CLVIII. THE PHOSPHORUS OF CASEINOGEN.

II. CONSTITUTION OF PHOSPHOPEPTONE.

By CLAUDE RIMINGTON.

(*Benn W. Levy Student, Research Scholar of Emmanuel College, Cambridge.*)

From the Biochemical Laboratory, Cambridge.

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IN the preceding paper [Rimington, 1927, 2] the isolation from caseinogen of a pure phosphorus-containing peptone has been described. This substance, which has been named *phosphopeptone*, and whose composition is represented by the formula $C_{37}H_{62}O_{33}N_9P_3$, has been isolated in a yield corresponding to 50 % of the organic phosphorus of the original tryptic digest. In the present paper evidence is adduced to show that the remaining organic phosphorus is present in similar if not identical form, and an account is given of the isolation and identification of the individual units of phosphopeptone, together with a suggested structure.

ISOLATION OF THE AMINO-ACIDS IN PHOSPHOPEPTONE.

20 g. of phosphopeptone were hydrolysed by boiling with 20 % hydrochloric acid for 36 hours. The resulting solution was concentrated and saturated with hydrogen chloride. After 4 days in the ice-box and a second saturation no material had crystallised except a small quantity of ammonium chloride. Hydrochloric acid was removed from the liquid by repeated concentration *in vacuo*; the resulting syrup was then diluted to about 200 cc. and the amino-acids were converted into their calcium salts by the method of Foreman [1914]. The phosphoric acid was at the same time converted into calcium phosphate and removed with the excess of lime by filtration.

After concentration *in vacuo*, the salts of the dibasic acids were precipitated by 97 % alcohol, about 2 l. being required. The sticky precipitate was dissolved in water and calcium removed quantitatively with oxalic acid. The solution was then concentrated and saturated with hydrochloric acid gas.

No glutamic acid hydrochloride could be separated even after long standing. Hydrochloric acid was therefore removed by repeated evaporation *in vacuo*, and the amino-acids converted into their copper salts by boiling with fresh copper hydroxide. The dark blue solution was concentrated and allowed to stand at 0° but nothing crystallised out. Aspartic acid was therefore absent.

Copper was removed from the solution by hydrogen sulphide and traces of chloride by acid silver nitrate, sodium hydroxide was then added to neutrality and the silver salt of hydroxyglutamic acid precipitated as described by Dakin [1918]. Thus obtained it was nearly pure, and, after one reprecipitation, the silver salt was analysed.

<i>Analysis.</i> $C_5H_7O_5NaAg_2$.				
	C	H	N	Ag
Found	15.42	1.75	3.5	57.5 %
Calculated	15.9	1.85	3.7	57.3 %

Yield: just over two molecules per molecule of phosphopeptone. For further identification the copper salt was prepared by boiling a solution of the acid, prepared from the silver salt, with excess of freshly precipitated copper hydroxide, filtering, concentrating, and precipitating the copper salt by alcohol.

<i>Analysis.</i> $C_5H_7O_5NCu$.				
	C	H	N	Cu
Found	25.1	3.50	5.8	27.4 %
Calculated	26.7	3.12	6.2	28.3 %

As the copper salt is not very suitable for analysis and as difficulty was experienced in getting the silver salt quite free from silver oxide, the free acid was also prepared by decomposing 1.5 g. of the silver salt with hydrogen sulphide and concentrating the resulting solution over sulphuric acid *in vacuo* at room temperature. It was obtained (0.7 g.) in microcrystalline form and corresponded with Dakin's [1918] description, giving all the colour tests described by him. When heated it softened indefinitely at about 100°. For analysis a sample was dried over phosphorus pentoxide at 35–40°.

<i>Analysis.</i> Ash = 1.2 %.			
	C	H	N
Found (ash-free)	36.5	5.60	8.60 %
Calc. for $C_5H_7O_5N$	36.8	5.52	8.59 %

The only dibasic acid present in the peptone is hydroxyglutamic acid. Its nitrogen corresponds to between two and three atoms out of the total of nine in the molecule.

The alcoholic solution of the calcium salts of the monobasic acids was concentrated *in vacuo* and calcium was removed by oxalic acid. The resulting solution gave a precipitate with neutral lead acetate, which proved to be only lead oxalate, due to a slight excess of oxalic acid in the solution. No lead salts were precipitated from the filtered solution by the addition of ammonia. Lead was removed from the filtrate as sulphide. The resulting liquid was concentrated to a small bulk *in vacuo* at 37° and the concentration continued over sulphuric acid *in vacuo* at room temperature. A syrup was obtained which gradually deposited a fairly large quantity of crystalline material.

Under the microscope two types of crystal were visible; in the greater proportion, very small platelets or prismatic needles, frequently grouped

together in dense aggregates, and among these large, rather irregular plates similar in appearance to crystals of serine.

A method of separation based upon chemical properties was sought without success. An attempt was therefore made to achieve separation by fractional crystallisation of the remainder of the material.

Differences in solubility were not great, but the more abundant substance was eventually obtained in a fairly pure state. It was very hygroscopic and had to be dried to constant weight over phosphorus pentoxide *in vacuo* at 50° before analysis. The figures obtained agree most nearly with the formula $C_4H_5O_3N$ which is that of hydroxyaminobutyric acid, a homologue of serine.

Analysis (micro.).

	C	H	N
Found	41.18	8.83	11.67 %
Calc. for $C_4H_5O_3N$	40.34	7.56	11.77 %

The copper salt was prepared by boiling a solution of the acid with freshly precipitated copper hydroxide. On concentration the liquid deposited small prismatic crystals having an intense deep blue colour. The copper salt of hydroxyaminobutyric acid was found by Schryver and Buston [1926] to contain two molecules of water of crystallisation.

Analysis. $(C_4H_5O_3N)_2Cu \cdot 2H_2O$.

	N	Cu
Found	8.60	19.73 %
Calculated	8.34	18.93 %

The dibenzoyl derivative was prepared by adding 3 cc. benzoyl chloride and 7 cc. 5*N* sodium hydroxide to a solution of 0.5 g. of the acid in 10 cc. water. After acidification, the precipitated product was extracted repeatedly with boiling light petroleum to remove benzoic acid and the insoluble residue was crystallised from a little hot benzene. It crystallised in fine needles having M.P. 109°. Schryver gives the M.P. as 112°.

The second fraction was small in amount and was consequently more difficult to obtain in a pure state. It crystallised in flat plates, was hygroscopic and was analysed after desiccation over phosphorus pentoxide.

Analysis. Nitrogen 12.60 %; Ash 2.83 %; Nitrogen (ash-free) 12.97 %.

The nitrogen content of serine is 13.33 %.

The substance melted with decomposition at 223°. The M.P. of *l*-serine is 224°. It also gave a benzoyl derivative which crystallised in fine needles, M.P. 121°. Dibenzoylserine melts at 124°.

Lack of material prevented further methods of identification, but from the above data it appears probable that the substance under investigation was an impure specimen of serine.

No other amino-acids were found.

The difficulty of separating the hydroxyamino-acids from one another and from other amino-acids is well known. In the above case the yield of hydroxyglutamic acid corresponded to between two and three molecules per molecule

of phosphopeptone, but the yields of hydroxyaminobutyric acid and of serine were considerably smaller.

The possibility exists that phosphopeptone may contain some hitherto unknown amino-acid, nevertheless, assuming that only the three which have been isolated are present, namely, hydroxyglutamic acid, hydroxyaminobutyric acid and serine, in the relative proportions depicted below, a formula can be arrived at for the peptone which agrees with that indicated by elementary analysis.

The molecule of phosphopeptone would thus appear to be made up of the following:

Hydroxyglutamic acid	3 molecules
Hydroxyaminobutyric acid	4 „
Serine	2 „
Phosphoric acid	3 „

Hydroxyaminobutyric acid has so far been isolated from only one protein, namely, from oat glutelin by Schryver and Buston [1926]. The above scheme would require its presence in caseinogen to the extent of 1.8 % or, if the whole of the phosphorus is similar to phosphopeptone, of about 3.6 %. Similarly the serine would amount to 0.8 % or 1.6 % of the protein. The best yield of serine from caseinogen so far recorded is 0.5 % but the amount actually present is undoubtedly greater [cf. Emil Fischer, 1903].

CONSTITUTION OF PHOSPHOPEPTONE.

In endeavouring to form a picture of the way in which the units of this peptone are united certain requirements, determined by experiment, must be fulfilled, namely:

- (i) The molecular formula shall be $C_{37}H_{62}O_{33}N_9P_3$.
- (ii) The substance must behave as a nine-basic acid.
- (iii) One-ninth of the total nitrogen shall be present in the form of amino-nitrogen, the remaining linkages must be peptide linkages.
- (iv) An explanation must be offered of the fact that two-thirds only of the total phosphorus is removed by bone phosphatase in the form of phosphoric acid, the remaining one-third being however almost certainly also in ester linkage as it is removed by the phosphoric esterase of kidney extracts [see Rimington and Kay, 1926].

Whilst it must be emphasised that as yet there is no possible means of deciding to *which* of the constituent amino-acids the phosphorus atoms are attached, nor in what sequence they occur, a structural formula can be constructed which satisfies *all* of the above conditions. This formula is depicted in Fig. 1 but it must be remembered that the positions of the amino-acids and phosphorus atoms are chosen arbitrarily.

It will be seen that two of the molecules of phosphoric acid are visualised as being united through one hydroxyl group only to the amino-acid chain, whilst the third forms a di-ester linkage. This affords a ready explanation of

the difference between the action of bone and of kidney phosphatase, a fact which was difficult to interpret upon any other basis¹.

Subsequent work may demonstrate the exact positions of the phosphorus groups, but in the meantime it was thought advisable to give the above formula for the sake of descriptive clearness.

Fig. 1.

(3 molecules of
hydroxyglutamic acid)

(4 molecules of
hydroxyaminobutyric acid)

(2 molecules of
serine)

THE RELATION OF PHOSPHOPEPTONE TO THE REMAINDER OF THE CASEINOGEN PHOSPHORUS.

As has been pointed out, the yield of pure phosphopeptone prepared from tryptic digests of caseinogen represents 50 % only of the original organic phosphorus. When however the action was investigated of bone and kidney phosphatase upon phosphopeptone and upon neutralised tryptic digests respectively a very striking similarity was observed. In both cases only two-thirds of the total organic phosphorus was hydrolysed by bone phosphatase preparations, the remainder being liberated by the kidney enzyme. These results are shown in the two figures previously published [Rimington and Kay, 1926, p. 783, Fig. 4; Rimington, 1927, 1, p. 278, Fig. 2].

It appears probable therefore that if other substances analogous to phosphopeptone exist, the form of the phosphorus linkages in them must be similar to those in phosphopeptone itself. The peptide linkages of phosphopeptone appear to be fairly resistant to tryptic hydrolysis; on the other hand, the peptone is split off from the rest of the caseinogen molecule with such rapidity that the linkages between it and the parent grouping must be of a labile nature and readily attacked by trypsin.

To the above considerations is due, no doubt, the fact that the material isolated from different preparations has, when pure, always the same composition.

¹ Martland and Robison [1927] have recently shown that bone phosphatase has very little action upon the diethyl ester of phosphoric acid while the monoethyl ester is readily hydrolysed.

DISCUSSION.

The association of phosphoric acid in the caseinogen molecule with a complex of hydroxyamino-acids raises several interesting questions. No evidence is as yet available concerning either the specific metabolic importance of the hydroxyamino-acids as a class, or the nutritional significance, if any, of phosphoric acid united with them in such a compound as the phosphopeptone just described. The behaviour of this peptone as a whole, its ready diffusibility and resistance to trypsin in no way exclude the possibility of its absorption from the gut into the blood-stream unchanged.

Its ready hydrolysis by the enzymes of ossifying cartilage also makes the suggestion not improbable that the phosphorus of caseinogen may by this means contribute in part to the inorganic phosphate laid down in bone formation. Experiments bearing upon this aspect of the problem are now in progress.

Caseinogen has frequently been regarded as a conjugated protein, the phosphorus it contained having been assumed to be present in some prosthetic group. The association seen in phosphopeptone cannot be regarded as of this kind, *i.e.* one in which constituents of a non-protein nature are characteristically present.

During the course of proteolysis, fragments, some large and some small, are formed by cleavage of the protein under the action of the enzyme. Some of these fragments show comparative resistance towards further hydrolysis, and phosphopeptone, however built into the general architecture of the caseinogen molecule, must be regarded as of this type.

Many proteins other than caseinogen contain phosphorus, although usually to a much smaller extent. It is, however, impossible to state whether or not in these cases also the association is one of phosphoric acid with hydroxyamino-acids. If such should prove to be the case, it might be permissible to regard the combination as a new unit constituent of proteins in general, such as, for example, phosphorylglutamic acid, which would be related to hydroxyglutamic acid in much the same way that cysteine is to serine.

SUMMARY.

1. From phosphopeptone after hydrolysis have been isolated hydroxyglutamic acid, hydroxyaminobutyric acid and serine.
2. From the above data a hypothetical structural formula has been constructed that is in agreement with all other known chemical properties of the peptone.

In conclusion I wish to thank Sir F. G. Hopkins for the interest he has taken in this work and also Dr H. D. Kay for encouragement and advice.

Note in conjunction with H. D. Kay.

S. Posternak recently called our attention [Posternak, 1927, 1] to work of his own on a patented process for "preparing the phosphorus nucleus of caseinogen" which had appeared (unaccompanied by the author's name) in a Swiss Patent Specification in 1923. Although we had examined the literature, this specification had unfortunately escaped our notice. Lately [1927, 2] he has claimed to have isolated from caseinogen a pure phosphopolypeptide which contains the following amino-acids: glutamic acid, 3 mols.; aspartic acid, 0.6 mols.; *isoleucine*, 3 mols.; serine, 3 mols.; phosphoric acid, 4 mols. From these findings he comes to the rather surprising conclusion that the phosphorus nucleus of caseinogen is composed of four serine-phosphoric acids.

Knowing some of the difficulties and pitfalls attending this work of purification and analysis, we are of opinion that it is only in the light of considerably more experimental and analytical detail than is presented by Posternak that his conclusions regarding the chemical composition of the "phosphorus nucleus of caseinogen" can be assessed.

If we take his statements at their face value we have no alternative but to conclude that the phosphopeptone first obtained by us in a crude form in 1924 (described at a meeting of the Biochemical Society on Feb. 9th, 1925 [Rimington and Kay, 1925]), and which has now been purified and analysed by one of us, is entirely different from the "pure" substance described by Posternak.

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