13. ENZYMIC PROTEOLYSIS

4. AMINO-ACIDS OF CASEIN PHOSPHOPEPTONE[1]

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Trypsin-resistant phosphopeptones from casein have been studied by Posternak [1927; 1928] and by Rimington [1927, 1, 2]. The former isolated three compounds which he designated 'lactotyrins' and showed them to be made up of 15 to 18 amino-acid units containing serine, glutamic acid, aspartic acid and *iso*leucine. Posternak's papers contain no experimental details, and although his conclusion that the P in casein exists in the form of phosphoserine has been confirmed by the isolation of this amino-acid by Lipmann [1933], the generally accepted view of the structure of phosphopeptone itself is that advanced by Rimington [cf. Jordan Lloyd & Shore, 1938], according to whom the enzyme-resistant polypeptide is made up of 9 hydroxyamino-acid units consisting of 3 mol. of hydroxyglutamic acid, 4 mol. of hydroxyaminobutyric acid and 2 mol. of serine. The discrepancies in the results obtained by these authors and the fact that Harington & Randall [1931] were unable to identify hydroxyglutamic acid as a constituent of casein prompted the reinvestigation of this problem.

To obtain a trypsin-resistant phosphopeptone of constant composition advantage was taken of the fact that peptic digestion of casein automatically causes the separation of an insoluble complex, the so-called 'paranuclein', which contains 50–60 % of the P of casein and only about 20 % of its N. After digesting the paranuclein till constancy of amino-N was reached, the P-containing complex was precipitated with neutral lead acetate, redigested with trypsin and the phosphopeptone finally separated as the Ba salt insoluble in 50 % alcohol. Preparations so obtained were constant in composition and unaffected by further treatment with trypsin. The amino-N value of 10 % and the atomic N/P ratio of 3·2 to 3·3 indicated a polypeptide of 10 amino-acids attached to 3 phosphoric acid residues. N distribution determined according to Damodaran [1931, 1] gave amide-N values varying from 10 to 13 % in different analyses, dicarboxylic acid-N 43–47 % and monoamino-N 43–45 %, and a complete absence of basic and non-amino-acids. For isolation of the amino-acids present a combination of the butyl alcohol [Dakin, 1918], the Ba-alcohol [Kingston & Schryver, 1924] and the copper salt [Damodaran, 1931, 2] methods were used. Serine, *iso*leucine and glutamic acid were found to be the only amino-acids present. It was also seen early in the investigation that the glutamic acid isolated accounted for less than two-thirds of the dicarboxylic acid-N as found by N-distribution. In view of the previously reported presence in phosphopeptone of dicarboxylic acids other than glutamic acid and of hydroxyamino-acids other than serine, it was decided to determine hydroxyamino-acids and dicarboxylic acids by indirect methods in all amino-acid fractions separated from the hydrolysate.

For the identification of the hydroxyamino-acids oxidation with chloramine-T and subsequent preparation of suitable osazones of the hydroxy-aldehydes

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formed [Dakin, 1916; 1917] was made use of. The *p*-nitrophenyl- and the 2:4-dinitrophenyl-osazones of glyoxal (from serine), of methylglyoxal (from hydroxyaminobutyric acid) and of malic semi-aldehyde (from hydroxyglutamic acid) have M.P. ranging between 299 and 319° and are therefore unsuited for identification; the phenylosazones, on the other hand, of methylglyoxal and glyoxal, though less insoluble, have M.P. 145 and 167° respectively, which make them useful for differentiation. Figures are not available for the M.P. of the phenyl- and the 2:4-dinitrophenyl-osazones of malic semialdehyde. By the use of these derivatives it was found that there was no evidence for the presence of any hydroxyamino-acid other than serine. As quantitative isolation of this amino-acid was naturally difficult the insolubility of the 2:4-dinitrophenyl-osazone formed from it was made use of for quantitative estimation.

For the indirect estimation of dicarboxylic acids at various stages of separation the titration method of Harris [1929] was used. It soon became obvious that the amount of glutamic acid isolated was much less than the amount indicated either by the Foreman method or by titration. This discrepancy, which could not be accounted for by losses in isolation, was finally found to be caused in the case of the Foreman method by the precipitation of serine along with glutamic acid and in the titration method by the presence of ether-extractable non-nitrogenous fatty acids. From the precipitate obtained by treatment with $Ca(OH)_2$ or $Ba(OH)_2$ and alcohol according to Foreman glutamic acid and serine could be separated by fractional crystallization. Further, working with pure serine it was found that from a 3·3 % solution treated by this method this amino-acid is precipitated to the extent of 63·8 %. In the titration method the high values for dicarboxylic acid obtained were eliminated if the hydrolysate were first submitted to prolonged extraction with ether, the residual titratable acidity being then equivalent to the glutamic acid that could be isolated.

The ether-extractable acidic material, which was free from nitrogen, was found to be a mixture, the main component of which was glyceric acid, the latter being isolated and identified as its quinine salt. Varying amounts of lactic and pyruvic acids were also usually present, the quantities of the two being determined by the methods of Leib & Zacherl [1932] and of Simon & Neuberg [1931] respectively. The nature of the non-nitrogenous fatty acids indicated their secondary origin in the decomposition of serine [Abderhalden & Broich, 1933], and suggested that the NH_3 formed during acid hydrolysis of the phosphopeptone also had the same origin. The amide-N did in fact vary in different analyses; on following the time course of NH_3 liberation during hydrolysis by dilute acid according to Vickery [1922] it was seen that the NH_3 did not arise from an amide group but from deamination. The equivalence of the ether-extractable acidity to the NH_3 present in any particular hydrolysate left no doubt that the NH_3 was formed by the secondary decomposition of serine.

The results of the analyses by direct and indirect methods are summarized in Table 1.

Table 1. *Amino-acid composition of phosphopeptone*

	N as % of total N	
Amino-acid	By isolation	By estimation
Glutamic acid	29·70	29·97
*iso*Leucine	27·90	—
Serine	13·10	28·90
Ammonia	—	10·90

The hydrolysate is seen to consist of 30 % each of the three amino-acids, serine, glutamic acid and *iso*leucine. Assuming, as we are justified in doing, that the NH_3 formed during hydrolysis represents decomposed serine, it is concluded that the phosphopeptone is a polypeptide of 10 amino-acids made up of 4 mol. of serine and 3 mol. each of glutamic acid and *iso*leucine.

EXPERIMENTAL

Materials and methods

Casein was prepared (from reconstituted milk made with Australian skimmed milk powder) by the method of Cohn & Hendry [1930] and contained 15·6 % N and 0·8 % P. B.D.H. pepsin and trypsin were used for digestion.

The following methods were frequently made use of:

Oxidation of the fractions with chloramine-T and preparation of the osazones	Dakin [1918] and Gulland & Morris [1934] with modifications
Titration of dicarboxylic amino-acids	Harris [1929]
Determination of lactic acid	Leib & Zacherl [1932]
Determination of pyruvic acid	Simon & Neuberg [1931]
Organic and inorganic P	Fiske & Subbarow [1925]

Preparation of enzyme-resistant phosphopeptone

(*a*) *Preparation of 'paranuclein'.* 5 kg. of wet freshly precipitated casein, corresponding to about 1 kg. dry weight, were uniformly dispersed in 15 l. of $N/10$ H_2SO_4 at 37°, the *p*H adjusted to 1·8 by the addition of $5N$ H_2SO_4 and the mixture incubated under toluene after addition of 25 g. of pepsin dissolved in 100 ml. of $N/10$ H_2SO_4. After 48 hr. the *p*H, which had risen to 2·2, was brought back to 1·8 and a further 25 g. of pepsin added. Amino-N reached constancy at about 20 % in 7 days. No inorganic P was formed at this stage in the digest. From the paranuclein formed as a compact precipitate at the bottom most of the clear yellow supernatant liquid was siphoned off and the remaining suspension was poured into large fluted filters and allowed to drain overnight in the refrigerator. The pasty solid was collected, stirred up with large volumes of water and washed free from H_2SO_4 by decantation and filtration as before. The product obtained after drying contained 15·2–15·6 % N, about 2 % P and had an atomic N/P ratio of 15–18 (compared with a ratio of about 44 for casein). Amino-N by the Van Slyke method was 12 %. The usual yield of dry paranuclein from 1 kg. of casein was 200–220 g., representing about 20 % of the N and 50–60 % of the P of casein.

(*b*) *Digestion of paranuclein with trypsin.* Trial experiments showed that digestion with trypsin at *p*H 8·3 caused only minimal liberation of inorganic P. At the end of 7 days when tryptic hydrolysis had reached completion (at 50–52 % of total hydrolysis) the inorganic P present was less than 5 %. The wet paranuclein from the peptic digestion was suspended in about 3 l. of distilled water and N NaOH added slowly with vigorous mechanical stirring till solution was complete. The solution, the *p*H of which was usually about 7 at this stage, was brought to 8·3 by the further addition of NaOH and incubated at 37° under toluene with 5 g. of trypsin. After 24 hr. amino-N of the solution was 40 % of the total N and the *p*H 6·3. No inorganic P was present. After readjusting the *p*H and adding a further 5 g. of trypsin, digestion was allowed to continue till amino-N failed to increase. This happened usually in 7 days when amino-N was about 53 % and about 5 % of the P was present in inorganic condition.

(c) *Precipitation of phosphopeptone as Ba salt.* The digest was now brought to *p*H 4·6 with glacial acetic acid and after keeping overnight in the ice-chest was filtered from the small amount of suspended material. To the filtrate a concentrated solution of neutral Pb acetate was added with vigorous stirring till precipitation was complete. The Pb precipitate, which contained the whole of the organic P of the digest, was separated by centrifuging, suspended in water and decomposed by H_2S. The filtrate (about 1·5 l.), freed from Pb and H_2S, was redigested with 2 g. of trypsin and reprecipitated with Pb acetate as before. The solution obtained after decomposing the precipitate and freeing from H_2S was treated with 30 g. of Ba acetate, made alkaline with $Ba(OH)_2$ and rapidly filtered into a beaker containing acetic acid. After adjusting the *p*H to 7 by the addition of $Ba(OH)_2$ an equal volume of alcohol was added with stirring. The fine precipitate obtained was separated on the centrifuge, washed repeatedly with 50% alcohol, then with absolute alcohol and ether and finally dried *in vacuo* over H_2SO_4.

Redigestion of the Pb precipitate with trypsin was found to be an essential condition for obtaining a Ba salt of constant composition which was resistant to further enzyme action. In earlier experiments in which the decomposed Pb salt was directly converted into the Ba compound, amino-N values ranging from 7·96 to 9·72% and N/P ratios varying from 2·6 to 3·72 were obtained. However, preparations obtained as described above showed little variation in composition, as will be seen from the analyses of different samples (Table 2).

Table 2. *Analyses of Ba phosphopeptones*

Preparation no.	Amino-N as % of total N	N %	P %	N/P atomic	Ba %	Opt. rotation $[\alpha]_D^{31°}$ in H_2O
III	9·94	6·48	4·53	3·20	33·45	+34·81°
IV	9·84	6·44	4·26	3·35	33·12	
V	9·98	6·39	4·58	3·16	34·02	
VI	9·96	6·46	4·34	3·30	33·31	

Properties of the phosphopeptone

The Ba salt was extremely soluble in water, giving a water-clear solution which was neutral in reaction. The free peptide obtained by removing the Ba quantitatively with H_2SO_4 was strongly acidic. It was not precipitable by trichloroacetic, phosphotungstic or tannic acid, but was precipitated by heavy metal salts such as Pb, Cu and U acetates. It gave none of the usual colour reactions for individual amino-acids and was free from inorganic P. It underwent no hydrolysis on treatment with trypsin or intestinal erepsin. The whole of the P was split off by 1% alkali in 24 hr. When an aqueous solution, made alkaline with $Ba(OH)_2$, was kept at 37° for a few hours and filtered from the precipitated Ba phosphate, the filtrate after being quantitatively freed from Ba was slowly acted upon by trypsin, amino-N increasing from 10 to 25% in the course of a week. The resistance of the phosphopeptone to enzyme action is thus obviously due to the presence of the phosphoric acid groups.

Nitrogen distribution

N-distribution was determined according to Damodaran [1931, 1]. The results are given in Table 3.

Table 3. *Distribution of N in the phosphopeptone*

	Nitrogen	
	mg.	%
Total N	171·10	100·00
Humin-N	0·00	0·00
Ammonia-N	18·65	10·90
Dicarboxylic acid-N	77·59	45·37
Monoamino-N	73·41	42·90
Basic-N	0·00	0·00
Non-amino-N	0·00	0·00
Total	169·65	99·17

Separation of amino-acids in the phosphopeptone (*scheme I*)

Outline. The phosphopeptone was hydrolysed with H_2SO_4 and the hydrolysate (A), after being freed from H_2SO_4, phosphoric acid and NH_3, was treated with $Ba(OH)_2$ and alcohol. From the precipitate (B), after removal of Ba quantitatively, glutamic acid (Bi) and serine (Bii*a*) were obtained by fractional crystallization. A small amount of uncrystallizable syrup was left. The filtrate (C) from the Ba-alcohol precipitate, after being freed from Ba quantitatively, gave on fractional crystallization two or three crops of *iso*leucine (Ci) and finally serine (Cii*a*).

In all the fractions serine was determined by conversion into the dinitrophenylosazone of glyoxal and the apparent dicarboxylic acid content by titration according to the method described by Harris [1929] for the determination of unbalanced carboxyl groups in dicarboxylic monoamino-acids.

Hydrolysis. 15 g. of the Ba salt of the phosphopeptone were dissolved in 37·5 ml. of water, 14 ml. of conc. H_2SO_4 added and the mixture boiled under reflux for 18 hr. After making up to volume and removing an aliquot for analysis, H_2SO_4 and phosphoric acid were removed by means of $Ba(OH)_2$, the former at neutrality to Congo red and the latter at strongly alkaline reaction. The $BaSO_4$ and the Ba phosphate contained respectively 5·23 and 1·0 % of the total N. The alkaline filtrate from the Ba phosphate was concentrated *in vacuo* at 40° to about 1/5 vol. (about 20 ml.) for the removal of NH_3 and treated with 200 ml. of alcohol little by little with vigorous shaking to precipitate the dicarboxylic acids. The mixture was centrifuged and the sticky precipitate washed twice with 20 ml. portions of 95 % alcohol. The precipitate was dissolved in 20 ml. of hot water and after adding a small amount of $Ba(OH)_2$ was reprecipitated by the addition of 200 ml. of alcohol. The precipitate (B) was dissolved in water and made up to volume. It contained 47·7 % of the total N of the hydrolysate. The filtrate (C) from the Ba-alcohol precipitate was freed from alcohol by evaporation *in vacuo* and worked up as described later.

Dicarboxylic acid fraction (*B*). The solution was quantitatively freed from Ba, the $BaSO_4$ thoroughly washed with boiling water and the washings added to the main fraction. A portion of the solution was oxidized with chloramine-T and the serine-N was found to be 33·18 % of the N of this fraction. Titration of another portion of the solution showed an acidity corresponding to 100·89 % of dicarboxylic acid-N. The main solution was concentrated in the desiccator when thick prismatic crystals were obtained. The first crop weighed 1·8 g., while a second crop of 0·5 g. was obtained after further concentration. The N in these fractions amounted to 29·7 % of the total N of the hydrolysate and 63 % of the N of the Ba-alcohol precipitate. The two crops were combined (Bi) and recrystallized from water and the substance was identified as glutamic acid having N 9·53 %,

amino-N 9·57 % (for glutamic acid, N 9·56 %) and M.P. 199°, which was not lowered by admixture with pure glutamic acid. 41 mg. of the acid required 2·82 ml. of 0·98 $N/10$ NaOH for neutralization to bromothymol blue, giving mol. wt. 148·4 (calc. 147·0). By saturating a concentrated solution of the substance with HCl gas in the cold it was quantitatively converted into glutamic acid hydrochloride with M.P. 213° and N 7·57 % (calc. for glutamic acid hydrochloride, N 7·63 %, M.P. 214°).

The mother liquor from the glutamic acid (fraction Bii) contained 0·1322 g. of N, of which 88 % was serine-N, and had a titratable acidity corresponding to 109 % of dicarboxylic acid-N. By treatment with alcohol as described in detail under the monocarboxylic acid fraction (C), 0·1 g. of crystalline amino-acid was obtained (Bii*a*). The needle-shaped crystals on recrystallization from aqueous alcohol yielded beautiful hexagonal plates melting at 228° and containing 13·23 % N (for serine, N 13·33 %, M.P. 228°). For further identification a portion of the substance was oxidized with chloramine-T; the resulting solution was divided into three portions and treated respectively with phenylhydrazine, *p*-nitrophenylhydrazine and 2:4-dinitrophenylhydrazine. The osazones obtained were recrystallized, the phenylosazone from aqueous alcohol and the nitrophenylosazones from hot nitrobenzene. A portion of serine was similarly oxidized and the osazones were prepared. The M.P. and the mixed M.P. are given in Table 4.

Table 4. *Melting points of osazones*

	M.P. of osazones		
	From Bii*a*	From serine	Mixed
Phenylosazone	167°	167·5°	167°
p-Nitrophenylosazone	311°	311°	311°
2:4-Dinitrophenylosazone	318°	319°	318°

The uncrystallizable mother liquor (Bii*b* containing 0·1109 g. N) contained 84·7 % of serine as determined by oxidation and was also strongly acidic. But persistent attempts failed to show the presence of a dicarboxylic acid in this fraction.

Monocarboxylic acid fraction (*C*). The filtrate from the Ba-alcohol precipitate contained 0·3054 g. N or 40·44 % of the total N of the hydrolysate. The solution was freed from Ba quantitatively and allowed to concentrate in the desiccator. A white amorphous material separated on the sides of the dish and on the surface of the liquid. The material was broken up and filtered and the mother liquor on further concentration gave two more crops of a similar substance. The N contents were 10·63, 10·68 and 10·98 % respectively. The first crop did not contain any serine-N, while the other two crops contained 3 and 10 % serine-N respectively. The crops were mixed together (Ci) and recrystallized from water in which they were only sparingly soluble, when the same amorphous material as before was obtained (1·54 g.) but without contamination with serine. When the substance was dissolved in a small amount of hot water and alcohol was added to turbidity, needle-shaped crystals aggregated in bunches were obtained. The M.P. of the original amorphous material was 265°, while the crystals melted at 275°. The N content of 10·68 % indicated that the substance was one of the leucines.

For identification the substance was converted into its Cu salt. 1 g. of the material was dissolved in a small amount of water on the water bath and copper carbonate was added little by little till no more dissolved. The mixture was heated for about 30 min. on the water bath and filtered. The residue of copper

carbonate was thoroughly washed with hot water till the washings were colourless. The combined filtrate and washings were evaporated to dryness on the water bath and the powder dried to constant weight at 100° in the air oven. It was repeatedly extracted by shaking with dry methanol freshly distilled over Ca, when almost the whole of the substance went into solution leaving a negligible residue. The methanol-soluble Cu salt was freed from alcohol by evaporation and recrystallized from water, from which it was obtained in the form of light blue needles. The crystals contained N 8·63 and Cu 19·63 % (calc. for Cu *iso*leucine, N 8·47 and Cu 19·51 %). A small amount of the Cu salt was decomposed by H_2S and the solution on concentration gave beautiful hexagonal plates of *iso*leucine melting at 275°. A 3·42 % solution of the substance in 20 % HCl had $[\alpha]_D^{31^\circ} + 26{\cdot}5^\circ$.

The mother liquor from Ci was heated on the water bath and alcohol was added drop by drop to turbidity. The sides of the dish were well scratched and the dish was left in a desiccator in the ice-chest overnight, when a mass of needle-shaped crystals was obtained. These were filtered quickly under centrifugal force, washed with alcohol and dried (Cii*a*). Another crop was obtained by concentration and addition of alcohol as before. The combined crops (0·448 g.) were recrystallized from aqueous alcohol. The hexagonal plates resembled fraction Bii*a* closely in all respects: N 13·32 %, M.P. 228°. The osazones were also identical with those of Bii*a*. The substance was therefore serine.

Table 5. *Analyses of fractions in scheme I*

	Fractions	N of fractions		
		In g.	As % of N of fraction	As % of T.N. of hydrolysate
A.	Hydrolysate	0·7554	—	100·00
	Dicarb.-N	0·3358	—	44·46
	Serine-N	0·2099	—	27·80
	Ammonia-N	0·0825	—	10·92
B.	Ba-alcohol precipitate	0·3603	—	47·70
	Dicarb.-N	—	100·89	—
	Serine-N	—	33·18	—
Bi.	*Glutamic acid* (2·3 g.)	0·2244	—	29·70
Bii.	Filtrate from (Bi)	0·1322	—	17·50
	Dicarb.-N	—	109·30	—
	Serine-N	—	88·00	—
Bii*a*.	*Serine* (0·1 g.)	0·0132	—	1·75
Bii*b*.	Mother liquor from above: uncrystallizable	0·1109	—	14·67
	Dicarb.-N	—	110·00	—
	Serine-N	—	84·72	—
C.	Filtrate from Ba-alcohol precipitate	0·3054	—	40·44
	Dicarb.-N	—	0·00	—
	Serine-N	—	34·72	—
Ci.	iso*Leucine* (1·54 g.)	0·1644	—	21·78
Cii.	Filtrate from above	0·1280	—	16·94
	Dicarb.-N	—	0·00	—
	Serine-N	—	82·56	—
Cii*a*.	*Serine* (0·45 g.)	0·0585	—	7·75
Cii*b*.	Uncrystallizable mother liquor	0·0629	—	8·33
	Dicarb.-N	—	0·00	—
	Serine-N	—	76·23	—

No crystals were obtained from the mother liquors Bii*b* and Cii*b* left from the dicarboxylic and the monocarboxylic acid fractions respectively. The total N in the fractions amounted to about 23 % of the hydrolysate-N. The fractions consisted mainly of serine, the amounts of which in the two fractions were 84·7 and 76·2 % respectively. They probably contained small amounts of the other amino-acids and also serine-decomposition products which prevented the amino-acids from crystallizing. On oxidation of the fractions only glyoxal osazones were obtained, which showed that serine was the only hydroxyamino-acid present in the hydrolysate. Conversion into Cu salts failed to show the presence of any aspartic acid. The analyses of the various fractions are given in Table 5.

Separation of the amino-acids in the phosphopeptone (scheme II)

Outline. The hydrolysed phosphopeptone (A) freed from H_2SO_4, phosphoric acid, NH_3 and Ba was acidified with H_2SO_4 and continuously extracted with ether for 48–72 hr. The H_2SO_4 was removed quantitatively and the solution after concentration was extracted with butyl alcohol 6–8 times. The butyl alcohol extracts (B) were concentrated *in vacuo* and the solids which crystallized out (Bi) were converted into the Cu salts. The Cu salts were separated into two fractions, Bi*a* soluble in methanol and Bi*b* insoluble in methanol. *iso*Leucine was isolated from the former and serine from the latter. The butyl alcoholic mother liquor (Bii) contained little nitrogenous material.

The aqueous solution (C), after extraction with butyl alcohol, was saturated with HCl gas and glutamic acid hydrochloride (Ci) was obtained. The filtrate from this (Cii), after removal of chloride, was concentrated and treated with alcohol to turbidity and cooled, when crystalline serine (Cii*a*) separated out. An uncrystallizable mother liquor (Cii*b*) containing 15·95 % of the total N was left.

Hydrolysis-ether extraction. 60 g. of the Ba salt were hydrolysed with H_2SO_4 and Ba, PO_4, SO_4 and NH_3 were removed as before. The $BaSO_4$ and Ba phosphate contained respectively 4·17 and 1·2 % of the total N. The solution was concentrated *in vacuo* to about 50 ml., acidified with about 5 ml. of 10N H_2SO_4 and extracted with ether in a continuous extractor for 72 hr., after which the dicarboxylic acid-N determined on an aliquot after quantitative removal of H_2SO_4 was found to be 30·0 % of the total N. Further extraction did not reduce the titratable acidity. The ether extract was evaporated in the presence of about 30 ml. of water and the aqueous solution was made up to a volume of 50 ml. It contained only 2·1 % of the total N but required 258 ml. of $N/10$ NaOH for neutralization, corresponding to a dicarboxylic acid-N value of 13·6 %.

Butyl alcohol extraction—iso*leucine as Cu salt.* The ether-extracted hydrolysate was quantitatively freed from H_2SO_4, concentrated to 100 ml. and extracted six times by shaking for 6 hr. each with 300 ml. portions of butyl alcohol. The butyl alcohol extracts were evaporated in three lots and the crystalline material which separated contained respectively 0·5723 g., 0·1168 g. and 0·0549 g. of N (5·45, 1·12 and 0·5 g. of solid respectively). The N contents were 10·51, 10·62 and 10·98 %. They showed no titratable acidity but contained respectively 9·61, 16·43 and 16·9 % of serine-N. The solids were combined and converted into the Cu salts as already described and separated into two fractions, one of Cu salts soluble in methanol (7·6 g.) and the other insoluble (0·98 g.). The methanol-soluble substance on recrystallization from water readily yielded crystalline Cu *iso*leucine: N 8·45 %; Cu 19·58 % (calc. for Cu *iso*leucine, N 8·47 and Cu 19·51 %). After removal of Cu by H_2S the amino-acid was obtained in pure condition with N 10·67 % and $[\alpha]_D^{32^\circ} = +25{\cdot}76^\circ$ in 20 % HCl for a 3·88 % solution.

The methanol-insoluble, water-soluble Cu salt after decomposition with H_2S was found to consist chiefly of serine as determined by the oxidation method; it was therefore added to the next fraction (C).

The material extracted by butyl alcohol was almost completely crystallizable, the final mother liquor (Bii) containing only 3·4 % of the total N.

Glutamic acid hydrochloride. The aqueous solution, (C) after extraction with butyl alcohol (which contained 51·82 % dicarboxylic acid-N and 41·62 % serine-N), was concentrated to a small volume, cooled in ice and saturated with dry HCl gas. After 24 hr. in the refrigerator the separated glutamic acid hydrochloride (Ci) was filtered; from the concentrated filtrate a second crop was similarly obtained. The two fractions (9·18 g.) together contained 27·28 % of the total N. The N content of the mixed solid was 7·7 %. A portion of the substance was recrystallized by dissolving in water and saturating in the cold with dry HCl gas when pure crystals were obtained: N 7·63 %, M.P. 213°. 0·1100 g. of the substance gave 0·0867 g. of AgCl. The molecular weight of the substance was 181·8. The molecular weight of glutamic acid hydrochloride is 183·5.

Serine. The filtrate from the glutamic acid hydrochloride (Cii) was freed from HCl by repeated evaporation to dryness *in vacuo* with water and finally by means of Ag_2SO_4. After removal of H_2SO_4 quantitatively by means of $Ba(OH)_2$, serine was crystallized out by the addition of alcohol as described before. The mixed crops (Cii*a*) weighed 2·2 g. and contained 12·98 % N. A sample recrystallized from aqueous alcohol contained 13·23 % N and melted at 228°. 40 mg. of the substance gave 120·7 mg. of 2:4-dinitrophenylosazone, while the same

Table 6. *Analyses of fractions in scheme II*

	Fractions	N of fractions: In g.	As % of N of fraction	As % of total N of hydrolysate
A.	Hydrolysate	2·5460	—	100·00
	Dicarb.-N	0·7610	—	29·97
	Serine-N	0·7358	—	28·90
	Ammonia-N	0·2774	—	10·90
Bi.	iso*Leucine* (5·84 g.)	0·6229	—	24·46
Bii.	Butyl alcohol mother liquor	0·0860	—	3·38
C.	Aqueous fraction after butyl alcohol extraction	1·5561	—	61·12
	Dicarb.-N	—	51·82	—
	Serine-N	—	41·62	—
Ci.	*Glutamic acid hydrochloride* (9·18 g.)	0·6942	—	27·28
Cii.	Filtrate from above	0·8467	—	33·25
	Dicarb.-N	—	10·45	—
	Serine-N	—	75·04	—
Cii*a*.	*Serine* (2·2 g.)	0·2796	—	10·98
Cii*b*.	Mother liquor from above	0·5671	—	22·27
	Dicarb.-N	—	15·48	—
	Serine-N	—	62·74	—
	Amino-acids from above by scheme I method			
	Glutamic acid (0·2 g.)	0·0191	—	0·75
	iso*Leucine* (0·69 g.)	0·0878	—	3·45
	Serine (0·4 g.)	0·0540	—	2·12
	Final uncrystallizable syrup	0·4062	—	15·95
	Dicarb.-N	—	16·95	—
	Serine-N	—	74·30	—

amount of serine gave 118·4 mg. of the osazone. The three osazones were prepared and the M.P. were identical with those of the osazones obtained from Bii*a* and Cii*a* in scheme I.

From the mother liquor (Cii*b*), by adopting the method of separation described in scheme I, 0·2 g. of glutamic acid, 0·69 g. of *iso*leucine and 0·4 g. of serine were obtained. The final mother liquor, which contained 15·95 % of the total N of the hydrolysate, was uncrystallizable. The results are summarized in Table 6.

Effect of ether extraction on the 'dicarboxylic acid-N' of hydrolysates

The hydrolysate obtained from 20 g. of the Ba salt, after determination of NH_3, was carefully freed from NH_3 and all reagents quantitatively made up to 100 ml. and portions were used for the following experiments. (i) In 2 ml. aliquots the apparent 'dicarboxylic acid-N' was determined by titration to *p*H 7 against $N/10$ NaOH. (ii) 20 ml. aliquots were acidified with 2 ml. of $5N$ H_2SO_4, continuously extracted with ether and the residual acidity determined by titration after removal of H_2SO_4 quantitatively. The ether extract was evaporated, the residue taken up in water and titrated to *p*H 7. (iii) In a 50 ml. aliquot glutamic acid was isolated as the hydrochloride. The results obtained, expressed as $N/10$ BaOH for the whole of the solution, were:

	ml. of $N/10$ NaOH
Total acidity	418·0
Acidity after extraction with ether	286·4
Acidity in the ether-extractable material	122·0
Glutamic acid as hydrochloride	268·7
Ammonia	128·3

The approximate equivalence of the acidity after ether extraction to the glutamic acid isolated and that of the ether-extractable acidity to the ammonia formed during the hydrolysis are patent.

Nature of the ether-extractable material

The fatty acids in the ether extracts did not consist entirely or even mainly of the acids observed by previous workers among the decomposition products of serine. Abderhalden & Broich [1933], who studied the decomposition of serine by H_2SO_4, obtained pyruvic acid, lactic acid, oxalic acid, glycine, alanine and NH_3, while Daft & Coghill [1931] obtained all the above-mentioned products except pyruvic acid during the decomposition of serine by alkali.

The aqueous solution from the ether extract, obtained as above, contained pyruvic acid, but estimation as the 2:4-dinitrophenylhydrazone [Simon & Neuberg, 1931] showed that only 15 % of the total acidity of the extract was due to this acid. Lactic acid estimated by the method of Friedemann *et al.* [1927], as modified by Leib & Zacherl [1932], was found to be present to the extent of 7 %. Thus the major portion of the mixture remained unaccounted for. The extract did not give any colour reactions for the common fatty acids. Ammoniacal $AgNO_3$ was not reduced. A brucine salt was prepared but could not be obtained crystalline. A crystalline quinine salt identical in properties with those of the quinine salt of *l*-glyceric acid described by Nef *et al.* [1917] was, however, obtained in good yield by the following procedure: the aqueous solution of the ether-extractable material was treated with a slight excess of 2:4-dinitrophenylhydrazine dissolved in an equal volume of $4N$ H_2SO_4 to remove pyruvic acid. The precipitated hydrazone was filtered off, and the filtrate was made alkaline with

an excess of $Ba(OH)_2$ and filtered. The solution was extracted repeatedly with ethyl acetate to remove excess of the phenylhydrazine, boiled for a few minutes to remove ethyl acetate and freed from Ba quantitatively by H_2SO_4. The solution was concentrated *in vacuo* and treated on the water bath with an alcoholic solution of quinine drop by drop till the reaction was alkaline. After keeping on the water bath for about 30 min. the solution was cooled, and the excess of quinine was removed by extraction with ether. On concentration *in vacuo* a crystalline mass of the quinine salt was obtained, which was recrystallized from water.

The M.P. of the salt was 164° (165° given by Nef *et al.* [1917]), which was not affected by admixture with the quinine salt of authentic glyceric acid (prepared from serine by deamination with nitrous acid). The weight of the quinine salt obtained from the ether extract (0·35 g. from an aqueous solution requiring 12·6 ml. of $N/10$ NaOH for neutralization) suggests that the main fatty acid produced is glyceric acid. For a more exact determination of glyceric acid the method suggested by Rapoport [1935] was tried. According to this author serine is deaminated with nitrous acid to glyceric acid, which is then estimated from the blue colour formed on treatment with naphthoresorcinol dissolved in conc. H_2SO_4. In our experiments no blue colour was obtained with naphthoresorcinol either with the material from the ether extract or with serine itself treated according to the method described. Serine prepared in this laboratory from silk and a sample obtained from Hoffmann-la Roche were tried; the naphthoresorcinol was also obtained from the same firm.

As Friedemann *et al.* [1927] have shown that glyceric acid can give rise to lactic acid in their method, the lactic acid value mentioned previously does not provide conclusive evidence of the presence of this acid in the ether-extractable material.

The ether extract contained 2·1 % of the total N of the hydrolysate which, even if it was wholly a dicarboxylic acid, would not account for more than about 15 % of the total acidity. Oxidation of the ether extract with chloramine-T and treatment of the resulting solution with 2:4-dinitrophenylhydrazine did not give an osazone.

Table 7. *Time-course of NH_3 liberation during hydrolysis*

	NH_3 as % of total N	
Time in hr.	With 25 % H_2SO_4	With N HCl
1	—	0·34
2	—	1·05
4	—	1·60
6	7·88	1·89
8	8·73	2·70
12	9·80	3·97
18	10·92	5·91
24	15·20	6·86

Determination of serine by oxidation with chloramine-T

The method used was a modification of those of Dakin [1918] and Gulland & Morris [1934] for the estimation of 'hydroxyglutamic acid'.

5 ml. of a solution containing 40 mg. of serine (1 mol.) were treated with 0·11 g. of chloramine-T (1 mol.) and the mixture kept on a water bath (60–70°) for an hour. It was then cooled in ice, filtered and the precipitate washed with ice-cold water. The filtrate and the washings were diluted to about 40 ml. and 0·22 g. (3 mol.) of 2:4-dinitrophenylhydrazine dissolved in 40 ml. of warm 4N

HCl was added. The mixture was kept in a boiling water bath for about an hour with occasional stirring, after which the precipitated osazone was filtered hot through a weighed sintered glass crucible and washed thoroughly, first with $4N$ HCl and then with boiling water till the washings were colourless. The precipitate, dried to constant weight, weighed 0·1207 g., which was 75·5 % of the theoretical yield. This proportion was found to be constant in a large number of experiments, including those with mixtures of glutamic acid, *iso*leucine and serine, showing only a variation from about 74 to 76 %, so that using this factor the method can be used for the determination of serine in solutions free from other hydroxy-amino-acids with an experimental error of about 1·5 %.

In the case of mixtures of amino-acids the solution was first neutralized to litmus with alkali before the addition of chloramine-T.

Precipitability of serine by $Ca(OH)_2$ or $Ba(OH)_2$ and alcohol

0·20 g. of serine (containing 26·66 mg. N) was dissolved in 6 ml. of saturated $Ba(OH)_2$ solution in a centrifuge bottle and 60 ml. of alcohol were added slowly from a dropping funnel with vigorous shaking. The mixture was centrifuged for 5 min., the sticky precipitate washed twice with 6 ml. portions of alcohol, dissolved in 6 ml. of water and precipitated once again by gradual addition of 60 ml. of alcohol as before. On dissolving the precipitate and making up to volume (50 ml.) it was found to contain 63·78 % (17·03 mg.) of the N. The filtrate from the Ba-alcohol precipitate was freed from alcohol by evaporation *in vacuo* and contained 37·24 % (9·93 mg.) of the N.

Repetition of the experiment with $Ca(OH)_2$ and alcohol gave similar results.

SUMMARY

By digestion of 'paranuclein' from casein with trypsin an enzyme-resistant phosphopeptone of constant composition has been isolated in the form of its Ba salt.

The phosphopeptone has been shown to contain 10 amino-acid units, viz. 3 mol. of glutamic acid, 3 mol. of *iso*leucine and 4 mol. of serine. The absence of other hydroxy- or dicarboxylic-amino-acids has been demonstrated by indirect methods.

A method is described for the approximate estimation of serine in the absence of other hydroxyamino-acids.

It is shown that the presence of serine in high concentration interferes with the estimation of the dicarboxylic acids both by titration and by precipitation according to Foreman, in the former by the formation of secondary acidic decomposition products and in the latter on account of the partial precipitation of serine under the same conditions as the dicarboxylic acids.

REFERENCES

Abderhalden & Broich (1933). *Biochem. Z.* **262**, 321.
Cohn & Hendry (1930). *Organic Synth.* **10**, 16.
Daft & Coghill (1931). *J. biol. Chem.* **90**, 341.
Dakin (1916). *Biochem. J.* **10**, 319.
—— (1917). *Biochem. J.* **11**, 79.
—— (1918). *Biochem. J.* **12**, 290.
Damodaran (1931, 1). *Biochem. J.* **25**, 2123.
—— (1931, 2). *Biochem. J.* **25**, 190.

Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
Friedemann, Cotonio & Shaffer (1927). *J. biol. Chem.* **73**, 335.
Gulland & Morris (1934). *J. chem. Soc.* p. 1644.
Harington & Randall (1931). *Biochem. J.* **25**, 1916.
Harris (1929). *J. biol. Chem.* **84**, 298.
Jordan Lloyd & Shore (1938). Chemistry of the Proteins. London: J. and A. Churchill.
Kingston & Schryver (1924). *Biochem. J.* **18**, 1070.
Leib & Zacherl (1932). *Hoppe-Seyl. Z.* **211**, 211.
Lipmann (1933). *Biochem. Z.* **262**, 3.
Nef, Hedenberg & Glattfeld (1917). *J. Amer. chem. Soc.* **39**, 1638.
Posternak (1927). *C.R. Acad. Sci., Paris*, **184**, 306.
—— (1928). *C.R. Acad. Sci., Paris*, **186**, 1762.
Rapoport (1935). *Biochem. Z.* **289**, 406.
Rimington (1927, 1). *Biochem. J.* **21**, 1179.
—— (1927, 2). *Biochem. J.* **21**, 1187.
Simon & Neuberg (1931). *Biochem. Z.* **232**, 479.
Vickery (1922). *J. biol. Chem.* **53**, 495.