Under these conditions edestin agglutinated the washed red corpuscles of sheep's blood at a dilution of 1-10000, whereas under the same conditions gliadin had no such action. These experiments show that agglutination is not a property peculiar to the proteins of the water extracts of some seeds, and leave little doubt as to the protein nature of phasins.

Phasins have been found in seeds of many species of plants, particularly in those of leguminosæ. Landsteiner and Raubitschek [1907] first described such from Phaseolus, Pisum, Ervum, and Vicia. Similar results were obtained by Mendel [1909, 2], and Wienhaus [1909], who examined the seeds of a large number of different species of plants, in many of which they found phasins. These investigations confirmed the earlier observations which indicated that the corpuscles of different species of animals were not equally sensitive to one and the same phasin. Wienhaus [1909] and Kobert [1913] reviewed the work of previous investigators and further studied the action of several phasins on blood corpuscles from many species.

In view of the character of the preparations which have been used for these investigations, it is impossible to reach any satisfactory conclusion respecting their mode of action or their chemical nature.

Neuberg and Rosenberg [1907] and Neuberg [1908] have raised the question as to whether or not the agglutination caused by the phasin was due to lipolysis, but the evidence furnished by Mendel [1909, 2] and by White and Avery [1913] makes this view untenable.

Some of the more important literature of this subject can be found in the following papers: Schaer [1891], Tichomiroff [1895], Werhovsky [1895], Stepanoff [1896], Cornevin [1897], Ehrlich [1897], Elfstrand [1898], Heuseval [1900], Kobert [1900, 1913], Jacoby [1901; 1902, 1, 2; 1903], Kraus [1902], Rehns [1902, 1, 2], Braun and Behrendt [1903], Brieger [1903], Arrhenius [1904], Fraenkel [1904], Jodlbauer and v. Tappeiner [1905], Pascucci [1905], Liebermann [1905; 1907, 1, 2; 1908], Sachs [1905], Woronzow [1907], Raubitschek [1911], v. Eisler and v. Portheim [1908, 1911, 1912], Miessner and Rewald [1909], Field [1910], Lenze [1909], Assmann [1911], Reid [1913], Sommerfeld [1913], Felke [1913], Agulhon [1914], Ford [1913].

C. *Anaphylaxis*

Wells and Osborne [1915] showed that it is possible to isolate from extracts of seeds, as well as from cow's milk,[1] preparations representing different kinds of protein, each of which reacts specifically according to the protein used, but not according to the species from which it originated. It thus appears that the anaphylactogenic property of a protein is determined not by its biological origin but by its chemical structure.

We have in this reaction the best means now available for establishing the chemical individuality of proteins, and since it has been shown that identical proteins occur only in plants which botanists regard as very closely related, we may also have a new method for indicating the botanical relations of different species.

Wells and Osborne [1911] showed that carefully purified preparations of proteins from several different sources caused typical anaphylaxis reactions, but that the onset of the symptoms was somewhat slower than when proteins of animal origin were used, and that the minimum intoxicating dose was somewhat larger. This may be due in some cases to the vegetable diets of the guinea pigs used for these experiments, because it was found that when these animals were previously fed on maize meal they did not react to zein as did those which had never eaten this grain.

The close similarity, if not identity, of legumin from the pea with

[1] Wells, H. G., and T. B. Osborne. *Anaphylaxis Reactions with Purified Proteins from Milk.* J. Infect. Diseases, 1921, **29**, 200-216.

that from the vetch was indicated by these experiments, as was also the identity of gliadin from wheat with gliadin from rye. They further showed [1913] that guinea pigs sensitized with gliadin from wheat gave strong reactions with hordein from barley, although these two proteins are certainly not chemically identical. Complete protection to subsequent injections of the homologous protein was not afforded by a reaction to the heterologous protein, thus indicating the presence of two or more individual proteins in the preparations of gliadin and hordein, one being common to both, or else the presence in gliadin and hordein of both common and specific reactive groups. The chemical evidence strongly favours the latter view. Gliadin and glutenin, which together form the chief constituents of wheat gluten, react anaphylactically with one another. Since guinea pigs sensitized with glutenin do not react to hordein, as do those sensitized with gliadin, it is probable that the reaction between gliadin and glutenin is due to a common reactive group not present in hordein, rather than to an incomplete separation of these proteins.

The immunity reactions of edestin have been studied by White and Avery, who found that guinea pigs reacted fatally when the sensitizing dose was only 0·0001 milligramme and the intoxicating dose 50 milligrammes. Lake, Osborne and Wells [1914] studied the immunological reactions produced by hordein, gliadin, and some other vegetable proteins. The reader is referred to the account of their results because these cannot well be summarised here.

Wells and Osborne [1914] state that the minimum intoxicating dose of most vegetable globulins is from 1 to 2 milligrammes when administered intraperitoneally, but that from 5 to 10 milligrammes are usually required for severe intoxication. On the other hand the minimum intoxicating dose of the so-called vegetable proteoses which are readily soluble in water is much smaller, moderate to severe reactions being obtained with 0·05—0·1 milligramme and fatal results with 0·5—2 milligrammes. They also found [1915] that these so-called proteoses obtained from different seeds are not only different from one another but also from the other proteins of the seeds from which they originated [Cf. Wodehouse, 1917]. Unlike the proteoses produced by the action of enzymes on native proteins, these natural vegetable proteoses are strongly anaphylactogenic, their activity not being destroyed by heating to 100° for one half-hour, presumably because they are not coagulable.

After comparing the anaphylaxis reactions between proteins obtained from seeds of different genera, Osborne and Wells [1916]

concluded that the specificity of the anaphylaxis reaction depends upon the chemical structure of the protein molecule since chemically similar proteins from seeds of different genera may react anaphylactically with one another, while chemically dissimilar proteins from the same seed in many cases fail to do so.

D. *Biological Relations of Seed Proteins.*

If the chemical and physical properties of the different proteins obtained from seeds are compared with one another, it will be noticed that they show many relations which are in harmony with the recognised botanical relations of the seeds from which they were obtained. The most marked instance of this agreement is shown by the protein constituents of the seeds of cereals. These contain a relatively large proportion of prolamins, which, as we have already stated, are characterised by yielding very little or no lysine and much proline, as well as much glutaminic acid and ammonia and also by yielding relatively little arginine and histidine. The physical properties and general behaviour of all these proteins are much alike, and present marked differences from the proteins obtained from other groups of seeds. The proteins from leguminous seeds resemble one another in many respects, but differ noticeably from those of the cereals.

Preparations of legumin from the pea, horse-bean, lentil or vetch are so nearly alike that no difference has been detected between them. The proteins of these seeds, while in the main resembling those from *Phaseolus*, are not the same, for distinct but slight differences in properties, composition and products of hydrolysis have been established between them. The proteins of the cow-pea and soy-bean, though closely resembling, in many respects, the proteins of the legumes just mentioned, are not exactly the same, while the proteins from different species of *Lupinus* present common characteristics but differ from the proteins of the other leguminous seeds. The proteins of *Lupinus*, however, resemble proteins of other leguminous seeds more closely than they do those of non-leguminous seeds. The proteins of different species of *Juglans* appear to be identical so far as they have been examined, but differ in one respect or another from the proteins of other plants. We thus find similar proteins only in seeds which are botanically closely related, and it would seem that these differences in the reserve food substances of the seeds must have an important bearing on the development of the embryo which derives its first food from them. This food substance, as well as the tissues of the embryo

itself, are the final products of the metabolism of the plant which produced them. When the embryo first develops it is supplied with a definite food which for each individual of the same species is the same, but for those of different species is different. Each member of a species thus begins its individual life under similar chemical conditions, but under chemical conditions which are different from those of every other species. It seems probable, therefore, that when the plant has reached a stage of development at which its organs of assimilation are able to furnish it with nutriment from its external surroundings, its chemical processes have already been established along definite lines which it must follow throughout the rest of its life.

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