31. ANALYSIS OF PROTEINS

13. CASEO-PHOSPHOPEPTONE

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POSTERNAK [1927, 1, 2] first prepared a polypeptide from casein with a P : N ratio of 4 : 15 or 16, and isolated from it the equivalents of 3 mol. glutamic acid, 0.6 mol. aspartic acid, 3 mol. isoleucine, 3 mol. serine and 4 mol. H_3PO_4 . Evidently, on account of decomposition of serine during hydrolysis, he concluded that the phosphopeptone contained 4 mol. phosphoserine. Rimington [1927, 1, 2] isolated a nonapeptide which was considered to consist of 3 mol. hydroxyglutamic acid, 4 mol. hydroxyaminobutyric acid, 2 mol. serine and 3 mol. H_3PO_4 . The composition of the phosphopeptone was further examined by Levene & Hill [1933], who obtained from it a dipeptide of glutamic acid and phosphoserine. Previously, Lipmann & Levene [1932] had isolated the Ba salt of phosphoserine from a polypeptide from vitellin and Lipmann [1933, 1, 2, 3] isolated it from a phosphopeptone from casein. Glutamic acid and serine were again isolated by Schmidt [1934]. The presence of glutamic acid in a phosphopeptone from vitellin could not be established by Levene & Schormüller [1933]. Sorimati [1939] considered that he obtained the Ba salt of phosphoserylglutamic acid. The only definite products from caseo-phosphopeptone thus seem to be glutamic acid and serine. On account of the contradictory results of Posternak and Rimington and in connexion with our work on dephosphocasein and dephosphocaseose [Macara & Plimmer, 1940] experiments were carried out on caseo-phosphopeptone. A preliminary account of our results was communicated to the Biochemical Society in March 1940. Caseo-phosphopeptone appeared to be an octapeptide with 2 mol. serine, 2 mol. glutamic acid and 2 mol. H_3PO_4 . Rimington [1941] consequently re-examined his caseopeptone; he has consulted with us and kindly shown us his paper before publication. His product is now considered to be a nona- or deca-peptide with 5 mol. glutamic acid, 1 mol. isoleucine, 4 mol. serine and 3 mol. H_3PO_4 . Owing to unforeseen circumstances our work had to cease, but we feel that our data, so different from those of Rimington, should appear simultaneously. Damodaran & Ramachandran [1941] have given the composition of caseopeptone as a decapeptide with 3 mol. glutamic acid, 4 mol. serine, 3 mol. isoleucine and 3 mol. H_3PO_4 . There is thus some general agreement about the composition of the phosphopeptone, but the number of mol. of each amino-acid needs further examination.

EXPERIMENTAL

Preparation of caseo-phosphopeptone

Crude Na-phosphopeptone was prepared from 'Rhenania' casein, purchased by one of us many years ago, according to the method of Levene & Hill [1933], using portions of 50 g. casein and 1.5 g. trypsin (B.D.H.). The casein contained

10.1 % H_2O , 13.44 % N and 0.82 % P. The average yield was 3.4 g. or 6.8 %, with a N:P ratio of 4.5-5 : 1. Na_2CO_3 was invariably present.

For purification, several crops were combined, dissolved in water, the Na_2CO_3 neutralized with acetic acid, the Pb salt again precipitated and converted back into the Na salt. This was carried out three times and the Na salt thrown down with alcohol. The alcohol decanted from the syrupy precipitate contained only traces of N and P, and the syrup had a consistent N:P ratio of 4 : 1. The free acid was then made by again precipitating the Pb salt, which was washed and decomposed with H_2S . The filtrate from PbS was concentrated *in vacuo* to a small volume, filtered from the last traces of PbS, further concentrated, transferred to a basin, treated with alcohol and acetone, dried *in vacuo*, and ground to a fine powder. The yield of purified phosphopeptone was approx. 4 %. Its P content corresponded with approx. 25 % of the original casein. Inorganic phosphate liberated by trypsin corresponded with 11-12 %. The remaining 63 % not precipitated by lead acetate or alcohol awaits further investigation.

Dried *in vacuo* over CaCl_2 , the purified caseo-phosphopeptone contained 10.56 % N and 5.77 % P. Hence N:P=4:1. Amino-N determination showed 12.6 % of the total N. The phosphopeptone thus appears to be an octapeptide with 2 atoms of P.

Sulphur and diamino-acids are absent, tyrosine and tryptophan reactions are negative. Hydrolysis with 20 % HCl for 24 hr. raised the amino-N to 88 % of the total N, for 36 hr. to 98 %, and for 48 hr. to 100 %, which suggests a more resistant peptide linkage in the molecule, and shows the absence of cyclic N.

The solution of phosphopeptone is very acid in reaction:

0.2704 g. = 13.9 ml. $N/10$ NaOH to either phenol- or thymol-phthalein. On then adding neutralized formalin, the figure was raised to 15.7 ml. 0.2704 g. = 28.35 mg. N is thus equivalent to 1.39 mg. H and after formalin to 1.57 mg. H.

$$\begin{aligned}\text{Ratio of acidic H to N atoms} &= 1.39 : 28.35/14 = 0.686/1 \text{ or } 5.5/8 \\ &= 1.57 : 28.35/14 = 0.775/1 \text{ or } 6.2/8.\end{aligned}$$

The octapeptide would thus have six replaceable H atoms. Two will belong to the two H_3PO_4 radicles, two to 2 mol. glutamic acid (see below), one to a COOH group at the end of the chain, and the sixth to another COOH group.

Liberation of P by $N/4$ NaOH at 37° . For comparison with caseinogen which loses all its P as PO_4 ions in 24 hr. at 37° , 0.4240 g. phosphopeptone was dissolved in 100 ml. $N/4$ NaOH and placed in an incubator at 37° . Samples were taken at intervals and inorganic PO_4 precipitated as NH_4MgPO_4 and estimated by our usual procedure:

At 0 hr. inorg. PO_4 = 0.421 mg. P = 17.9 % hydrolysis.			
4	"	= 0.570	" = 24.3 "
8	"	= 1.566	" = 66.7 "
12	"	= 1.810	" = 77.1 "
24	"	= 2.040	" = 87.0 "
48	"	= 2.048	" = 87.3 "
Total P		= 2.346	

Rather more than half the P is separated in 8 hr. and the hydrolysis is not complete in 24 hr. and not increased in 48 hr. The difference in rate may be due to the position of the phosphoserine in the chain, one more easily hydrolysed in the middle portion, the other at the end of the chain.

The results contrast with the stability of phosphoserine (later publication) which is not hydrolysed by $N/4$ NaOH at 37° and at 100° loses 66 % in 48 hr.

Separation of H_3PO_4 by hydrolysis with acid. For future work on the isolation of amino-acids and confirmation of the production of a dipeptide of glutamic acid and phosphoserine hydrolysis by HCl at 100° was investigated:

With 5.5 N HCl for 24 hr. inorg. PO_4 separated was 53.85 % of total P					
5.5	"	48	"	100	"
2	"	10	"	15.4	"

In comparison with phosphoserine which lost 60 % of its P as inorg. PO_4 by boiling with N HCl for 24 hr. the phosphopeptone is more stable.

Estimation of glutamic acid. The opportunity was taken of estimating glutamic acid by the micro-enzymic method of Cohen [1939] at the same time as in dephosphocasein and dephosphocaseose [Macara & Plimmer, 1940].

1 g. phosphopeptone was hydrolysed with 5.5 N HCl for 24 hr. The solution was evaporated *in vacuo* several times to remove HCl, the resulting syrup dissolved in water, neutralized to pH 3 and adjusted to 50 ml. A nitrogen estimation showed the concentration to be 2 %. The procedure as described by Macara & Plimmer was then carried out:

4 ml. hydrolysate contained 22.1 mg. glutamic acid	= 27.6 %
5 " " 25.9 " "	= 25.9 %
Mean 26.8 %	

The mean value is 24.2 % of the total N, which indicates the presence of 1 mol. per 4 atoms of N or 2 mol. in 1 mol. of octapeptide.

Isolation of glutamic acid. 5 g. phosphopeptone were hydrolysed with 50 ml. 5.5 N HCl. The solution was concentrated *in vacuo* and saturated with dry HCl gas. Glutamic acid hydrochloride slowly crystallized out. Dried *in vacuo* the crude salt weighed 0.8 g. = 16 % of the phosphopeptone. The yield is far below the figure by estimation. Precipitation is probably inhibited by the presence of serine and its decomposition products.

Estimation of threonine. To establish the presence and determine the amount of this amino-acid the method of Block & Bolling [1939] as modified by Macara & Plimmer [1940] was used.

1.3824 g. phosphopeptone were hydrolysed with 5.5 N HCl for 36 hr. and the volume adjusted to 100 ml.; 2 ml. of this solution after treatment gave only a mere trace of colour with the reagent.

It had to be concluded that threonine was absent from the octapeptide.

Estimation of total β -hydroxyamino-acids. According to Van Slyke *et al.* [1940] oxidation of β -hydroxyamino-acids with KIO_4 completely separates the amino group as NH_3 . The same hydrolysate was used as for threonine.

5 ml. gave 7.3 ml. N/14 NH_3	= 10.56 % total N
5 ml. gave 1.1 ml. N/14 NH_3 by aeration with Na_2CO_3	= 15 % of total N
and 1.2 ml. N/14 NH_3 on oxidation with KIO_4	= 16 % of total N

Assuming that amide groups are not present in the phosphopeptone and that the NH_3 liberated by Na_2CO_3 arises from decomposition of serine by boiling with HCl (see below) the total of 31 % of the total N indicates the presence of 2 mol. serine in the octapeptide if hydroxyglutamic is not present. Harington & Randall [1931] could not isolate this compound from caseinogen and Gulland & Morris [1934] obtained a doubtful 0.33 %.

Estimation of serine. Attempts to estimate serine by Rapoport's method [1937] were not successful; only brown charred solutions resulted [cf. Macara & Plimmer, 1940].

Liberation of NH_3 by hydrolysis. Posternak [1927, 1] noted the liberation of NH_3 during acid hydrolysis of phosphopeptone and regarded it as indicative of serine peptides and later [1928] found 12 % evolved from caseopeptone and 20 % from vitellopeptone. As higher amounts were evolved with greater concentrations of acid he considered it did not arise from amide groups. Serine under the same conditions gave 8 %. Hydrolysis of caseopeptone with NaOH gave higher amounts. Further examination of the liberation of NH_3 seemed advisable with serine, phosphoserine and caseopeptone.

Serine. (a) 0.5 g. serine was boiled under reflux with 60 ml. 5.5 *N* HCl. Samples were taken every 12 hr., made alkaline with Na_2CO_3 and the NH_3 aerated into standard acid.

(b) A similar experiment was made with the addition of 1 equiv. of H_3PO_4 to the solution.

With HCl only				With H_3PO_4			
After 12 hr. NH_3 = 0.294 mg. N = 2.7 % total N				= 0.294 mg. N = 2.7 % total N			
24	"	= 0.399	" = 4.0	"	= 0.370	" = 3.3	"
36	"	= 0.588	" = 5.3	"	= 0.955	" = 8.6	"
60	"	= 0.785	" = 6.7	"	= 1.920	" = 17.1	"

(c) Plimmer & Lowndes [1938] found 17.9 % N liberated on boiling serine for 6 hr. with 5*N* NaOH.

Phosphoserine. 0.11 g. was dissolved in 100 ml. 2*N* NaOH. Total N in 10 ml. = 0.7 mg. The remaining 90 ml. (= 6.3 mg.) diluted with 90 ml. H_2O were distilled and the NH_3 collected at 15 min. intervals in standard acid:

In 15 min. NH_3 evolved	= 0.07 mg.
In next 15 min. NH_3 evolved	= 0.07 mg.
" "	= 0.07 mg.
After 75 min.	total = 0.30 mg. = 4.76 % of total N

Caseopeptone. (a) As stated above under KIO_4 oxidation 15 % of the total N was evolved as NH_3 on boiling with 5.5 *N* HCl for 36 hr.

(b) 0.2694 g. was dissolved in 200 ml. *N* NaOH and distilled for 1 hr. The NH_3 evolved was 21.3 % of the total N.

(c) 0.3 g. was dissolved in 100 ml. 2*N* NaOH. 10 ml. were taken for total N = 3.55 mg. The remaining 90 ml. were diluted with 90 ml. H_2O and distilled and the NH_3 collected in standard acid and titrated at 15 min. intervals. The volume was kept constant by occasional addition of water.

After 15 min. NH_3 = 5.00 mg. N = 15.7 % of total N (31.95 mg.)					
30	"	= 6.02	"	= 18.8	" "
45	"	= 6.53	"	= 20.4	" "
60	"	= 7.04	"	= 22.0	" "
75	"	= 7.40	"	= 23.2	" "
90	"	= 7.62	"	= 23.9	" "
105	"	= 7.84	"	= 24.5	" "
120	"	= 8.13	"	= 25.5	" "

Serine and phosphoserine are comparatively stable to hydrolysis by acid and alkali. Caseopeptone is unstable to both. On plotting the figures of NH_3 liberation by alkali on a curve, the rapid evolution in the first 15 min. is equivalent to 1 atom of N out of the 8 atoms in the octapeptide (12.5 % of total N). A slower liberation follows up to 25.5 % in 120 min., which is equivalent to 2 atoms of N of the octapeptide. The difference in rate suggests loss of NH_3 from phosphoserine groups at the end or middle of the chain, a free amino group at the end being very labile.

Our preparation of caseo-phosphopeptone, judging from its N and amino-N contents, is an octapeptide containing two H_3PO_4 groups, 2 mol. glutamic acid and 2 mol. serine. Its acidity indicates the presence of another mol. of a dicarboxylic acid, leaving 3 mol. of simple amino-acids.

As laboratory work has been interrupted, calculations of molecular weight and deductions therefrom may be allowed:

Mean = 1076

$$\begin{array}{r} \text{Less 2 ester linkages and 7 peptide linkages } 9 \times 18 = \underline{162} \\ 538 \end{array}$$

Mol. wt. of remaining 4 mol. = 538

Mean mol. wt. of amino-acids = 134

Mol. wt. of leucines	=131
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Mol. wt. of aspartic acid = 134

The deductions are confirmatory of the isolation of 3 mol. of isoleucine by Posternak, and by Damodaran & Ramachandran. The latter authors find caseo-phosphopeptone to be a decapeptide with 3 mol. glutamic acid, 3 or 4 mol. serine and 3 mol. H_3PO_4 . Rimington's phosphopeptone is a nona- or decapeptide. The isolation of a dipeptide of glutamic acid and phosphoserine by Levene & Hill points to the combination of these two amino-acids. In view of the rapid liberation of 1 mol. NH_3 by the action of $N/4$ NaOH and a difference in the rate of separation of H_3PO_4 we suggest that one phosphoserylglutamic group is at the end of the chain with a free amino group easily liberated, and the second group is in the middle of the chain, and picture the molecule of the octapeptide thus: phosphoserylglutamic-*X-X*-phosphoserylglutamic-*X-X*, where *X* is the unknown amino-acid, probably 3 mol. isoleucine and 1 mol. aspartic acid.

The difference between our octapeptide and the decapeptide seems to be 1 mol. of dipeptide of serine and glutamic acid, possibly at the other end of the chain from which it may be more easily split off: phosphoserylglutamic-X-X-phosphoserylglutamic-X-X-phosphoserylglutamic.

There is no evidence whether the dipeptide is phosphoserylglutamic acid, or glutamylphosphoserine. Both isomers may be present, but the one at the left-hand end seems to be phosphoserylglutamic acid. Phosphoserine may also be in combination with *isoleucine* or other amino-acid, if present.

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