The Composition and Characterisation of Genuine Proteins.

HUGO MÜLLER LECTURE, DELIVERED BEFORE THE CHEMICAL SOCIETY ON OCTOBER 28TH, 1926.

By SØREN PETER LAURITZ SØRENSEN.

MR. PRESIDENT, LADIES, AND GENTLEMEN,

May I be permitted to express to you, Mr. President, and to the Council of the Chemical Society my hearty thanks for the great honour you have shown me in inviting me to deliver the Hugo Müller Lecture this year.

If for the subject of the lecture I am to give to-day I have chosen the problem of the composition and characterisation of genuine protein substances—a problem equally important to Chemistry and to Biology—it is certainly not for the reason that a satisfactory answer can now be given. On the contrary, it will be a long time, and much labour will be required, before this will be possible; but the interest generally taken in the matter, and the discussions concerning the way in which these highly important substances must be assumed to be built up are at present so animated and so many-sided in nature that a brief account of the various points of view from which these questions are dealt with seemed to me to offer some interest to an assembly of English chemists.

The inquiry into the composition of proteins is of a twofold character. In the first place it may be asked which elements enter into their composition, and in what proportions, that is to say, what is the "elementary" composition? When this question has been answered, the next will be the manner in which the different elementary atoms constituting the protein molecule, or the complex of protein molecules, are joined together; in other words, of what compounds of simpler structure are the proteins made up? We shall first contemplate this aspect of the matter.

In the first half of the last century the view advanced by the Dutchman Mulder was generally adopted, according to which all albuminoid matter was composed of one and the same organic substance, which, in consequence of the vital process, underwent various changes, whilst being at the same time combined with different proportions of inorganic substances. Assuming that this nitrogenous substance, this *prima materia* common to all albuminoids, formed the basis of the maintenance of the vital process, Mulder gave it the name of "protein," derived from the Greek verb signifying "to occupy the first place."

As knowledge of the different properties of the different protein substances increased, Mulder's theory of a fundamental substance common to all proteins receded into the background and chemists began to fix their attention upon the decomposition products which can be obtained by the splitting-up of proteins on treatment with acids or alkalis or as a result of enzymatic action.

As far back as 1820 Proust and Braconnot had isolated, from the acid-decomposition products of muscle and glue, leucine and glycine, respectively, to which was added by Liebig, towards the end of the 'forties, a substance obtained from cheese and from horn, namely, tyrosine. It was not until 1865 that the next decomposition product, serine, was isolated by Cramer from silk, and after that time new decomposition products were discovered—often at intervals of many years—so that we now know about twenty different decomposition products of protein substances. Of these products, I shall mention one only, namely, tryptophan, a very important substance isolated by Hopkins and Cole in 1901 from the mixture of products formed by the tryptic digestion of casein.

The decomposition products of proteins are widely different in character, but, with the exception of proline and hydroxyproline, they all have one property in common, in being α-amino-acids, $NH_2 \cdot CHR \cdot CO_2H$.

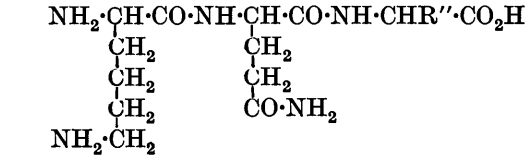
As was shown systematically by Emil Fischer at the commencement of this century, such amino-acids can be united by means of suitable chemical reagents, with separation of water and formation of the so-called peptides :

$$NH_2 \cdot CHR \cdot CO \cdot NH \cdot CHR' \cdot CO_2H$$

Dipeptide.

$$NH_2 \cdot CHR \cdot CO \cdot NH \cdot CHR' \cdot CO \cdot NH \cdot CHR'' \cdot CO_2H$$

Tripeptide.



Residue of lysine. Residue of the amide of glutamic acid.

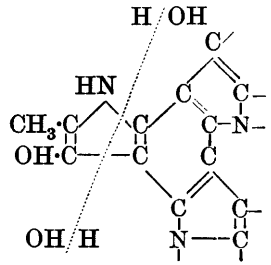
Tripeptide.

Such synthetically-prepared peptides, and more particularly those of complicated structure, resemble in many points the less degraded decomposition products of proteins, the so-called peptones, and consequently it is an obvious conclusion that the proteins consist

essentially of amino-acids, bound together by peptide linkings, –CO·NH–.

This conception of the structure of proteins, which was chiefly based on the very extensive investigations of Emil Fischer, furnishes the most reasonable and plausible explanation of the principal properties of proteins, more especially of the many analogies existing between proteins and amino-acids, and also of the formation of the latter by the decomposition of the former. The view taken by Fischer has indeed, during many years past, formed the general foundation of all researches and reflexions on the composition and structure of protein substances, and only the work done in the most recent years would seem to suggest the necessity of modifying it.

In this connexion, I may first mention the Danish chemist N. Troensegaard, who five or six years ago proposed the view that protein substances are in the main built up of hydroxyl-containing, heterocyclic rings, more especially pyrrole rings. Troensegaard holds that in this way a bridge can be thrown between protein substances on the one hand and the pyrrole-containing compounds —the hæmin of blood and the chlorophyll of plants—on the other. The following formula is given by Troensegaard as representing such a provisional model of a protein ring system :



Troensegaard regards the formation of amino-acids by hydrolysis as taking place in the manner shown by the dotted line in the figure. It will be seen that such a hydrolysis, which requires the entire disruption of the pyrrole ring, will lead to the formation of alanine or, in the case of substitution in the methyl group, of substituted alanines such as phenylalanine, tyrosine, tryptophan, and histidine. By elaborate and ably conducted experiments (chiefly reductive decomposition experiments of different kinds), Troensegaard has of late years endeavoured to prove the correctness of his views; but in my opinion he has not yet succeeded in furnishing conclusive evidence. There can, however, be no doubt—as was also clear to Emil Fischer—that in the protein molecule there exist bindings other than the simple peptide linking, and it is highly probable that a part of the nitrogen which it has not yet been possible to isolate in the

form of known compounds from the hydrolytic decomposition products of a protein substance may exist in such, or similar, easily decomposable, heterocyclic rings as those mentioned by Troensegaard; but it is surely going too far to replace all peptide bindings by heterocyclic rings of this kind.

More compatible with the usual conception, according to which protein substances are complicated polypeptides, is the supposition that diketopiperazine rings may exist in the protein molecule. The simplest of such compounds is, as you know, glycine anhydride (I), and the general formula of the anhydrides of different amino-acids may be written as (II).

(I.) $NH<\begin{matrix}CO-CH_2\\CH_2-CO\end{matrix}>NH$ $NH<\begin{matrix}CO-CHR'\\CHR''-CO\end{matrix}>NH$ (II.)

The possibility of such groups being present in the protein molecule was recognised by Emil Fischer and repeatedly examined by Emil Abderhalden, but its present great interest dates more particularly from the time when, shortly after 1920, R. O. Herzog and his collaborators, basing their views upon Röntgen-spectrographic investigations of high-molecular organic substances, including silk fibroin, tried to prove that the essential substance of silk fibroin is a polypeptide of simple construction or an anhydride of the same, containing only glycine and alanine. They consider that this compound is in all probability glycylalanine anhydride, and consequently is possessed of a diketopiperazine ring. The molecules of the fundamental substance are supposed by Herzog to be combined with one another by secondary valencies.

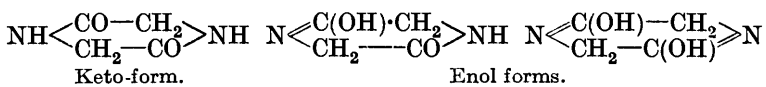
Since then, the question of the existence of diketopiperazine and other similar rings in the protein molecule has been the subject of much discussion and of thorough experimental investigation by Abderhalden, M. Bergmann, and P. Karrer. It would carry us too far to go into all the details regarding this very difficult question, which is as yet far from being cleared up; I should, however, like to mention a few particularly interesting details.

It is Abderhalden who has made the greatest endeavours towards demonstrating the presence of diketopiperazines in proteins. He has succeeded in isolating such substances from the products of protein decomposition and has sought to prove that these anhydrides are not formed from polypeptides by a secondary process. Abderhalden has further studied colour reactions, and oxidation and reduction processes, towards which he finds polypeptides and diketopiperazines behave differently. For example, diketopiperazines, if suitably reduced, yield piperazines—and so also do the protein substances studied by him—whereas the polypeptides do not; and

from these facts he draws the conclusion that protein substances must contain the diketopiperazine ring.

Abderhalden's experiments and his interpretation of the results have been the subject of a good deal of criticism, and, as it seems to me, not without reason. In my opinion, the most that can be said is that his researches go to show that at any rate a part of the protein nitrogen is probably present in the form of diketopiperazine rings.

A very important point concerning the structure of protein material is the behaviour of the compounds towards proteolytic enzymes. Waldschmidt-Leitz and Schäffner (*Ber.*, 1925, **58**, 1356) have shown that none of the usual proteolytic enzymes attacks diketopiperazines, whereas polypeptides, as is well known, are readily decomposed by erepsin. This observation implies that no great part of the protein molecule can consist of diketopiperazines, at all events in the usual keto-form. On the whole, the reactivity of the commonly occurring diketopiperazines is too slight and these substances are far from being labile enough to justify the assumption that in the usual keto-form they constitute the main part of such easily changeable substances as the proteins. In fact, Abderhalden has repeatedly pointed out the possibility that the diketopiperazines may exist in proteins not in the usual keto-form, but in some tautomeric, more labile form; indeed, particularly by heating them with aniline, he has succeeded in transforming common diketopiperazines into tautomeric compounds possessing a greater power of reaction and exhibiting properties that suggest the presence of an ethylenic linking. The following are the formulæ given by Abderhalden for these desmotropic—as they are called—2 : 5-dioxopiperazines :



Keto-form. Enol forms.

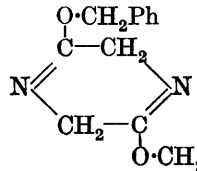
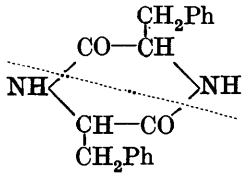
New forms.

Abderhalden believes that the desmotropic form prepared by him has a double bond between the carbon atoms, but he has no decisive reason for preferring this formula to the usual enol-form. Such proteins as silk fibroin, keratins, and the like, which are very resistant, contain, he supposes (*Z. physiol. Chem.*, 1925, **152**, 89), the stable keto-form, whereas the more labile proteins contain one of the anhydride forms with the double bond. He points out as particularly characteristic that the labile glycine anhydride prepared by him is converted into the keto-form by simple heating in aqueous

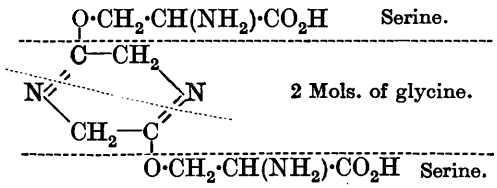
solution at 90—100°, and it is undeniable that such a reaction reminds one of the denaturation of proteins by heating.

The question of labile nitrogenous rings forming the fundamental structure of proteins has been dealt with also by other investigators from similar standpoints and on the basis of the results obtained in Röntgen-spectrographic researches on substances of high molecular weight. I will mention only a few of the most prominent of these inquirers.

Karrer, Gränacher, and Schlosser (*Helv. Chim. Acta*, 1923, **6**, 1108) have shown that the silver compound of glycine anhydride, if treated with benzyl chloride on a boiling water-bath, yields the *OO'*-dibenzyl ether of dihydroxydihydropyrazine (III), which possesses quite different properties from those of the well-known anhydride of phenylalanine (IV). In contradistinction to phenylalanine anhydride, the new compound can be very easily decomposed

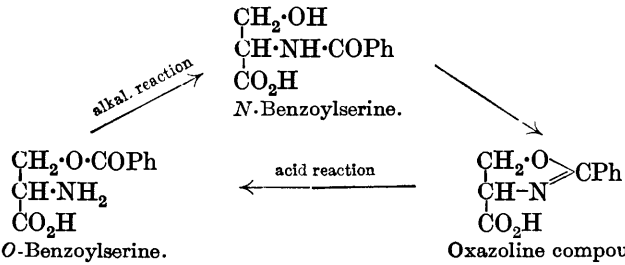
(III.)   (IV.)

by being heated in an acid liquid, with formation of glycine and benzyl alcohol. The authors, directing attention to the great lability and power of reaction possessed by such compounds in comparison with common amino-acid anhydrides, say: "Die Vorstellung, dass ein physiologisch so ungemein aktiver und wandelbarer und chemisch labiler Stoff wie das Eiweiss nur aus verhältnismässig reaktionsträgen und beständigen Polypeptidketten und Diketopiperazinringen aufgebaut sei, kann schwerlich befriedigen." More particularly can hydroxyamino-acids be easily formed by hydrolysis of compounds of the type here referred to, as will appear from the formula given below of a substance which will yield serine and glycine by hydrolysis.



In this connexion the wide-ranging experimental researches of M. Bergmann and his collaborators are of paramount interest. As a perspicuous example, I may mention the investigations of Bergmann and Mickeley (*Z. physiol. Chem.*, 1924, **140**, 128) on the

displacements which take place in benzoylserine. They may be expressed in the following form :



If *N*-benzoylserine is treated with diazomethane, the methyl ester will be formed, and this compound, on treatment with thionyl chloride, even in the cold, yields, by loss of water, an oxazoline compound which is stable in alkaline solution and can therefore be saponified with formation of a salt of the oxazoline compound as depicted in the table. As soon as the liquid is rendered acid, however, hydration begins, with formation of *O*-benzoylserine. This compound is stable only in acid solution; as soon as the liquid becomes alkaline, a transformation to the original *N*-benzoylserine takes place. I will not go into the question as to the cause of these transpositions, but will only mention that if the benzoyl group in *N*-benzoylserine is replaced by the radical of glycine, $NH_2 \cdot CH_2 \cdot CO$–, we arrive at the formula of common glycylserine, on which also Bergmann has been working. The state of things here is rather more complicated, yet even in this case it has been proved possible to prepare compounds which by a mere alteration of the reaction of the solution are converted into isomeric substances. Bergmann says indeed : " Ich halte es für sehr wahrscheinlich, dass solche Oxazoline oder analoge instabile Ringsysteme am Aufbau der natürlichen Proteine beteiligt sind " (*Naturwiss.*, 1924, **12**, 1158).

The researches of which I have now given a somewhat condensed and necessarily incomplete account, and all of which date from the last few years, are extremely interesting. It has been emphasised with perfect justice how desirable, indeed how necessary, it is for the comprehension of many of the transformations of protein substances to be able to point out labile bindings—I need here mention only the denaturation process, still obscure, which many soluble proteins undergo on heating or on treatment with alcohol.

But a question now arises which in my opinion it is absolutely necessary to subject to a closer examination before we may draw decisive conclusions as to modern ideas regarding the structure of protein substances; this is the question how compounds having

the assumed structure will behave towards proteolytic enzymes. Concerning this problem, as far as I am aware, this only is known with certainty, that diketopiperazines, as I have mentioned above, in contradistinction to polypeptides, are not split up by erepsin. The question can now be put with all the more stringency because the investigations made by Waldschmidt-Leitz and his fellow-workers in Willstätter's laboratory at Münich have thrown more light on the specific effects of proteolytic enzymes. By means of a suitably effected adsorption of the enzymes (the adsorbent used was aluminium hydroxide prepared in a definite way) and subsequent convenient elution it has been proved possible to prepare the different proteolytic enzymes in a state of more perfect purity than was hitherto known. By this procedure it has now been shown—as stated in the table below—that all the peptides experimented upon

Enzyme-specificity.

— = no action. + = action. ++ = marked action.

		Enzyme.		
		Erepsin.	Trypsin.	Trypsin + enterokinase.
1.	Alanylglycine	+	—	—
2.	Glycyltyrosine	+	—	—
3.	Glycylglycine	+	—	—
4.	Glycylalanine	+	—	—
5.	Leucylglycine	+	—	—
6.	Leucylalanine	+	—	—
7.	Leucylglycylglycine	+	—	—
8.	Peptone (*ex* albumin, Merck)	—	+	++
9.	Clupein	—	+	++
10.	Thymus histone	—	+	++
11.	Caseinogen	—	—	+
12.	Fibrin	—	—	+
13.	Gelatin	—	—	+
14.	Gliadin	—	—	+
15.	Zein	—	—	+

are split up by erepsin, whereas none of them—not even a tripeptide which was formerly considered to be decomposable by trypsin—is split up by the enzyme trypsin. It will further be seen that the field of activity of erepsin is confined to the simple peptides: neither peptone, protamine histone nor any of the comparatively complex proteins can be split up by erepsin. On the other hand, all these substances are split up by trypsin after the latter has been activated by means of enterokinase: in no case, however, can trypsin be replaced by erepsin, or *vice versa*.

This view must, however, undergo some alteration in consequence of the investigations recently published by E. Waldschmidt-Leitz, A. Schäffner, and W. Grassmann (*Z. physiol. Chem.*, 1926, **156**, 68) on the effects successively produced on clupein by different proteolytic enzymes. Indeed, it is shown that if the consecutive

order of the enzymes employed is changed, results are obtained which are inconsistent with the supposition that each enzyme possesses an absolutely specific character. The degree of specificity seems, on the contrary, to be dependent not solely on the different amino-acids which enter into the polypeptide complex concerned, but also on some other factors, as yet unknown.

In one point, however, the two enzymatic scissions resemble each other. In both of them free carboxyl groups are formed (measurable by formol titration or by Willstätter's titration) and amino-groups (measurable by van Slyke's or by Folin's method), and in the case of the simple polypeptides, where such a test is practicable, the formation of carboxyl and amino-groups takes place in equivalent quantities. This may be, and has hitherto been, accounted for by assuming a splitting-up of peptide linkings, and no evidence has yet been furnished to prove that the new cyclic compounds assumed to enter into the composition of proteins admit of being split up by trypsin or erepsin with formation of carboxyl and amino-groups.

In peptic scission, it has long been known that the effect is far less radical. Most of the natural protein substances are attacked, at any rate after denaturation, by pepsin; but the decomposition, measured, say, by means of formol titration, is only slight, whilst on the other hand the properties of the substances are greatly altered. What really takes place here is not yet known with certainty; it would seem, however, that it is something more than a splitting-up of a few peptide linkings. In this respect some experiments published this year by H. Steudel and his collaborators (*Z. physiol. Chem.*, **154**, 21, 198) are of considerable interest. In the peptic scission of a number of proteins these investigators find a far greater increase of carboxyl groups than of amino-groups; but they too are unable to give a definite explanation of this fact. From some very recent experiments of E. Waldschmidt-Leitz and E. Simons (*ibid.*, 1926, **156**, 114) it appears, however, that in the peptic scission of certain proteins (casein, egg-albumin, and *Ricinus* globulin), in the first stages at least, equivalent quantities of carboxyl and amino-groups are formed.

Regarding the actual state of affairs, it seems to me premature to assume that the protein molecule is composed in the main of heterocyclic rings. Besides the well-known heterocyclic rings (the indole ring in tryptophan, the iminazole ring in histidine, and the proline ring) it is highly probable that there exist smaller proportions of other heterocyclic rings; however, I still consider the peptide linking the principal one.

It is quite another question whether or no the protein molecule may reasonably be supposed to consist of one large polypeptide

complex. According to the Röntgen-spectrographic researches, the fundamental substance in the crystalline part of silk fibroin appears to have a simple structure; but it may just as well be a dipeptide as a diketopiperazine ring. Something similar, though often considerably more complicated, may be the case with other proteins, which accordingly would have to be considered as consisting in the main of larger or smaller polypeptide complexes, bound together by secondary bindings or by mutual salt formation taking place, without any real chemical reaction, between the complexes. Such complexes loosely knit together would act in solution as a co-ordinated whole and consequently exert but a very slight osmotic pressure, as, in fact, is the case. The view here advocated also accounts for the remarkable behaviour with regard to solubility and fractional precipitation of the albumins and globulins in salt solutions.

In an earlier paper (*Compt. rend. Trav. Lab. Carlsberg*, 1923, **15**, No. 11; *J. Amer. Chem. Soc.*, 1925, **47**, 457) I accounted for the solubility of serum globulins and tried to show that these remarkable phenomena can be explained, not on the assumption that we have to do with mixtures of different globulins, but only by assuming these different globulin complexes to be loosely combined with one another in a larger whole, to which I assigned the formula E_pP_q. This was intended briefly to express that the different globulins contain different numbers of euglobulin complexes (E) and pseudoglobulin complexes (P), thus accounting for all that seemed strange in the solubility and precipitation relations.

Later on, in explaining the results of a series of fractionating experiments on casein, Linderstrøm-Lang at the Carlsberg laboratory has successfully used the same view.

If, however, we are to get to the bottom of the matter in the examination of these complexes, without splitting them up, the first thing to be done is an intensive fractionation, and it is of supreme importance that the protein to be used for this purpose shall be really pure or at least as pure as it can be obtained without loss of its characteristic properties.

We here encounter the second question upon which I should like to touch to-day, namely, the characterisation of the individual protein substances. As this question is bound up with that of the elementary composition of proteins, I beg leave to say a few words about it.

As is generally known, all proteins contain the elements carbon, hydrogen, oxygen, and nitrogen; and most of them also small quantities of sulphur and phosphorus. The different proteins have approximately the same percentage composition, it is true; but

nevertheless the differences, and more particularly those in the nitrogen, sulphur, and phosphorus contents, are so considerable that when we come to characterise the individual groups the percentage composition is of valuable assistance. The content of sulphur is but slight, and that of phosphorus generally quite minimal, and the question therefore arises whether we have here to do with integral constituents of the protein molecule itself or of the molecular complex, or whether the sulphur- or phosphorus-containing compounds are only loosely bound, say by adsorption, to the real protein, with the characteristic properties of which these foreign substances have nothing to do. It is quite natural to make such reflexions, particularly if dissolved proteins are considered as composed of a number of complexes loosely knit together, the mutual binding relations of which are liable to be altered by alterations in the temperature, hydrogen-ion concentration, salt concentration, etc., of the solution. It would take too long to go into this question in great detail; I shall have to confine myself to trying to throw some light upon the subject by the aid of a few examples relating to the phosphorus content of proteins.

Setting aside nucleoproteins and their degradation products, the nucleic acids, which, as you know, occupy a peculiar position in the chemistry of proteins, the phosphorus content of proteins shows variations from infinitesimal amounts up to 1% of the dry substance. Those among these proteins which contain the highest amount of phosphorus, and which in several points exhibit similar or identical properties, have been united under the common designation of phosphoproteins or phosphoproteids, the principal representative of which is casein. In regard to the other proteins, it is, rather implicitly, believed that the phosphorus content originates in impurities, probably adhering phosphatides (lecithin and the like). I doubt, however, whether this question can be settled in so simple a manner, and I should like to make good my doubt.

Common casein, prepared and carefully purified by Hammarsten's method, contains about 0·8% of phosphorus and about 15·6% of nitrogen, *i.e.*, about 50 milligrams of phosphorus per gram of nitrogen. The inquiry now arises whether the whole of this phosphorus belongs to the casein molecule as an integral constituent, or whether, by further purification or by an appropriate fractionation of the casein, the phosphorus content can be diminished without the casein losing its characteristic properties. This brings us to the question what is the best definition of casein, or, in other words, how this substance is to be most correctly and most exhaustively characterised. For this purpose we may choose the old familiar reaction—the conversion of casein into the insoluble calcium salt of

paracasein by addition of a soluble calcium salt and a rennet solution (an extract of the mucous membrane of calf's stomach)—which is specific and particularly suitable for the characterisation of casein.

As I have already mentioned, Linderstrøm-Lang has succeeded, by suitable fractionation, in separating casein into several fractions having differing properties, *e.g.*, different solubilities in hydrochloric acid containing sodium chloride, the phosphorus contents being likewise different, and varying from a little more than 50 mg. down to less than 20 mg. of phosphorus per gram of nitrogen. Yet all the fractions gave the characteristic rennet reaction. Which phosphorus content is, then, to be considered as the normal one of casein? Or still more precisely: Is it possible to prepare a casein free from phosphorus and nevertheless able to give the rennet reaction?

If as examples of proteins poor in phosphorus we take globulin and albumin derived from white of egg and horse's serum, it will be found that these substances present a most peculiar picture, which I have broadly outlined in the table below. All the substances had

Phosphorus content of albumins and globulins.

	Mg. P per g. of total N.		Solubility in Am_2SO_4 solution under certain conditions.
	P coag.	P precipitable by alcohol.	Increasing content of P.
Egg-globulin	2	—	—
Egg-albumin	7·5	Whole amount of coag. P.	Constant.
Ordinary serum-euglobulin	2—40	$\frac{1}{10}$—$\frac{1}{60}$ amount of coag. P.	Decreasing.
Ordinary serum-albumin ...	0·3—1·5	$\frac{1}{10}$—$\frac{1}{30}$ amount of coag. P.	Increasing.
Serum-albumin after treatment with alcohol–ether at −4°.	0·04	Almost the whole amount of coag. P.	

been carefully purified by a long succession of precipitations and dialyses, or in the case of albumins by repeated crystallisation. As to the egg-proteins, you will notice that the albumin has a higher phosphorus content than globulin; with respect to serum proteins, the case is quite the reverse. In the egg-albumin, prepared and recrystallised in the usual manner, the phosphorus content is practically constant and appears to be intimately bound up with the rest of the molecular complex of the egg-albumin, as the whole amount of phosphorus is precipitated along with the latter, whether the precipitation be effected by heating or by means of alcohol. The case is quite different with the serum proteins, where only a small part of the whole amount of coagulable phosphorus is precipitable by alcohol. Hence it is probable that—in harmony with

what was formerly assumed—the substances concerned are but loosely associated with the serum-protein complexes. The question whether these substances are to be looked upon as impurities or as belonging to the albumin or globulin complex must, in my opinion, be settled by an inquiry into the possibility of removing them without depriving the albumin and euglobulin of their characteristic properties. In this connexion, I regard it as particularly important that the albumin should be capable of crystallising (a property which it is apt to lose at the slightest alteration) and that the euglobulin should preserve its well-known property of being only slightly soluble or quite insoluble in water, but soluble in weak salt solutions, from which it can again be precipitated by addition of water.

Fortunately we possess a trustworthy method by which practically the whole amount of coagulable phosphorus can be removed from serum without detriment to the above-mentioned characteristic properties of the albumin and the globulin. The procedure was first indicated by W. B. Hardy and Mrs. S. Gardiner (*J. Physiol.*, 1910, **40**, 68) and later employed by E. G. Young (*Proc. Roy. Soc.*, 1922, *B*, **93**, 15), and consists in precipitating the serum at −4° by alcohol, after which the precipitate is thoroughly treated at the same temperature with alcohol and ether. With respect to the serum proteins, the materials in question must therefore be nothing more than impurities, and not integral constituents at all, so that the phosphorus content can scarcely be held responsible for the rather strange fact that under certain conditions euglobulin becomes less soluble in an ammonium sulphate solution the greater its phosphorus content is, whilst the serum-albumin behaves conversely.

I have tried to purify egg-albumin too by treatment with alcohol and ether at —4° and even lower temperatures, but could not extract any phosphorus; the egg-albumin was completely denatured, and the denaturated substance contained, just as it did before the treatment, 7·5 mg. of phosphorus per g. of nitrogen. I may add that by such a treatment casein also fails to give up phosphorus-containing substances to the alcohol.

In this respect, therefore, there seems to be an essential difference between the two albumins, serum-albumin having to be regarded as free from phosphorus, whilst egg-albumin is to be looked upon, for the present at least, as a phosphorus-containing substance.

In some memoirs on egg-albumin published by me some ten years ago, I tried, upon the basis of my measurements of the osmotic pressure of egg-albumin solutions, to determine the weight of the molecule or molecular complex of egg-albumin in the anhydrous state, and at a rough estimate the molecular weight was found to be approximately 34,000, corresponding to a content of about 380

nitrogen atoms. This molecular weight agrees well with that (33,200) found last year by E. J. Cohn (*Physiol. Reviews*, 1925, **5**, No. 3, p. 359), who supposed two tryptophan groups to be present in the molecule, and also tallies with that recently obtained by I. B. Nichols, who found the molecular weight of well-purified egg-albumin to be between 33,000 and 35,000 by Svedberg's centrifugal method (*Z. physikal. Chem.*, 1926, **121**, 76).

If, then, the molecular weight is taken to be 34,000 and the number of nitrogen atoms to be 380, a simple calculation based on the assumption that there is one atom of phosphorus in the molecule would demand a content of 5·8 milligrams of phosphorus per gram of nitrogen. As you will see, the order of magnitude of the phosphorus content found (7·5) agrees fairly well with this estimate; the discrepancy, however, much exceeds the possible error of analysis. I have therefore tried in different ways to reduce the phosphorus content of egg-albumin without depriving it of its characteristic capacity of crystallising. As I have already mentioned, the reduction is not practicable by treatment with alcohol and ether at low temperatures. Neither have I succeeded by fractional crystallisation at different hydrogen-ion concentrations; the phosphorus contents of the different fractions varied only from 7·52 to 7·78 milligrams per gram of nitrogen.

Phosphorus content of egg-albumin.

	Mg. of P per g. of N.
Ordinary egg-albumin	7·5
Fractionated „	7·52—7·78
Electrodialysis:	
Anode liquid	*ca.* 8·5
Inner liquid	7·0—5·0
Stored in ice	7·3—4·7
Calculated for 1 atom of P per 380 atoms of N	5·8

Better results were achieved by electrodialysis, in Pauli's apparatus, of egg-albumin solutions poor in electrolytes. I used two collodion membranes and placed the albumin solution between them. The membranes were impermeable to egg-albumin when no current was passing through the liquid; but as soon as the circuit was closed, egg-albumin began to penetrate through the membrane near the anode, whereas the cathode liquid remained free from albumin for several days. I shall not have sufficient time to go into this remarkable phenomenon at greater length, but will only mention that I always found, relative to the nitrogen content, a larger quantity of phosphorus in the anode liquid than in the remaining inner liquid. When care was taken that the egg-albumin solutions contained but minimal amounts of electrolytes during the electrolysis, the albumin

both in the anode liquid and in the inner liquid could be made to crystallise, and even after repeated crystallisations the phosphorus content of the albumin in the anode liquid was found to be a little higher than usual (about 8·5 mg. P per g. N), whereas the albumin from the inner liquid showed a lower phosphorus content (varying from about 7·0 to 5·0 mg. P per g. N).

Finally, I may mention that, when egg-albumin solutions—especially if dialysed and consequently poor in electrolytes—are stored in ice and saturated with toluene (in order to prevent putrefaction), a part of the albumin will separate in a denaturated state after some time. After the precipitate is filtered off, the albumin remaining in the solution shows normal properties and a normal osmotic pressure: it crystallises readily in the normal manner. The egg-albumin thus obtained and repeatedly crystallised, contains, however, less phosphorus than that newly prepared, and the less the longer it is kept. The phosphorus contents of the samples examined by me varied from 7·3 to 4·7 milligrams per gram of nitrogen; the latter figure refers to a dialysed egg-albumin which had been stored in ice for eleven or twelve years and yet crystallised very nicely and readily after filtration. Thus, as you see, it is possible to prepare well-crystallised egg-albumin with a lower phosphorus content than the normal. The phosphorus content, however, is always very different in order of magnitude from that of serum-albumin, and, as I have already said, I am still of opinion that the large molecule of egg-albumin contains one phosphorus group.

In conclusion, for the sake of preventing any misunderstanding, I may add that I do not regard the crystallisability of albumins and the capacity of casein of forming a coagulum with rennet as affording a complete characterisation of the proteins concerned. As I have lately remarked in a brief note * dealing with this problem, I look upon these properties as *necessary* but not, when taken alone, as "*sufficient*" criteria.

I have been able to give here but a brief account of some of the diverse problems which, at the present moment, occupy the attention of those engaged in a study of the chemistry of proteins, but I trust that it has conveyed an adequate impression of the perseverance with which the intense endeavours are being made in many branches of Science to add to our knowledge of these extremely important substances.

* S. P. L. Sørensen: "Proteins, Lectures given in the United States of America in 1924" (The Fleischmann Laboratories).