CLXXXIII. THE NATURE OF PARANUCLEIN

II. A COMPARISON OF THE PEPTIC DIGESTION PRODUCTS OF VARIOUS PHOSPHOPROTEINS

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It appears to be a fairly constant characteristic of phosphoproteins in general, that on digestion with pepsin there is formed a white precipitate containing a higher percentage of P than the original phosphoprotein. Caseinogen yields a material known as paranuclein,¹ which has been fairly extensively studied; vitellin yields haematogen; and ichthulins prepared from various fish roes all give a pseudonuclein. The sole exception recorded is the caseinogen of human milk [v. Moraczewski, 1895], to which we may now add the batrachiolin of frog's eggs.

The cause for the precipitation of pseudonuclein is rather obscure; and it was decided to undertake the preparation of this material from a number of different sources, in the hope that comparison of the products might show a common relationship.

In a previous communication [Herd, 1936] it was shown that paranuclein was not a single substance, but that it consisted of at least two fractions, one of which was soluble in water and in trichloroacetic acid. This suggested a simple method of comparison of the various pseudonucleins, and we have used it to compare paranuclein and haematogen. With ichthulin from cod roe and batrachiolin from frog's eggs, there is not formed sufficient pseudonuclein for similar analysis, but the digest as a whole has been analysed by trichloroacetic acid precipitation analogous to the experiments already carried out with caseinogen by Stirling & Wishart [1932], and with vitellin by Blackwood & Wishart [1934].

Caseinogen

The investigation of caseinogen has already been recorded by Stirling & Wishart, and that of paranuclein by Herd. For the sake of comparison with the results obtained with other phosphoproteins, the results may be briefly summarized here.

When caseinogen of N/P *ca.* 20 is digested with pepsin, there separates a white precipitate of N/P 8. This material contains some 15–20 % of the caseinogen N, and 25–30 % of the caseinogen P. When dissolved in weak alkali and reprecipitated by trichloroacetic acid, a part of the paranuclein remains in solution. This soluble fraction has N/P about 10, somewhat higher than paranuclein, the acid-insoluble fraction has N/P about 6. Extraction with water alone at pH 2 serves to fractionate the paranuclein into the same two fractions, only the acid-soluble fraction being soluble in water at this pH. This acid-soluble fraction differs from the acid-precipitate in its amino-acid content, being deficient in the aromatic amino-acids.

¹ It was suggested by Hammarsten [1894] that the precipitates as a class should be known as "pseudonucleins", and that the name "paranuclein" should refer specifically to the material derived from caseinogen; and we shall use the names according to this scheme.

When the caseinogen digest as a whole is precipitated by trichloroacetic acid, the acid-precipitate has N/P 12.8.

Vitellin

It was shown by Blackwood & Wishart that digestion of vitellin of N/P 19.2 by pepsin results in the separation of a pseudonuclein known as haematogen of N/P 4.3, containing some 20 % of the vitellin N and 77 % of the vitellin P.

Precipitation of the digest as a whole by trichloroacetic acid gives an acid-precipitate of N/P 6.5.

For comparison with paranuclein, some haematogen was prepared, centrifuged off, washed and dried, and examined as described below.

A weighed amount of haematogen, *ca.* 0.35 g., was suspended in 25 ml. water, and allowed to extract at 37.5°, the pH being approximately 4.2. After 30 min., 10 ml. of the suspension were filtered, and the filtrate analysed for N and P. To another 10 ml. sample, alkali was added to dissolve the haematogen, and the volume made up to 20 ml. Part of this was analysed for total N and P; a 5 ml. sample was precipitated with 5 ml. of 10 % trichloroacetic acid, filtered and the filtrate analysed.

The results of this experiment, and a subsequent duplicate, are given in Table I, and amino-acid colour reactions in Table II. N and P are expressed throughout as % of the totals present.

Table I

Experiment	...	1		2			
Total N/5 ml.	...	12.08 mg.		11.50 mg.			
Total P/5 ml.	...	2.70 mg.		2.60 mg.			
N/P ratio	...	4.5		4.4			
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		N%	P%	N/P	N%	P%	N/P
Water-soluble fraction		22.1	3.9	25.0	30.1	3.2	41.2
Acid-soluble fraction		29.8	18.2	7.7	24.2	14.8	7.2
Acid-insoluble fraction		70.2	81.8	3.8	75.8	85.2	3.9

Table II

Test	Haematogen	Water-soluble fraction	Acid-soluble fraction
Biuret	+	+	+
Xanthoproteic	+	+	-
Millon	+	Faint	-
Glyoxylic	+	Faint	-
Labile S	+	Faint	Faint

It is clear that haematogen shows distinct points of difference from, and resemblance to, paranuclein. Haematogen also contains acid-soluble and acid-insoluble fractions; and the soluble fraction has a N/P ratio higher than the acid-insoluble. Further, as with paranuclein, the acid-soluble fraction appears to be deficient in the aromatic amino-acids. But unlike paranuclein, the acid-soluble and water-soluble fractions differ markedly in N/P ratio; and consideration of the relative distribution between them of N and P renders it unlikely that either is part of the other.

Ichthulin

The production of pseudonuclein from ichthulin has been studied by several workers, including chiefly Walter [1891], Levene [1901] and McCrudden [1921]. Levene, however, used a different method of preparation of his "ichthulinic acid", and it appears certain that his material is not the typical pseudonuclein.

The ichthulin used in the experiments reported below was prepared from cod roe in three stages of maturity, prep. I representing that from the youngest roe,

prep. III that from the oldest. The method of preparation of the ichthulin was that described by Levene. (Ichthulinic acid we have not studied.)

Digestion of ichthulin with pepsin gave a surprisingly small quantity of pseudonuclein. Too little was obtained for its separation in bulk, sufficient being obtained in each digest for only one direct analysis, and insufficient to establish any quantitative difference between the whole digest and the supernatant liquor. Precipitation of the digest liquor with trichloroacetic acid was also carried out in the same way as with caseinogen and vitellin.

1 g. ichthulin was ground up in a mortar with a little water, and some 2.5 ml. *N* HCl added, to give pH 2. The volume was made up to 80 ml., and 20 ml. of 2% pepsin (B.D.H.) were added. The mixture was incubated at 37.5° till precipitation and flocculation of pseudonuclein occurred; several 5 ml. samples were meanwhile precipitated by addition of 5 ml. of 10% trichloroacetic acid.

The results of N and P estimations are given in Table III.

Table III

Preparation	Ichthulin N/P	Time min.	Acid-soluble			Acid-insoluble			Pseudo- nuclein N/P
			N%	P%	N/P	N%	P%	N/P	
I	62.8	45	75.7	31.8	150	24.2	68.2	22.4	10.3
II	105	40	58.3	48.9	125	41.8	51.1	85.8	—
		60	67.8	51.1	139	32.2	48.9	69.2	15.5
		80	73.6	54.0	143	26.4	46.0	60.3	—
		120	77.3	57.7	142	22.7	42.3	56.4	—
III	75.0	45	82.1	83.0	74.5	17.9	17.0	79.0	8.4

Batrachiolin

The preparation of a phosphoprotein from frog's eggs has been recorded by McClendon [1909], who did not, however, investigate its reaction to pepsin.

The batrachiolin used below was prepared in a different manner from that of McClendon, being an adaptation of that used for the ichthulin.

Several hundred ml. of frog's eggs were ground up in a mortar with broken glass and some 5% saline. The resulting thinly viscous product was squeezed through coarse linen in a hand press and an equal volume of saline added to the liquor obtained. The crude solution was extracted six times with an equal volume of ether, each extraction involving 3 hr. shaking. The saline suspension was then precipitated by pouring into 20 vol. of water, with adjustment of the pH to 5. The supernatant liquor was syphoned off, the protein redissolved in saline and again precipitated in 20 vol. of water. The protein was then washed thoroughly with 75%, 95% then absolute alcohol, washed again with ether and dried *in vacuo*.

The resulting black powder contained a considerable amount of melanin; attempts to remove the latter were not, however, readily successful, and in subsequent work its presence was ignored.

1 g. batrachiolin was ground in a mortar with 1 ml. *N* NaOH, then 3 ml. *N* HCl were added gradually and the volume made up to 80 ml. Little visible solution occurred. 40 ml. of 2% pepsin were added and the mixture incubated at 37.5°. No visible separation of pseudonuclein occurred, though digestion certainly took place. The course of digestion was followed by precipitation of 5 ml. samples with 5 ml. trichloroacetic acid solution in the usual manner.

The results are given in Table IV.

There appears at first to be no relationship between the various phosphoproteins with regard to their pseudonucleins; there is no evidence of a component common to all. If, however, the N/P ratios of the various fractions are

Table IV

N/P batrachiolin = 28.2

Time	Acid-soluble			Acid-insoluble		
	N%	P%	N/P	N%	P%	N/P
30 min.	82.8	59.7	38.8	17.2	40.3	12.4
45 ..	84.9	64.9	36.8	15.1	35.1	12.1
1 hr.	91.0	73.6	32.9	9.0	22.4	11.3
3 ..	91.8	80.6	32.0	8.2	19.4	11.9
5 ..	93.4	83.6	31.4	6.6	16.4	11.4

tabulated, one characteristic feature does nevertheless emerge. In Table V are set forth some typical results of fractionation of the peptic digests by (a) natural separation of the pseudonuclein, (b) precipitation of the digest by trichloroacetic acid and (c) fractionation of the separated pseudonuclein by trichloroacetic acid.

Table V. *N/P ratios*

	Caseinogen	Vitellin	Ichthulin	Batrachiolin
Undigested protein	15.2	16.8	99.2	—
Pseudonuclein	6.5	4.7	12.8	—
Supernatant liquor	16.4	63.9	99.2	—
Undigested protein	19.7*	19.2†	99.2	28.2
Trichloroacetic ppt.	12.8	6.5	33.1	11.3
Trichloroacetic filtrate	30.5	46.1	120.0	32.9
Pseudonuclein	6.5	4.5	—	—
Trichloroacetic ppt.	5.0	3.8	—	—
Trichloroacetic filtrate	10.4	7.7	—	—

* Stirling & Wishart [1932].

† Blackwood & Wishart [1934].

The above results show clearly that fractionation of the digests is invariably accompanied by cleavage into products of different, often widely different, N/P ratio; and further, that in every case the less soluble material has a lower N/P ratio, arising from a relative concentration of P. Viewed from another standpoint, it might be suggested that the presence of P increases the resistance of the degradation product to digestion by pepsin.

In order to determine whether the presence of P or phosphoric acid *per se* was responsible for this resistance, we have studied the effect of the introduction of further P into caseinogen, and of the phosphorylation of certain other proteins such as globulin and albumin.

Phosphorylated proteins

Phosphorylation of proteins has been previously accomplished by Neuberg & Oertel [1914] and also by Rimington [1927]. We have adopted the method used by the latter, viz. phosphorylation with POCl_3 in carbon tetrachloride of a mildly alkaline solution of the protein, the temperature throughout being kept below 5° , and the product being purified by some six reprecipitations. The method has been applied to caseinogen and serum globulin, and also extended to serum albumin.

In each case, the product obtained was more resistant to pepsin than the untreated protein. In the case of phosphorylated caseinogen, the time required for digestion to the point of paranuclein precipitation was twice as long as previously, in a solution containing only half the concentration of protein. Paranuclein was, however, produced in quantity comparable with that normally obtained, i.e. in some 20% yield. With phosphoglobulin, there was produced a

pseudonuclein in roughly 5% yield; with phosphoalbumin, there was a slight precipitate, but it was too small in quantity to affect the N and P analyses.

The results of digestion of these phosphorylated proteins are given in Table VI.

Table VI

Phosphorylated protein	N/P undigested protein	N/P pseudo-nuclein	N/P supernatant liquor
Caseinogen	7.1	4.4	10.5
Globulin	5.2	5.1	4.9
Albumin	5.9	—	5.3

Once more, there occurs with phosphorylated caseinogen a marked fractionation into products of widely different N/P ratio; and it is rather striking that the N/P ratios of the various fractions bear the same relationship to each other in the case of the phosphorylated product as they do with the natural protein. With phosphorylated globulin and albumin, however, there is no such fractionation, the N/P ratios for all fractions being within experimental error.

DISCUSSION

The precipitation of pseudonuclein on digestion of almost all phosphoproteins suggests that there must be some common reason for this phenomenon. Examination, however, of the various individual pseudonucleins does not disclose the existence of any common component. On the contrary, the various pseudonucleins have each a characteristic N/P ratio; and on fractionation yield products apparently different in each case.

The one feature common to peptic digestion of all the natural phosphoproteins is the appearance of cleavage products of different N/P ratios. As has been already pointed out by Wishart and co-workers, this necessitates the assumption that P is unevenly distributed throughout the molecule. This uneven distribution may be the result of (a) the uneven distribution of certain hydroxyamino-acids to which the P is attached, or (b) the preferential attachment by the maternal organism of phosphoric acid to certain particular regions of the molecule, or both. In either case, the presence in the molecule of further groups to which P may be attached is shown by the capacity of caseinogen to take up as much P again as it already contains.

Although a discussion on the actual nature of the attachment of phosphoric acid is hardly relevant here, it may be remarked that the newly phosphorylated proteins, like the natural phosphoproteins, are stable to acid and unstable to alkali, and it may be assumed that the phosphoric acid introduced by POCl_3 has been attached again to hydroxy-groups, not to nitrogen. The artificial introduction of P by means of POCl_3 differs, however, from that effected naturally in the even distribution of the P. When we digest phosphorylated caseinogen with pepsin, we find that the N/P ratios of all fractions have been equally reduced; but since the new P is superimposed on a previous uneven distribution, fractionation into products of different N/P ratios still occurs. With globulin and albumin, the newly introduced P is not superimposed on any uneven distribution, the digestibility of all parts is equally reduced, and there is little tendency for pseudonuclein formation.

Examination of the different cleavage products referred to above shows, however, that the less soluble fractions contain a higher proportion of P. This would suggest that the presence of P is inhibitory to pepsin activity; and this is

directly confirmed by the much slower rate of digestion of proteins to which more P has been added.

Considered from this standpoint, the initial separation of pseudonuclein, and the fact that it is composed of several components, no longer seems such a mystifying phenomenon. It would appear that pseudonuclein is essentially an accumulation of those products of digestion of a phosphoprotein which contain a high proportion of P and are thereby rendered resistant to peptic attack. The various component substances are presumably held together in loose combination, and precipitate together for reasons not yet understood, but probably connected with the insolubility of a highly polar complex in the acid digestion medium.

SUMMARY

Several different phosphoproteins, caseinogen, vitellin, ichthulin and batrachiolin (from frog's eggs), were digested with pepsin; the products were fractionated by (a) separation of the precipitated pseudonuclein, in the case of caseinogen and vitellin, and (b) trichloroacetic acid precipitation, with all four proteins. In every instance, digestion was accompanied by the appearance of cleavage products of different N/P ratios, with a higher concentration of P in the less soluble fractions. That the presence of P was responsible for the resistance to pepsin was confirmed by experiments with proteins phosphorylated with POCl_3 .

The results suggest that pseudonuclein is a loosely bound mixture of those products of digestion which contain a high concentration of P and are resistant to peptic attack.

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