CCXLIV. THE NATURE OF PARANUCLEIN.

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THE traditional misnomer "paranuclein" has been somewhat indiscriminately applied to a variety of phosphorus-containing degradation products of caseinogen; here paranuclein will refer specifically to the material deposited during the digestion of caseinogen by pepsin.

The experiments of the earlier workers (summarized by Rimington & Kay [1926]) showed that the material varied considerably, according to the experimental conditions, both in total amount and in its content of the original caseinogen-phosphorus. To explain these variations in phosphorus content, Robertson [1907], in work in which he claimed to have synthesized paranuclein from lower degradation products of caseinogen, expressed the opinion on somewhat slight evidence that paranuclein must be a mixture of at least two substances. Thereafter for a time the subject lapsed until Rimington & Kay, in their well-known studies on caseinogen, investigated also the derived paranuclein, and definitely confirmed its resistance to pepsin and its ready degradation by trypsin and by 1 % NaOH.

Holter *et al.* [1932] analysed paranuclein or "phosphorpepton" with regard to its identity as a single compound, and found a N/P ratio¹ of 8.3 to 8.7 among some half-dozen specimens, the divergence being assumed to be within the limits of experimental error. On prolonged digestion, they found that the N/P ratio fell to 7.1. Stirling & Wishart [1932] investigated the organic phosphorus produced on peptic digestion of caseinogen, using the technique of precipitation by trichloroacetic acid, and found a fairly constant N/P ratio for the insoluble residues of 12.8. This work, however, is difficult to correlate with the previous since the acid-insoluble residues and paranuclein are clearly not identical. Breese-Jones & Gersdorff [1934] analysed various fractions produced in the peptic digestion of caseinogen, of which paranuclein was one; as prepared by them, it had a N/P ratio of 6, and showed several interesting changes in amino-acid composition from the original caseinogen, markedly the absence of cystine.

Certain work on the other phosphorus derivatives of caseinogen is also of interest. Salkowski [1901] prepared a "paranucleic acid" by precipitation of the digest liquor with ferric ammonium sulphate, which as finally purified had a N/P ratio of 3.1, and was precipitated by tannic and phosphotungstic acids though but slightly by trichloroacetic acid. Dietrich [1909], following up some work by Reh, obtained several phosphorus-containing peptides from peptic digests, which had N/P ratios varying from 0.45 to 1.75. A well-defined phosphopeptone of basic character was prepared by Rimington [1927] from tryptic digestion of caseinogen; it had a N/P ratio of 1.44 and gave no precipitate with phosphotungstic or trichloroacetic acid. Posternak [1927] has described a phosphopeptone of N/P ratio 2; it appears to differ from any others described. Utkin [1936],

¹ The N/P ratios mentioned here are in every case those of absolute, not atomic proportion, calculated where necessary from the original data.

in an investigation of the rate of liberation of soluble phosphorus from caseinogen by pepsin, finds evidence suggestive of two different phases; the first concerns the liberation of 19–20 % of the material as a compound of N/P ratio 7, recalling paranuclein; the second phase is characterized by liberation of material of N/P ratio 3.5, which he considers may be related to Salkowski's "paranucleic acid".

From the above it is obvious that there is no unanimity of opinion as to whether paranuclein is an individual substance, or what role it may play in the degradation of caseinogen. The following work was undertaken in the hope that time-course studies of its hydrolysis by enzymes and by alkali might throw some light on its nature.

EXPERIMENTAL PROCEDURE.

Preparation of paranuclein. 10 g. caseinogen (*nach Hammarsten*, N/P:17.25) were rubbed in a mortar with water until a fairly smooth paste was obtained. *N* HCl, 1.2 ml. per g. caseinogen, was then added gradually with constant stirring. The mixture was made up to 300 ml. and allowed to stand overnight in the refrigerator. At first, this solution was centrifuged and the supernatant liquor decanted; the residues were dissolved in a further 150 ml. water, filtered, and the filtrate added to the original liquor. Later, the more dilute solution was made up directly, it being found that any undissolved material disappeared during digestion before the paranuclein began to precipitate.

2 g. pepsin (B.D.H.), dissolved in 100 ml. water, were added to the above caseinogen solution and incubated at 37.5°. Simple measurements showed that the point of maximum precipitation occurred at the end of one hour. This was found to be fairly coincident with the point at which the precipitate began to settle into flakes [cf. Holter *et al.* 1932], and the latter was used as the end-point of the reaction. The precipitate was now centrifuged off, washed once with water, recentrifuged, and dried *in vacuo*. The yield is just over 20 %; the N/P ratio has been found to vary from 7.1 to 8.8.

Time-course of hydrolysis.

1% NaOH. A weighed amount of paranuclein was suspended in water, immersed in the thermostat, and at a noted time *N* NaOH was added in the requisite quantity to give a final concentration of 1 %. The paranuclein dissolved immediately. 5 ml. samples were removed at noted intervals and treated with an equal volume of trichloroacetic acid. The filtrate after precipitation and the residue of the original solution were then subjected to analysis for determination of acid-soluble and total N and P respectively.

That no loss of N as ammonia occurred during incubation was established in the first experiment by the identity of values obtained for total N at the beginning and end of the incubation.

Trypsin. A weighed amount of paranuclein (about 0.5 g.) was suspended in 20 ml. water and immersed in the thermostat. To it were added at a noted time 10 ml. of trypsin solution (Difco) and immediately sufficient *N*/10 NaOH to give a faint pink with phenolphthalein. The mixture was then diluted to 50 ml. Samples of 5 ml. were removed at noted times, precipitated by trichloroacetic acid and analysed. Noted amounts of *N*/10 NaOH were added during the incubation to maintain the pH at 8.5; it was felt that this procedure was preferable to the use of a buffer since salt effects might complicate the reaction.

Pepsin. Preliminary experiments with dilute solutions of pepsin, similar to those with trypsin, indicated that hydrolysis was extremely slow. With high pepsin concentrations measurable hydrolysis occurred, but the large values of the N and P of the controls increased the experimental error considerably. The data recorded for this enzyme are therefore limited to one experiment in which the following method was used.

5 g. pepsin (B.D.H.) were dissolved in 100 ml. *N*/10 HCl; 50 ml. of this solution were added to 1 g. paranuclein, the remaining 50 ml. served as control. Samples were taken only (*a*) as soon as possible after the addition of the enzyme and (*b*) after 20 hours' incubation; they were precipitated and analysed in the usual way.

Papain. The literature is rather contradictory with regard to the optimum pH for papain action. Plimmer & Bayliss [1905] used it in slightly acid or neutral medium on caseinogen. Willstätter & Grassmann [1924; 1926, 1, 2] found an optimum pH for various proteins corresponding in each case to the isoelectric point. The presence of certain salts has, however, an important effect [Ringer, 1935]. Further complications arise when the use of activators is considered.

Preliminary experiments with citrate-NaOH buffers at different pH values showed that maximum activity was obtained at pH 6.7, and that the enzyme was sufficiently active for the purpose without the use of an activator. One experiment with the addition of an activator was however carried out for comparison. The final procedure was as follows.

Approximately 0.5 g. paranuclein was suspended in 30 ml. water and 15 ml. citrate buffer pH 6.7 were added. After 5 min. immersion in the thermostat, 5 ml. enzyme extract were added; the extract was prepared by allowing 1 g. papain powder (B.D.H.) to stand overnight at 0° with 10 ml. water, centrifuging and decanting, the liquor being diluted as required to give final concentrations of approx. 1, 0.2 and 0.1% papain.

The experiment with 0.1% papain was repeated with an enzyme extract which had been activated by cysteine hydrochloride, as described by Purr [1935].

All incubations were carried out at $37.5 \pm 0.1^\circ$ and the trichloroacetic acid was invariably used in 10% concentration. Nitrogen was estimated by micro-Kjeldahl and phosphorus by the Fiske-Subbarow method throughout. The values of N and P given in the tables have been corrected for the N and P of the enzyme solution present. It may be remarked incidentally that whilst pepsin-P was entirely acid-soluble, papain-P was partially so and trypsin-P was entirely non-acid-soluble. In all cases, the N was partly acid-soluble.

Three blank experiments were also carried out—two in dilute NaOH at pH 8 in the absence of trypsin, and one in citrate buffer of pH 6.7 in the absence of papain.

RESULTS.

Since the results of the blank experiments play a large part in the interpretation of the enzymic hydrolyses, they are given first in Table I, Exps. 1, 2, 3.

Table I.

			pH 8		pH 6.7 (citrate)	
			1	2	3	
Exp.	...	...				
Total N/5 ml.	...		9.26 mg.	6.30 mg.	6.59 mg.	
Total P/5 ml.	...		0.842 mg.	0.786 mg.	0.842 mg.	
N/P	...	...	8.6	8.0	7.8	

			% acid-soluble		% acid-soluble		% acid-soluble	
			N	P	N	P	N	P
Time								
5 min.			—	—	40.5	35.6	39.5	39.0
15			39.3	35.6	—	—	—	—
30			39.7	35.9	41.1	36.0	42.9	40.4
1 hr.			40.0	36.9	41.6	36.5	46.6	42.5
2			—	—	—	—	48.1	44.9
3			41.5	37.1	42.2	36.5	—	—
4			—	—	—	—	49.2	46.0
7			—	—	—	—	51.1	48.9

From them, the surprising fact emerges that about 40 % N and 35 % P are already soluble in trichloroacetic acid. No appreciable further hydrolysis occurs at pH 8; at pH 6.7 there is a slight further hydrolysis.

Omitting further consideration of this point for the moment, it is obvious that enzymic digestion of paranuclein as followed by the action of trichloroacetic

acid will show only the rate of digestion of the insoluble fraction of the par-nuclein. This fact is, indeed, confirmed by the time curves of enzymic hydrolyses given below, since the best-fitting curves to the gross amounts of acid-soluble N and P (with the exception of pepsin) cut the ordinate at values approximating to those obtained in the blank experiment.

Digestion with 1% NaOH.

The individual results of this series are shown in Table II and Exp. 4 is depicted graphically in Fig. 1 (upper pair of curves).

Table II.

Exp.	...	...	4	5	6	
Total N/5 ml.	...	...	6.04 mg.	6.67 mg.	6.13 mg.	
Total P/5 ml.	...	...	0.746 mg.	0.777 mg.	0.700 mg.	
N/P	...	...	8.1	8.6	8.8	
			% acid-soluble		% acid-soluble	
Time			N	P	N	P
2 min.			53.3	37.5	43.9	30.3
15			63.9	50.0	51.7	34.1
30			66.7	55.6	56.1	41.3
1 hr.			72.3	70.5	59.5	49.0
1.75			79.1	83.4	64.8	63.5
2.5			80.0	91.6	—	—
4.5			—	—	76.8	78.3
*7			80.0	99.9	79.3	83.4
24			81.0	101.6	—	—
72			82.8	103.2	—	—

* 6 hr. in the case of Exp. 1.

Fig. 1. Action of 1% NaOH. N' and P' represent the hydrolysis of the originally acid-insoluble material.

Digestion by trypsin.

The results with various enzyme concentrations are shown in Table III and Exp. 9 in Fig. 2 (upper pair of curves).

Table III.

Exp.	...	...	...	7	8	9	10	11
Final trypsin conc.	...	...	...	0.1%	0.02%	0.02%	0.004%	0.004%
Total N/5 ml.	...	...	...	8.79 mg.	7.36 mg.	7.23 mg.	4.76 mg.	10.32 mg.
Total P/5 ml.	...	...	...	1.14 mg.	0.993 mg.	0.937 mg.	0.624 mg.	1.197 mg.
N/P	...	...	...	7.7	7.4	7.7	7.6	8.6

Time	% acid-soluble		% acid-soluble		% acid-soluble		% acid-soluble		% acid-soluble	
	N	P	N	P	N	P	N	P	N	P
5 min.	81.3	83.3	53.1	39.2	45.1	42.3	49.0	43.8	52.2	28.0
15	92.5	100.9	70.3	69.6	77.6	70.8	61.1	55.5	60.6	33.1
30	98.3	102.7	78.5	90.6	82.9	90.3	75.2	69.1	67.7	42.4
45	—	—	83.4	98.0	90.2	96.6	84.1	87.2	71.8	52.5
1.25 hr.	98.5	103.5	93.9	97.8	97.5	99.0	87.6	94.1	78.1	62.3
2	—	—	98.0	96.6	—	—	91.4	96.5	—	—
3	—	—	99.6	99.9	100.0	99.8	98.1	102.4	87.3	77.5

Fig. 2. Action of 0.02 % trypsin. N' and P' represent the hydrolysis of the originally acid-insoluble material.

By this deduction, the crossing of the curves in the tryptic hydrolysis is entirely removed; with papain the crossing is much less marked and may be due to experimental error involved in estimating the small quantities of acid-soluble N and P in the early stages; with 1 % NaOH however the crossing involves larger values than are probably explicable in this way. In general, with both trypsin and 1 % NaOH, the P is more rapidly liberated in acid-soluble condition than the N. The experiments with 1 % NaOH disclose the existence of a residue containing some 20 % N and no P which is resistant to the action of alkali.

With peptic hydrolysis, there is again evidence from the early samples of the existence in the solution of preformed acid-soluble N and P, but in much smaller amounts than in the trypsin or papain hydrolysis—about 20 %. At first, this was assumed to be due to the acid reaction but, as will be shown below, the behaviour of paranuclein in the presence of pepsin is peculiar. Since, however, the amounts of preformed N and P are practically identical, their deduction would not influence the relative positions of the N and P curves, and it may be definitely stated that pepsin differs from the other hydrolytic agents in liberating acid-soluble N more quickly than P.

With non-activated papain, there is little difference in the relative rates at which acid-soluble N and P are liberated. With the activated papain (Exp. 16), the rates of hydrolysis of both N and P are accelerated, particularly that of P. It is hoped to investigate this acceleration further.

The most interesting disclosure is, however, the presence of a preformed acid-soluble fraction in the paranuclein, and we returned to a further investigation of its nature. The caseinogen itself was shown, as expected, to have no acid-soluble fraction, the results obtained being of the order of 0.002 % N and no detectable P. Experimentation was then directed towards discovering whether the two fractions of the paranuclein could be separated by simple solution at different pH values.

Some paranuclein was suspended in water in the thermostat and left for 30 min. to come to equilibrium. The pH of this suspension was 4.2. Samples of 5 ml. were then brought to different pH values by addition of HCl or NaOH as required, the pH being controlled by indicators. After standing a further 30 min. in the thermostat, the samples were filtered without further treatment and the N and P of the filtrate determined (Exp. 17).

Another series was treated in similar fashion, but before filtration 5 ml. trichloroacetic acid were added to each sample (Exp. 18).

Table VI.

Exp.	17		18 (Precipitated by trichloroacetic acid)	
Total N/5 ml. ...	5.89 mg.		7.36 mg.	
Total P/5 ml. ...	0.806 mg.		0.903 mg.	
N/P	7.3		8.2	
	Filtrate		Filtrate	
pH value	% N	% P	% N	% P
2.0	49.1	42.4	51.9	48.9
3.5	44.5	41.3	45.9	44.5
4.2	44.1	42.7	46.1	44.0
5.5	70.3	68.0	47.8	43.6
7.0	87.9	88.8	45.6	43.4
8.5	94.4	93.7	—	—

As the results show, separation of the paranuclein can be produced by mere adjustment of pH. The trichloroacetic acid-insoluble fraction of paranuclein is also insoluble in aqueous solution at pH 4.2 and below; above this pH the solubility of the fraction rapidly increases until, at pH 8.5, the paranuclein is almost entirely soluble.

Although unconnected with the main argument, comment may be made on the fact that in Exp. 17 there is evidence of slight hydrolysis at pH 2. More important, there exists even at this pH a soluble fraction containing almost half of the N and P. Why, then, is the paranuclein precipitated at this pH during its formation by peptic digestion? The original digestion mixture from which the paranuclein was prepared differed from that in Exp. 17 only in the presence of undigested caseinogen, degradation products other than paranuclein and of pepsin. The influence of these materials was tested by precipitating paranuclein with trichloroacetic acid from solutions containing in addition (a) caseinogen and (b) pepsin. The final concentration of each was 1 %. There was no lowering in (a) of the acid-soluble material, but (b) showed a fall in soluble N to 25 % in one case and 33.6 % in another, with comparable P values. It may be recalled also that in the original time-course experiments on peptic hydrolysis, in which 5 % pepsin was used, the immediately acid-soluble N and P were approximately 20 %. Apparently the mere presence of the pepsin preparation used influences the acid-solubility of the paranuclein. To complete the analogy with Exp. 17, another test was made on the effect of pepsin on the solubility of paranuclein as shown by simple filtration, no trichloroacetic acid being used. The soluble N amounted to 37 %, the P to 30 %. It is hoped to investigate this effect further.

Returning to the question of the dual nature of paranuclein, from its physical behaviour the existence of two loosely bound fractions seems undeniable and attention was turned towards a possible chemical differentiation of these fractions.

A suspension of paranuclein was filtered, and to the filtrate and insoluble residue the tests given below were applied; the two fractions were also combined with iodine according to the method of Blum & Strauss [1920] by solution in sodium carbonate and addition of a solution of iodine in potassium iodide.

The results of the qualitative tests and the percentages of iodine bound are shown in Table VII.

Table VII.

	Residue	Filtrate
Biuret		
Xanthoproteic	Violet	Violet
Labile S	+	+
Millon	+	—
Glyoxylic	+	Very faint
Iodine %	+	Very faint
	10.30	26.19

These results suggest a deficiency in the soluble material of tyrosine and tryptophan, and an absence of labile S. The power of combination with iodine also clearly distinguishes the two fractions.

DISCUSSION.

The crossing of the original time-course curves for acid-soluble N and P is obviously mainly due to the fact that, in a solution of paranuclein, there exist already in acid-soluble form considerable fractions of the N and P, approximately 5 % more of the former than the latter. The deduction of this "preformed" soluble N and P in all cases diminishes, and in some abolishes, the crossing of the original curves. It is possible that the slight degree of crossing which remains is in some manner due to experimental error.

After correction of the curves by this deduction, they show that, in hydrolyses by alkali and trypsin, the P is liberated in acid-soluble form more rapidly than the N. With pepsin, the reverse holds, so that its mode of attack on the substrate

must be different. Similar findings were obtained by Stirling & Wishart [1932] for the peptic and tryptic digestions of caseinogen. Furthermore, digestion of paranuclein by trypsin is very rapid, that by pepsin very slow. Papain takes an intermediate place both in regard to activity and the relative rates of N and P liberation; the acceleration on activation is rather strikingly greater for P liberation than for N. The action of NaOH differs however from that of the enzymes in leaving unattacked a fraction of the paranuclein containing some 20 % of the original N and no P.

The most important disclosure from these time-course experiments was the indication they gave of the existence in paranuclein of two fractions; and the question of the constancy of composition of this substance is reopened. There seems little doubt from a study of the literature that the total amount of paranuclein produced is dependent on the experimental conditions, though there is equally little doubt that under similar conditions a fairly constant product is obtained. Linderstrøm-Lang and his co-workers find their paranuclein to contain $\frac{2}{3}$ to $\frac{3}{4}$ of the total caseinogen phosphorus, and to have a N/P ratio of 8. Breese-Jones & Gersdorff's product contained about $\frac{1}{3}$ of the caseinogen phosphorus and had a N/P ratio of 6. Our own experience is similar to that of Breese-Jones & Gersdorff with regard to phosphorus content, but the average N/P ratio of our preparations is in the neighbourhood of 8. It is quite possible that the lower ratio of their material is due to the repeated washing of the centrifuged precipitate, thus removing a certain amount of material of higher N/P ratio, that which we have found acid- and water-soluble.

The variations in phosphorus content do not invalidate the hypothesis that a single substance is being formed, and this hypothesis is supported by the comparative constancy of composition of the samples prepared by Linderstrøm-Lang and co-workers from widely different preparations of caseinogen. On the other hand, the variations of composition found by the early workers led Robertson to suggest that paranuclein may be a complex of more than one substance, the components being precipitated together under the conditions of peptic digestion.

The present results seem to point definitely to the latter conclusion, or at least to the existence of two loosely bound fractions, since on mere solution at different pH values almost half of the paranuclein is not precipitated by trichloroacetic acid. Further, a quantitatively similar separation is effected by simple filtration of the suspension at acid pH values, and the fractions so obtained differ in their amino-acid composition and power of combination with iodine.

But whilst it is clear that, in the paranuclein as separated from the original peptic digest, there are these two fractions, the fact that one of them is soluble in water at pH 2 is very difficult to reconcile with its appearance in insoluble form during peptic digestion. The necessarily low solubility of the paranuclein as originally formed can be reproduced to a certain extent, however, by the addition of pepsin to solutions of the separated and dried material, and three possibilities suggest themselves. First, the soluble material is precipitated in combination with a component of the commercial pepsin used; the combination of a component of the enzyme with one of its degradation products is well within precedent. Second, the soluble material is liberated from the caseinogen in loose combination with the other, insoluble, fraction of the paranuclein; the very ready hydrolysis of this loose complex might then be reversed only by the synthetic action of pepsin, which would explain why the reversal obtained was never complete. Third, the paranuclein itself is a synthetic by-product of the original reaction; and we may refer again to the ready synthesis by Robertson [1907] of what he claimed was paranuclein.

With regard to the relation between paranuclein and the various other phosphorus-containing degradation products of caseinogen which have been described in the literature, nothing definite can yet be said. Presumably the rise in phosphorus content of paranuclein on prolonged peptic digestion noted by various workers is due to the loss of the fraction of higher N/P ratio which we have found water-soluble.

It appears an attractive hypothesis that paranuclein is formed during tryptic digestion of caseinogen but does not become evident because of the alkalinity of the medium, and that Rimington's phosphopeptone is one of its further degradation products. The present findings, however, and the previous ones of Stirling & Wishart [1932] indicate that the modes of attack of pepsin and trypsin on caseinogen are fundamentally different, and it becomes unlikely that paranuclein is formed by trypsin. At the same time, it cannot be denied that phosphopeptone may have analogues in peptic digestion; this is suggested in fact by Dietrich's finding of acid peptones of N/P ratio similar to that of phosphopeptone during peptic digestion.

Similarly with the "paranucleic acid" prepared by Salkowski [1901] and believed to have been obtained also by Utkin, the relation between these products and paranuclein will not be clarified until they have been prepared by digestion of paranuclein itself, or isolated from a caseinogen digest from which all paranuclein has been previously removed.

SUMMARY.

Time-course studies are reported of the rate at which the N and P of paranuclein are rendered soluble in trichloroacetic acid by the action of pepsin, trypsin, papain and 1 % NaOH. The commercial preparation of papain is intermediate in action between pepsin and trypsin; the fully activated form is different. The action of 1 % NaOH discloses the existence of a nucleus containing 20 % of the paranuclein N and no P, which is resistant to alkaline hydrolysis.

These time-course studies indicated in addition that paranuclein contains at least two fractions. This was confirmed by the finding of differences in physical behaviour, in amino-acid composition and in iodine-combining power of the two fractions.

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