XXXIX. PHOSPHORYLATION OF PROTEINS.

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THE peculiarity of the phosphoproteins in containing relatively large quantities of phosphorus, organically combined, and the unique importance of these substances in the nutrition of the young organism has led in the past to a good deal of speculation as to the mode of combination by which the phosphorus is held in the molecule, and also to attempts on the part of one or two workers to produce similar phosphorus-containing bodies by the action of various reagents upon proteins.

Liebermann [1888] and Pohl [1889] investigated the action of metaphosphoric acid upon proteins. Phosphorus oxychloride appears to have been used first by Bechhold [1901] who treated egg-albumin, dissolved in aqueous alkaline solution, with this reagent. There was, however, great development of heat during the addition of the phosphorus oxychloride and a consequent destruction of the protein. The resulting reaction-mixture was found to contain substances, some of which were precipitable by acid, which contained phosphorus, and exhibited properties characterising them as derived proteins resembling albumoses in complexity.

A modification of the method was attempted by Neuberg and Pollak [1910] and later by Neuberg and Oertel [1914], the phosphorus oxychloride being dissolved in chloroform. Lactalbumin, serum globulin and caseinogen were "phosphorised" in this way and the phosphorised products precipitated by acetic acid and washed until no free acid remained in the filtrate. They contained amounts of phosphorus varying from 1.28 % in lactalbumin to 1.77 % in serum globulin. These authors also made some experiments of a purely qualitative nature upon the behaviour of the compounds towards the digestive enzymes. Neuberg and Oertel [1914] also state that phosphorised globulin is coagulated by rennin, an observation which the present author has not been able to confirm, as in his experience the calcium salt of the phosphorised protein is already insoluble under the conditions in which the test would be carried out.

Undoubtedly the technique employed by Neuberg affords a method of bringing about organic combination of phosphorus with the protein, but no

conclusive evidence was brought forward that the products were free from admixture with inorganic phosphoric acid. The difficulty of removing small quantities of this substance from protein precipitates is very great, and it seems that a couple of reprecipitations, even accompanied by thorough washing, is insufficient to secure complete freedom from inorganic impurities.

In the present work, a method is used by which the progressive purification of the product is followed by determining the P/N ratio of the *filtrates* from each reprecipitation. The process of reprecipitation and washing is repeated until the P/N ratio of the filtrate remains constant and is then equal, it is found, to the P/N ratio of the precipitated substance itself. By this procedure absolute certainty is achieved that all the phosphorus remaining in the product is in definite chemical union, while at the same time none of the substance itself is used up in analyses during its purification—a point of undoubted advantage when small quantities of material are being dealt with.

The method of carrying out the phosphorylation is analogous to the Schotten-Baumann reaction for the benzylation of organic substances in aqueous solution. By this means the high concentrations of alkali employed by Neuberg are avoided and the risk of destructive changes in the proteins thereby diminished, for it has already been shown by Rimington and Kay [1926] that under the action of 1 % sodium hydroxide at 37° caseinogen rapidly loses both phosphorus (as phosphoric acid) and ammonia.

The proteins used in this investigation are caseinogen, serum globulin, prepared from horse serum, carefully purified and subsequently denatured by alcohol, and “dephosphorised caseinogen,” the phosphorus-free protein obtained by the action of 1 % sodium hydroxide for 24 hours upon caseinogen at 37°. The globulin was chosen for the following reasons. It was shown by Crowther and Raistrick [1916] and confirmed by Woodman [1921] that the globulins of serum, colostrum and of milk are identical, whilst the albumin of serum differs from that present in colostrum and milk. The proportion of lactoglobulin in colostrum is very high, 6–12 %, and falls immediately to the low figure of 0.005 % as soon as secretion of true milk commences.

In considering the problem of mammary secretion, the possibility has always to be kept in mind that one or other of the blood proteins might conceivably furnish the starting point for the synthesis of caseinogen. The ready permeability of the cells of the mammary gland to serum globulin, as evidenced by the identity of the protein in serum, colostrum and milk respectively, taken together with the difference in the amounts occurring in the two secretions makes this possibility worthy of further consideration. Moreover the distribution of nitrogen in caseinogen and in lactoglobulin shows some similarity as will be seen from the figures given by Crowther and Raistrick [1916].

It was felt that phosphorylation of serum globulin would be of interest not only in so far as it helped to throw light upon the manner in which the synthetically introduced phosphorus atoms are held in combination with the protein but also with reference to the problem of the origin of caseinogen in

the mammary cell. The stability of the phosphorus in phosphorised globulin towards acids, alkalis and enzyme preparations has been investigated upon lines similar to those adopted by Rimington and Kay in the study of caseinogen, and a striking similarity has been found to exist between these two proteins, suggesting a similar type of linkage. It is hoped that the accumulation of additional facts will afford evidence, in the light of which the hypothesis outlined above may be examined and a clearer knowledge obtained of the materials entering into the synthesis of caseinogen *in vivo*.

PHOSPHORYLATION OF PROTEINS.

In all cases the proteins were first thoroughly purified by solution and reprecipitation at the isoelectric point several times in succession. Caseinogen was directly prepared from separated milk. Care was taken to avoid undue exposure to alkali, the sodium hydroxide being added drop by drop, over a period of several hours to the suspension of protein in water which was vigorously agitated by a mechanical stirrer. A quantity of the protein, between 7 and 10 g., was then dissolved in water with the aid of the requisite quantity of sodium hydroxide, the total volume being brought up to 125–150 cc.

Phosphorus oxychloride (20–25 g.) dissolved in eight times its volume of carbon tetrachloride was allowed to drop into the ice-cold, rapidly stirred protein solution, to which had been added phenolphthalein to serve as an indicator, and the reaction was kept faintly alkaline by addition of 4*N* sodium hydroxide. The addition of the oxychloride extended over 6–8 hours and the temperature of the reactants never rose above 5°.

After separating the aqueous layer from the carbon tetrachloride, the phosphorised protein could be precipitated by addition of 2*N* hydrochloric acid until maximum separation occurred. This corresponded to a p_H between 3 and 4. The filtrate was kept for nitrogen determination and the precipitate purified as outlined above by dissolving in 0.1*N* sodium hydroxide and reprecipitating by hydrochloric acid, the filtrates from each reprecipitation being analysed for nitrogen and phosphorus. The data thus obtained served for the calculation of the P/N ratios (see Table I) and when this became constant the product was dehydrated by means of alcohol, dried over sulphuric acid *in vacuo* and a portion taken for analysis.

Phosphorised caseinogen.

In view of the fact that evidence has been obtained by Rimington and Kay [1926] suggesting the existence of an ester linkage in caseinogen between phosphoric acid and some other constituent of the molecule, it became of interest to find out whether by artificial means still further quantities of phosphorus could be induced to combine with the protein and, if this took place, to ascertain if possible the nature of the combination.

A quantity of 18 g. was dissolved in sufficient sodium hydroxide to give a neutral solution and the volume made up to 250 cc. Using the precautions described above, 50 g. of phosphorus oxychloride dissolved in 200 cc. of carbon tetrachloride were then added to the ice-cold solution in such a way that the operation was completed in 9 hours, sodium hydroxide solution being added as required to maintain a faintly alkaline reaction.

The phosphorised product was precipitated by addition of 59.5 cc. of 2*N* hydrochloric acid, filtered off, washed with a litre of distilled water and redissolved in the minimum quantity (87 cc.) of 0.2*N* sodium hydroxide. The exact equivalent of hydrochloric acid (0.2*N*) was then added very slowly to the rapidly stirred solution and the process of filtration, washing, etc., repeated until purification was complete as shown by the constancy of the P/N ratio in the filtrate from the precipitated protein. As a rule, five or six reprecipitations were found necessary before this stage was reached.

Table I.

Purification of phosphorised caseinogen. Nitrogen and phosphorus contents of the filtrates from each reprecipitation.

Filtrate	Volume cc.	Total nitrogen mg.	Total phosphorus mg.	P/N
1	300	31.2	—	—
2	242	4.55	5.79	1.27
3	320	6.53	2.83	0.43
4	248	6.35	1.24	0.20
5	266	5.43	1.21	0.22
6	300	3.83	0.60	0.16

The product was finally dehydrated by alcohol, and dried over sulphuric acid *in vacuo*. The yield was 61 %. Upon analysis it gave the following results, from which it will be seen that a further 0.97 % of phosphorus had been introduced into the molecule.

Analysis

H ₂ O lost at 107° (% of wet weight)	...	...	...	7.72
Total N (Kjeldahl, % of dry weight)	...	...	...	13.53
Total P (Neumann, % of dry weight)	...	...	...	1.77

Ratio P/N = 0.130. A second preparation gave P/N = 0.131.

The substance in all appearances resembles caseinogen but requires nearly twice as much alkali to bring it into solution.

It was shown by Rimington and Kay [1926] that whereas the phosphorus of caseinogen was quite stable to 0.25*N* hydrochloric acid at 37° and to bone phosphatase preparations, the action of 0.25*N* sodium hydroxide at 37° brought about the liberation of the whole of the phosphorus as inorganic sodium phosphate in 24 hours. Phosphorised caseinogen when tested in the same way gave the following results:

Trypsin	Slow liberation of inorganic phosphorus.
Pepsin	Very slow liberation of inorganic phosphorus.
Bone phosphatase ...	No liberation of inorganic phosphorus.
0.25 <i>N</i> hydrochloric acid	Very slow liberation of inorganic phosphorus. Slightly slower than with pepsin.
0.25 <i>N</i> sodium hydroxide	Complete liberation of phosphorus as phosphate in 24 hours.

Phosphorised caseinogen is also coagulated by rennin in the presence of calcium chloride, a fact which seems to show that whatever modification the treatment with phosphorus oxychloride may have produced, no radical structural change can have taken place, such as, for example, destructive hydrolytic cleavage.

Re-phosphorised caseinogen.

The name "dephosphorised caseinogen" was given by Rimington and Kay to the phosphorus-free protein obtained by allowing 1 % sodium hydroxide to act upon caseinogen for 24 hours at 37°. The substance so formed resembles caseinogen, but has an appreciably lower nitrogen content. An analysis of dephosphorised caseinogen by the Van Slyke N-distribution method (see Rimington [1927]) has shown that the loss has occurred chiefly in the amide nitrogen, but also to some extent in the arginine fraction, this amino-acid having presumably been partially converted into ornithine and ammonia.

Dephosphorised caseinogen is not coagulated by rennin (one sample did, however, show slight coagulation after many hours), but appears in all other respects to be very similar to caseinogen, except for less ready hydrolysis by proteolytic enzymes, due no doubt to partial racemisation having occurred during the action of the alkali.

4.7 g. of a preparation of dephosphorised caseinogen were phosphorised by means of 13 g. of phosphorus oxychloride in 75 cc. of carbon tetrachloride and yielded a substance, here referred to as "re-phosphorised caseinogen."

Analysis

Total N (Kjeldahl)	...	...	...	14.16 %	} P/N=0.124
Total P (Neumann)	...	...	...	1.75 %	

It was not appreciably acted on by proteolytic enzymes nor by bone phosphatase preparations, but lost all its phosphorus as phosphoric acid in 24 hours when acted upon by 0.25*N* sodium hydroxide at 37°. Towards 0.25*N* hydrochloric acid it displayed the same behaviour as phosphorised caseinogen.

Re-phosphorised caseinogen was found to be coagulated by rennin in the presence of calcium chloride, although less rapidly than is caseinogen under the same conditions. Possibly the property of coagulability by rennin is a feature dependent upon the presence in the molecule of organically bound phosphorus. That the nature of the protein is also of importance is what would have been expected *a priori*. Phosphorised globulin prepared by the phosphorylation of alcohol-denatured serum globulin yields insoluble precipitates at once on the addition of soluble calcium salts and one is led to wonder whether the obscure modification of physical properties brought about by the action of rennin upon caseinogen solutions is not, in reality, a process analogous to the denaturation of albumins and globulins by such agents as alcohol, heat, etc.

Phosphorised globulin.

All samples of protein used in this preparation were denatured by addition to their aqueous solutions of 5 volumes of alcohol and 30 cc. per litre of a salt buffer mixture¹. The protein was then purified by frequent reprecipitation, brought into solution in dilute sodium hydroxide and phosphorised in the usual way. It was found, however, that during the phosphorylation of globulin there is persistently formed a small quantity of material which is only soluble with difficulty in dilute acids and alkalis. This substance, unless removed by centrifugation, renders the subsequent operations involved in the purification of the phosphorised globulin somewhat difficult. It contains phosphorus in about the same proportion as phosphorised globulin but has not been further investigated.

The dried phosphorised globulin is a fine, white powder, insoluble in water, but dissolved by addition of sodium hydroxide. Its solutions give an immediate precipitate on addition of calcium chloride solution.

Analysis

H ₂ O lost at 107° (% wet weight)	...	...	...	10.19
Total N (Kjeldahl, % dry weight)	...	...	...	13.85
Total P (Neumann, % dry weight)	...	...	...	0.71
Ratio P/N = 0.051				

In order to investigate the stability of the combined phosphorus towards enzyme preparations, etc., solutions of phosphorised globulin were incubated at 37° with the different enzymes at the requisite p_H and in presence of suitable buffers. Specimens were taken at intervals, and inorganic phosphorus was determined colorimetrically by the method of Bell and Doisy.

The results, with the exception of the action of trypsin, are summarised below:

Bone phosphatase	...	...	No inorganic P formed.
Pepsin	...	...	Very slow liberation of inorganic P, 40 % as PO ₄ in 10 days.
Bone phosphatase after pepsin...	...	...	No liberation of inorganic P.
0.25N hydrochloric acid	...	...	Very slow liberation of inorganic P, 35 % as PO ₄ in 10 days.
0.25N sodium hydroxide	...	...	Complete liberation of P as PO ₄ in 24 hours.

The action of trypsin on phosphorised globulin.

It was shown by Rimington and Kay that trypsin very rapidly liberates the whole of the phosphorus from caseinogen in the form of a diffusible, organic compound which is not precipitated by trichloroacetic acid. This substance is very slowly acted upon by trypsin with the formation of inorganic phosphoric acid, but is hydrolysed with extreme rapidity by bone phosphatase preparations, which split off approximately 67 % of the phosphorus as PO₄. The remaining 33 % may be liberated by means of kidney phosphatase

¹ Made up as follows:

Sol. A. 40 g. potassium sulphate, 202.5 g. sodium sulphate (cryst.), 360 cc. water heated up to effect solution then cooled.

Sol. B. Normal acetic acid.

Sol. C. Normal sodium acetate.

Equal volumes of A, B and C are mixed: $p_H = 4.7-4.8$.

preparations. Neither kidney nor bone phosphatase, however, has any appreciable action upon the phosphorus of caseinogen itself.

Phosphorised globulin was investigated in a similar manner. It was found that, in this case also, *trypsin brought about a very rapid liberation of the entire phosphorus in the form of a soluble organic compound*, but only brought about the subsequent hydrolysis to free phosphoric acid at an extremely slow rate. Bone phosphatase preparations, although they have, as just mentioned, no action upon phosphorised globulin, produced, when added to the solutions containing this organic, soluble derivative, *a liberation of the entire phosphorus as phosphoric acid within 10 hours*. This is shown in the accompanying curve, Fig. 1, the curve obtained from caseinogen using the same enzyme preparations being shown for comparison in Fig. 2.

Fig. 1. Action of trypsin and of bone phosphatase on phosphorised globulin.
 — trypsin. - - - bone phosphatase.

Fig. 2. Action of trypsin, bone and kidney phosphatase on caseinogen.
 At A a further quantity of bone phosphatase was added.
 At B kidney phosphatase (rabbit) was added,

It would appear therefore that a similarity exists between the nature of the phosphorus linkage in phosphorised globulin and in caseinogen, with the exception that, in the latter, a portion (1/3rd) of the total phosphorus differs from the remainder in being stable towards bone phosphatase, although hydrolysed by kidney phosphatase preparations.

The nature of the linkage involved in the phosphorus-containing proteins.

While as yet no decisive evidence is available, the work already published would render it highly probable that in caseinogen phosphorus is held, in part at least, by means of an ester linkage between phosphoric acid and some constituent of the caseinogen molecule. The existence of a possible NH-P linkage between an amino-group and phosphoric acid seems to have been disproved by the experiments of Rimington and Kay [1926], and evidence is also presented here against the view that such a combination takes place when proteins are phosphorised by phosphorus oxychloride¹.

Should such a phosphorus-nitrogen linkage exist, it should, on hydrolysis, give rise to a free amino-group titratable by means of formaldehyde according to the Sørensen technique. In order to test this possibility, suitable quantities of the phosphorised proteins were incubated at 37° with 0.25*N* sodium hydroxide for 24 hours under which conditions the whole of the phosphorus is removed as phosphate. The solutions were then aerated for two or three hours at room temperature in order to remove any ammonia [see Rimington and Kay, 1926] and the amino-nitrogen was determined by formaldehyde titration, a sample of the mixture having been titrated immediately after the addition of the sodium hydroxide. In no case was any increase in titratable groups found which was outside the error of the method. This fact in conjunction with the ready hydrolysis by the phosphoric esterase of bone would indicate that the phosphorus has entered the molecule in combination with some hydroxyl group.

Now the number of free hydroxyl groups in proteins is not known, although within the past few years several hydroxy-derivatives of previously known amino-acids have been found to be integral constituents of the protein molecule. Thus Dakin [1918] has estimated the amount of hydroxyglutamic acid in caseinogen as 10 % so that it is probable that the number of free hydroxyl groups is sufficient to account for the quantities of phosphorus which proteins are found capable of taking up. The method might even serve as some guide to the number of free hydroxyl groups present.

The possibility must be borne in mind, however, of the existence in the protein molecule of diketopiperazine rings which, in the "enol" form would be capable of taking up phosphorus just as the hydroxyl group of an amino-

¹ *p*-Chloroanilinephosphinic acid—in which phosphorus is directly linked to nitrogen—was found to be comparatively stable in alkaline solution but was decomposed rapidly with liberation of phosphoric acid by 0.25*N* hydrochloric acid at 37°. Neither trypsin nor phosphatases had any action on this substance.

acid. Whether these cyclic structures do exist, as maintained by Abderhalden and Komm [1924], in natural proteins, and if so in what form, are questions which are as yet undecided, but even supposing an equilibrium to exist between "keto" and "enol" modification, it is quite conceivable that phosphorus oxychloride, by combining with the "enol" form might so alter the equilibrium that eventually all the diketopiperazines pass over into this type. In this connection it is of interest to recall the stability of the diketopiperazine ring to trypsin and to compare it with the resistance offered by the organic, soluble phosphorus compounds of caseinogen and of phosphorised globulin towards this enzyme.

According to Bergmann [1924], a certain lability characterises the derivatives of the amino-acid serine, in which a benzoyl group has been introduced in place of hydrogen. Bergmann found that so slight a change as that of the medium from acid to alkaline reaction was sufficient to cause migration of the benzoyl group from union with the hydroxyl to the nitrogen atom.

As a result of his investigations, he is led to the view that such interchanges may take place in the protein molecule, thus furnishing a possible explanation of the extreme lability characteristic of protein material. One cannot discount the possibility that a phosphoryl group might behave in a manner at any rate analogous to the benzoyl group, although, up to the present, no experimental fact has been encountered supporting such an opinion.

SUMMARY.

1. Proteins may be phosphorised by means of phosphorus oxychloride in a manner analogous to the Schotten-Baumann reaction.
2. Caseinogen, serum globulin and dephosphorised caseinogen have been treated in this way. Phosphorised caseinogen contains 1.77 % P, corresponding to a ratio $P/N = 0.130$. "Re-phosphorised caseinogen," the product obtained from dephosphorised caseinogen, contains 1.75 % P and has a ratio $P/N = 0.124$. Both substances are coagulable by rennin.
3. Phosphorised globulin contains 0.71 % P with $P/N = 0.051$. It is precipitated by soluble calcium salts.
4. The phosphorised proteins lose phosphorus slowly as phosphoric acid under the action of pepsin or 0.25 *N* hydrochloric acid, but 0.25 *N* sodium hydroxide at 37° liberates the whole of the phosphorus as phosphoric acid in 24 hours. Bone phosphatase liberates *no* inorganic phosphorus.
5. Trypsin brings about a rapid separation of the whole of the phosphorus of phosphorised globulin in the form of an organic, acid-soluble substance. It only liberates phosphoric acid from the compound with extreme slowness.
6. Bone phosphatase liberates the whole of the phosphorus from this organic substance as phosphoric acid within a few hours. In this respect, phosphorised globulin exhibits a similarity to caseinogen.

7. Phosphorus appears to enter the protein by means of an ester linkage between phosphoric acid and some hydroxyl group.

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