32. NOTE ON THE AMINO-ACIDS PRESENT IN PHOSPHOPEPTONE

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SOME years ago Rimington & Kay [1926] gave the name 'phosphopeptone' to a substance present in tryptic digests of caseinogen and later isolated [Rimington, 1927, 1] by a process involving separation of inert material and then precipitation of the phosphorus-rich complex as an insoluble uranyl derivative and lastly as a copper salt.

The N/P ratio of the crude copper salt was approximately 3·3/1, but by fractional copper precipitation a main homogeneous constituent with N/P = 3·0 was obtained and used for detailed study of amino-acid composition, etc. The fractions with higher and lower N/P ratios were not analysed in this way, but it was realized that peptones of varying composition might easily be obtained as a result of more or less prolonged tryptic digestion before isolation was commenced. The main phosphopeptone was not completely stable towards activated pancreatic juice [Rimington & Kay, 1926].

Examination of phosphopeptone by the methods then available for the identification of amino-acids [Rimington, 1927, 2] revealed the presence of serine and a dicarboxylic acid considered to be hydroxyglutamic acid and also an incompletely identified substance thought, possibly, to be β-hydroxy-α-amino-butyric acid.

Since these early studies, investigations have been published by several workers. Schmidt [1934], after prolonged tryptic digestion of caseinogen, isolated a substance with N/P = 2, composed of glutamic acid and serine; fractions with higher N/P ratios were also encountered, when the digestion was less complete, in which a leucine was also present. Levene & Hill [1933], combining a preliminary tryptic digestion of caseinogen with subsequent hydrolysis by boiling acid, succeeded in isolating from the mixture a dipeptide of glutamic acid, serine and phosphoric acid, whilst Lipmann [1933] by boiling acid alone obtained from his hydrolysates serine phosphoric acid; peptides of higher N/P ratio were also present in the mixtures. More recently Damodaran & Ramachandran [1940], in a preliminary communication, claimed to have isolated a phosphorus-containing peptone from caseinogen by applying pepsin, separating the paranuclein formed and then digesting with trypsin. It had N/P = 3·3 and is reported to contain 3 mol. glutamic acid, 3 mol. *iso*leucine and 4 mol. serine.

Although it is only to be expected that the use of different chemical or enzymic hydrolyses (and for different lengths of time) should lead to phosphorus-containing materials of different constitution, it was thought desirable to re-examine the original 'phosphopeptone' preparations, now that more reliable methods are available for the detection and determination of amino-acids like threonine. The isolation of serine phosphoric acid after acid hydrolysis of caseinogen by Levene & Hill [1933] supports my conclusion that a part, at least, of the phosphorus in phosphopeptone is united to serine, but from the colorimetric determinations presented by Rapoport [1937] the question arises whether or not all the phosphorus in caseinogen is bound in this way.

Experimental

There was available for this work a small quantity of the original phosphopeptone described in 1927 and a larger quantity of crude copper salt prepared subsequently. From the latter the copper was removed as sulphide, employing fuller's earth to facilitate filtration [Rimington, 1927, 1], and the free peptone was precipitated by addition of excess of alcohol. After dehydration with absolute alcohol and ether it was dried *in vacuo* at 105° over P_2O_5. Both peptones were colourless powders, readily soluble in water to form strongly acid solutions.

Analysis. Determination of N/P ratios gave the values 3·01/1 and 3·33/1 for the original peptone and the larger sample respectively. Since amino-N determinations and other data indicate that there are 3 P atoms per mol., these substances contained 9 and 10 N atoms respectively and will be referred to as the N_9 and N_{10} peptones. Elementary analysis gave the following results:

	C	H	N	P
N_9 peptone	35·29	6·16	9·86	7·27
$C_{37}H_{60}O_{33}N_9P_3$ requires	35·50	4·80	10·07	7·44
N_{10} peptone	37·58	6·16	10·40	6·88
$C_{43}H_{71}O_{34}N_{10}P_3$ requires	37·83	5·21	10·27	6·82

It will be noticed that the empirical formulae, deduced from the analytical figures and assuming 3 phosphoric acid residues per mol., differ by one molecule of leucine or *iso*leucine, $C_6H_{13}O_2N$, in peptide linkage.

Determination of dicarboxylic acids and isolation of glutamic acid

Hydrolysates of the N_{10} and N_9 peptones were prepared by boiling for 48 hr. with 20 % HCl. Portions of these were worked up by Foreman's quantitative calcium salt method for the determination of dicarboxylic amino-acids. The result in each case corresponded to rather less than 4 mol. of dicarboxylic acid per mol. of peptone, thus:

N_9 peptone:	total N in hydrolysate	19·72 mg.
	4/9 of total N	8·76 ,,
	total N in Ca salt fraction	8·64 ,,
N_{10} peptone:	total N in hydrolysate	166·0 ,,
	4/10 of total N	66·4 ,,
	total N in Ca salt fraction	59·3 ,,

Since hydroxyglutamic acid had previously been suspected, in the dicarboxylic acid fraction, calcium was removed from a portion by means of oxalic acid and the crude amino-acid obtained oxidized with chloramine T as described by Harington & Randall [1931]. The mixture was treated with *p*-nitrophenylhydrazine and warmed on the water bath for 45 min., then filtered and the residue washed repeatedly with boiling methyl alcohol until a small dark red residue remained, a trace of which afforded an intense pure blue colour with alcoholic KOH. The substance was recrystallized from hot nitrobenzene, from which it separated in the form of dark red needles melting at 322–7°. The yield was only 4·4 mg. from 0·3 g. of starting material and the substance contained 22·6 % N. It was considered to be the *p*-nitrophenylosazone of glyoxal, since the M.P. was not lowered by admixture with this substance (N = 25·6 %). Mixed with the corresponding derivative of malic semi-aldehyde (N = 21·7 %), kindly supplied by Prof. Harington, the M.P. was depressed approximately 20°. Unfortunately the M.P. of these substances are not very suitable for their

characterization; however, since serine is known to be unstable under the hydrolytic conditions employed and could readily yield glyoxal, the conclusion is probably justified that serine was present as an impurity in the crude dicarboxylic acid fraction examined.

Further evidence against the presence of any α-amino-β-hydroxy-acid in this fraction was obtained by treating an aliquot portion with periodic acid [Van Slyke *et al.* 1940], which failed to liberate any ammonia.

The remainder of the crude dicarboxylic fraction was freed from calcium by oxalic acid and concentrated to a small volume. Crystals separated which were filtered off, washed sparingly and dried (crop 1) and, by further concentration of the mother liquors, a second deposit (crop 2) was obtained. The final mother liquors were treated with several volumes of alcohol, when a small amount of crystalline material (crop 3) slowly separated. These fractions were analysed with the following result:

	C	H	N
Crop 1 (M.P. 198°)	41·01	6·05	9·64
Crop 2	40·59	6·28	9·50
Crop 3 (corrected for ash)	36·45	6·49	—
Glutamic acid requires	40·85	6·17	9·52
Hydroxyglutamic acid requires	36·81	5·52	8·59

It would appear, in the light of the experiments already recorded, that crop 3 consisted only of impure glutamic acid. There was unfortunately insufficient material for N determination. In view of the similar C and H figures which were previously reported [Rimington, 1927, 2] for the dicarboxylic acid fraction of phosphopeptone, it is noteworthy that impure glutamic acid may give analytical results so closely approaching those required by hydroxyglutamic acid.[1]

The optical rotatory power of the material of crop 1 in 10 % HCl was $[\alpha]_{5461} = +37{\cdot}39°$. The hydrochloride was easily prepared in 88 % yield and, dissolved in water (3·2 %), had $[\alpha]_D = +22{\cdot}04°$, $[\alpha]_{5461} = +27{\cdot}11°$. Anslow & King [1927] give $[\alpha]_{5461}$ as 26·85° for a 2·5 % aqueous solution and 27·45° for a 5 % solution.

Past attempts to isolate glutamic acid as the hydrochloride directly from phosphopeptone hydrolysates had not succeeded and the separation has now only been achieved with difficulty and in poor yield. It would appear that the other products of hydrolysis, such as serine and phosphoric acid, exert an inhibiting effect upon the crystallization of the hydrochloride.

5 g. of N_{10} peptone were refluxed with 50 ml. of 20 % HCl for 30 hr. and the mixture then saturated with HCl gas. In spite of seeding, not more than a trace of material separated after 24 hr. at 0°. The liquid was therefore concentrated to small volume and again saturated with HCl, when 1·4 g. of crystals separated in the course of several days. Repeated concentration and saturation raised the yield to 1·48 g., which represents only between 2 and 3 mol. per mol. of peptone.

It must be concluded from the above findings that glutamic acid and not hydroxyglutamic acid is present in phosphopeptone.

Isolation and identification of isoleucine *from* N_{10} *peptone*

The filtrate from the calcium salts of a hydrolysate corresponding to 1·6 g. of N_{10} peptone was freed from calcium by oxalic acid, made slightly alkaline to litmus with ammonia and concentrated to about 2·5 ml. Upon cooling in the

[1] Prof. A. C. Chibnall informs me that he has often obtained analytical figures for impure glutamic and aspartic acids which correspond closely to those required for hydroxyglutamic acid.

ice-chest, crystals separated which, after recrystallization from 75 % alcohol, formed fragile glistening plates. Concentration of the mother liquor yielded a further small crop: total yield 73·5 mg. 1 mol. per mol. of peptone corresponds to 155 mg.

	C	H	N
Analysis: Found	54·88	9·98	10·73
*iso*Leucine ($C_6H_{13}O_2N$) requires	54·97	9·99	10·69

A 1·6 % solution in 20 % HCl had $[\alpha]_D = +34·38°$; $[\alpha]_{5461} = +40·62°$. The $[\alpha]_D$ of *iso*leucine in this solvent is +34·26° and that of leucine is +15·4°. For further identification, the phenylisocyanate derivative was prepared. It had M.P. 117°; that of *iso*leucine melts at 119°.

Examination for threonine

Block & Bolling [1939] have recently described a method for the micro-determination of threonine depending upon its oxidation with lead tetraacetate to acetaldehyde which is determined colorimetrically after reaction with *p*-hydroxydiphenyl; serine does not interfere. When this method was applied to a phosphopeptone hydrolysate only a trace of colour resulted, corresponding to less than 1/40 mol. threonine per mol. of peptone. It is therefore concluded that threonine is absent from phosphopeptone.

Determination of serine and total hydroxyamino-acids

Phosphoserine is remarkably resistant to hydrolysis with boiling acids (personal communication from Prof. R. H. A. Plimmer); this property evidently made possible the isolation of phosphoserine by Lipmann [1933] and by Schmidt [1934] from acid hydrolysates of caseinogen. Serine itself, however, is readily transformed by hot mineral acids into ammonia, glyceric acid, glyoxal and other products (compare Damodaran & Ramachandran [1940]), so that one is beset with the difficulty, in attempting its determination in phosphopeptone, of achieving complete liberation from phosphoric acid with the minimum of destruction. The use of alkali for this purpose was not satisfactory.

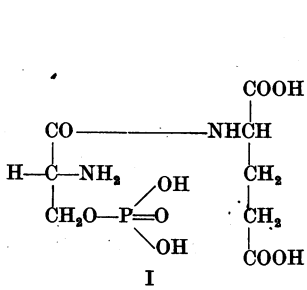
2 g. of N_{10} peptone were refluxed with 20 % HCl for 160 hr., by which time all the phosphorus was present as free phosphoric acid. Direct determination of ammonia by an aeration method showed 26·5 % of the total N to be present in this form. A periodate oxidation liberated another 13·6 %, making in all 40·1 % of the total N, or 4 mol. per mol. of peptone, derivable from hydroxy-amino-acids. On a portion of the hydrolysate, serine was determined by Rapoport's [1937] colorimetric method. The amount present corresponded to only 5·6 % of the total N, in contrast to the figure of 13·6 % of hydroxyamino-acid by periodate oxidation recorded above. This finding is similar to Rapoport's own results with casein hydrolysates, from which he concluded that at least one-third of the total phosphorus of the protein was bound to hydroxyamino-acids other than serine. In view of the known instability of serine under the conditions of hydrolysis it is doubtful, however, if much emphasis should be laid upon such negative data. It is possible that a portion of the serine originally present has been so changed that it is no longer capable of yielding glyceric acid. In fact, Damodaran & Ramachandran [1940] report that they found both lactic and pyruvic acids, presumably derived from serine, in their phosphopeptone hydrolysates.

Nature of the phosphorus linkage in phosphopeptone

The suggestion was made by Rimington [1927, 2] that two of the three phosphoric acid residues of phosphopeptone were held in mono-ester linkage whilst

the third was in the form of a di-ester. This suggestion was based upon the observed difference in action of bone and kidney phosphatases upon phosphopeptone, the former liberating only two-thirds of the phosphorus as free phosphoric acid, the latter producing complete hydrolysis. Since phosphoserine is relatively resistant to hydrolysis by boiling dilute alkali, whereas the phosphorus is rapidly split off as phosphoric acid from caseinogen by 1 % NaOH at 37°, another explanation appears possible. The action of alkali upon phosphopeptone was therefore studied again in detail.

It was found that only about two-thirds of the total phosphorus was removed as phosphoric acid by 1 % NaOH at 37° and the time curve of the hydrolysis bore a strong resemblance to that recording the action of bone phosphatase upon phosphopeptone. It appears that the relative stability towards alkali of phosphoserine, or of a terminal phosphoserine residue in peptone linkage with other amino-acids as in phosphopeptone, may be conditioned by the close proximity of the free amino group. Phosphocholine, which as Beznák & Chain [1937] point out, has a betaine structure, was similarly found by them to be remarkably stable towards boiling acids and alkalis although hydrolysed by both bone and kidney phosphatases. Plimmer & Burch [1937] reported the same behaviour on the part of phosphoaminoethanol and phosphocholine. It would be very interesting in this connexion to discover whether Levene & Hill's [1933] phosphodipeptide possessed structure I or II (Levene & Hill have formulated their substance as an aspartic acid derivative).



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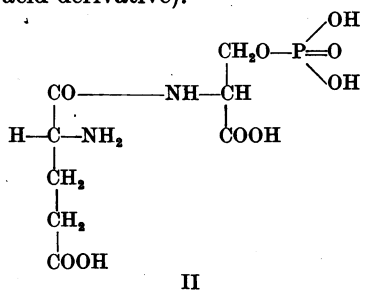

II

Discussion

On the assumption that only glutamic acid, *iso*leucine, serine and phosphoric acid are present in the N_{10} phosphopeptone, it is found possible to construct the following formulae, which are in good agreement with the elementary analysis of the peptone and roughly correspond with the yields of each amino-acid experimentally determined.

N_{10} peptone:

5 glutamic acid + 1 *iso*leucine + 4 serine + $3H_3PO_4 - 12H_2O = C_{43}H_{71}O_{34}N_{10}P_3$,

requiring	37·83 % C;	5·21 % H;	10·27 % N;	6·82 % P.
Found	37·58 % C;	6·16 % H;	10·40 % N;	6·88 % P.

Experimentally not more than 4 mol. of glutamic acid were found by the Ca salt method, but there were also found 4 mol. of serine and at least 1 mol. of *iso*leucine. Similarly for the

N_9 peptone:

5 glutamic acid + 4 serine + $3H_3PO_4 - 11H_2O = C_{37}H_{60}O_{33}N_9P_3$,

requiring	35·50 % C;	4·80 % H;	10·07 % N;	7·44 % P.
Found	35·38 % C;	5·14 % H;	10·03 % N;	7·41 % P.

Once more the glutamic acid determination fell short of the required figure, but the difference in elementary composition between the N_{10} and N_9 peptones corresponds to exactly 1 mol. of *iso*leucine, and it will be remembered that this amino-acid was not detected in the course of the original study made of the N_9 phosphopeptone [Rimington, 1927, 2].

Whilst, then, some reserve must still be exercised, it is felt that the compositions suggested above do probably represent the true nature of the two peptones which have been here subjected to study. Naturally, other conditions of preparation of the caseinogen hydrolysate, e.g. prolonged tryptic digestion, might lead to peptones with a different composition, as the work of Levene & Hill and others clearly indicates; most drastic hydrolysis leads to the comparatively stable phosphoserine.

Damodaran & Ramachandran [1940] considered the phosphopeptone which they prepared to be composed of 3 mol. of glutamic acid, 3 mol. of *iso*leucine, 4 mol. of serine and 3 mol. of phosphoric acid. The yields of these substances which they obtained were in fairly good agreement with such a conclusion, but no elementary analysis of the complete peptone is recorded. This is unfortunate, since elementary analyses would have afforded a check upon the conclusions arrived at. The composition demanded by such an assembly of amino-acids differs considerably from that determined experimentally for my N_{10} peptone, thus:

N_{10} peptone:	C	H	N	P
Found	37·78	5·30	10·25	6·81
Calculated formula $C_{43}H_{73}O_{34}N_{10}P_3$ requires	37·83	5·21	10·27	6·82
Damodaran & Ramachandran's peptone would require	40·54	5·93	10·51	6·98

One must conclude that Damodaran & Ramachandran's peptone, prepared as it is from paranuclein, differs from the phosphopeptone prepared according to my method by the action of trypsin only upon caseinogen. It appears that the essential phosphorus linkage in caseinogen is between phosphoric acid and serine and that this amino-acid is united in peptide linkage with other amino-acids, predominantly glutamic acid but possibly also *iso*leucine, and that tryptic digestion rapidly sets free these complexes from the rest of the protein molecule (compare the evidence of Rimington & Kay [1926]), but that they themselves being more resistant towards enzymic hydrolysis are only slowly degraded into peptones or peptides of ever decreasing complexity. A phosphopeptone unit containing 10 amino-acids and 3 mol. of phosphoric acid is comparatively readily isolated.

This reinvestigation of phosphopeptone is a result of communications made to the Biochemical Society (*Chem. and Ind.* **59**, 185, 1940) by Prof. R. H. A. Plimmer on 'Phosphoryl hydroxyamino-acids' and by Dr J. Lowndes on 'The composition of phosphopeptone'. I have enjoyed the benefit of frequent discussion with the authors and this paper is published with their approval. I desire to thank Prof. Plimmer for a specimen of *dl*-threonine and Prof. Robison for a specimen of the Ba salt of 3-phosphoglyceric acid.

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