THE NATURE OF PARANUCLEIN.

THESIS

presented for the Degree of Doctor
of Philosophy of Glasgow University

by

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INDEX.

Part I. The Paranuclein Derivative of Caseinogen.

Page.

Introduction 1

Time-Course Digestion Curves of Paranuclein ... 13

The Study of the Components of Paranuclein ... 30

A) The Solubility of Paranuclein under Different Conditions 30

B) The Precipitation of Paranuclein under Different Conditions 34

C) Precipitation of Paranuclein by Tannic and Phosphotungstic Acids 43

D) Dialysis of Paranuclein 44

E) Amino-Acid Composition of Paranuclein ... 46

Discussion 49

Part II. The Paranuclein Derivatives of Other Phosphoproteins

Introduction 56

Experimental Procedure and Results 67

Discussion 70

Part III. The Paranuclein Derivatives of Synthetic Phosphoproteins.

Introduction 73

Experimental Procedure and Results 75

Discussion 79

PART I.

The Paranuclein Derivative of Caseinogen.

INTRODUCTION.

When caseinogen is digested with pepsin, there appears in the course of digestion an insoluble white precipitate, gelatinous at first, but eventually settling out in flakes, which contains a much higher percentage of phosphorus than the original caseinogen. This material, first recorded by Lubavin (1871, 1877), has been called variously "paranuclein" and "pseudonuclein", since it seemed at first to bear some resemblance to the nucleins, compounds which also on digestion liberate protein and show a precipitate of a P-containing complex.

Although the distinction between the true and false nucleins had not yet been made, Lubavin (1877) observed many facts which anticipated later work. Showing first of all that "nuclein" was not a simple compound of caseinogen and phosphoric acid, he demonstrated next that it was not a chemical individual but a mixture of at least two different substances. He states, "Wird nämlich Nucleïn in einprocentiger Natriumcarbonatlösung aufgelöst und alsdann fractionsweise durch einprocentige Salzsäure ausgefällt, so werden Fractionen von verschiedener Zusammensetzung erhalten." The P content is greater in the more

insoluble fractions, the N/P ratios* of the fractions varying from 8.0 to 5.3. The fractions were also distinct in acid-equivalence and in the colour developed with Millon's reagent. A possible explanation of his results is, he suggests, the observation by Miescher that nucleins are highly unstable in alkaline solution. In the light of later knowledge, we know that Miescher was referring to the true nucleins; but nevertheless, Lubavin refutes this possibility himself by showing that his preparation was stable to sodium carbonate. Lubavin also demonstrated that the composition of paranuclein was dependent on the length of digestion of the caseinogen with stomach juice, and the concentration of the mixture; with more rapid digestion, the P content was in the region of 2 - 3%, with longer digestion, it might rise to 4.37%.

In a later paper, Lubavin (1879) adds some observations on the solubility of paranuclein, and its slow disruption on prolonged boiling in water. He mentions also that when it is dissolved in carbonate and reprecipitated by weak acid, there is formed a water-soluble fraction giving all the protein colour reactions, containing phosphorus, and non-dialysable through parchment.

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*The N/P ratios used here and subsequently are invariably those of absolute weight, not atomic proportion.

In the same year, there appeared a paper by Bokay (1877), who investigated the digestibility of paranuclein by pancreas. After incubation with pancreas in vitro, he obtained back most of the paranuclein unchanged. When fed to dogs, to determine its absorption in vivo, he recovered the paranuclein from the faecal material.

The important disclosure that paranuclein was chemically distinct from the true nucleins appeared in 1886, when Kossel demonstrated that paranuclein, and an analogous material "haematogen" which is obtained from vitellin, contained no purine bases. Further investigation has fully confirmed his work; but the name paranuclein, though proving in this respect unfortunate, has been traditionally maintained. It was suggested by Hammarsten (1894) that in view of certain differences between the paranuclein from milk, on the one hand, and that from vitellin and ichthulin on the other, that the compounds as a class should be called "pseudonucleins".

For some twenty years after Lubavin's publications, there appeared sundry papers concerning the digestion of caseinogen. These are admirably summarised by v. Moraczewski (1895) and need not concern us further here. In 1893, however, Salkowski (1893, 1) published results which are important chiefly for two observations. First, he definitely disproved the previous idea that all the phosphorus of caseinogen was precipitated in the paranuclein.

On an average, he found ca. 15% of the phosphorus in the paranuclein, the remainder being in the digestion-liquor attached to albumose and peptone. Secondly, he states emphatically that "Dieser Phosphor ist organisch gebunden: Orthophosphorsäure ist in der Lösung nicht nachweisbar, auch Metaphosphorsäure ist auszuschliessen." In a later communication (1893, 2), he adds that although under certain conditions large quantities of paranuclein may be produced, he himself has never succeeded in isolating them. "Augenscheinlich wurde der grösste Teil des Paranucleins, sobald es aus dem Casein abgespalten war, alsbald weiter zersetzt. Setzt man die Verdauung längere Zeit, über 5 - 6 Tage fort, so verschwindet das noch restirende Nuclein vollständig und man erhalt eine ganz klare Verdauungslösung."

Following on Salkowski's work, v. Moraczewski confirmed the former's findings, though he could not agree that no paranuclein might be produced under certain conditions. He himself found present in the paranuclein a minimum of 6.75% of the caseinogen P, and a maximum of 63.21%; and he points out "Wenn nämlich ein präformiter Nucleïnkern im Caseïn vorhanden wäre, so sehe ich nicht ein, warum das Nucleïn so wenig vom Caseïn-phosphor enthält, obgleich es nur ... 24 Stunden verdaut wurde, wenn es in anderen Versuchen bei längerem Verdauen mehr davon enthielt."

Though the significance may not appear till later, there is one other point in v. Moraczewski's paper which may be noted here. Quoting Wildenow (1893), he remarks that when a solution of paranuclein is precipitated with acetic acid in the presence of ovalbumin, the amount of the precipitate is doubled, and suggests, "Es ist hiernach wohl möglich, dasz diese Nucleïnsäure, nachdem sie vom Caseïn abgespaltet ist, eine gewisse Eiweiszmenge auszufällen vormag."

Immediately following the above paper, came a contribution from Sebelien (1895). Confirming first of all v. Moraczewski's results, he points out that the occurrence of an appreciable amount of the phosphorus of a valuable human food in such an insoluble compound as the nuclein must afford considerable surprise; and he finds it difficult to believe that the nuclein is not further digested by the intestinal juice, the alkaline reaction of which is capable of readily bringing it into solution. While admitting that this question could not be decided by experiments outside the body, yet he considered it would be interesting to determine to what extent pseudonuclein really could be digested by a pancreatic extract, and to what extent it was merely held

in solution by the alkaline medium. To this end, he digested, not pseudonuclein itself, but caseinogen, with trypsin. The caseinogen, he found, was almost completely broken up by trypsin, including necessarily what would be the pseudonuclein portion of the molecule, for by precipitation of the liquor on acidification, the small amount of material obtained could in no way be identified with pseudonuclein. The previous findings by Bokay were thus shown to be erroneous, certain in vivo experiments on the absorption of true nucleins carried out by other workers affording confirmatory evidence.

Meantime, Salkowski had still been studying the conditions of precipitation of paranuclein, and in 1899, he published these further results. The quantity of paranuclein produced he found to be affected directly by conditions favouring digestion; thus in a concentrated caseinogen solution, with low pepsin concentration and incubation at room temperature, the yield was very high; whereas under favourable conditions of low caseinogen concentration, high pepsin concentration, and incubation at room temperature, the yield was very small, and under ideal conditions might disappear altogether. Salkowski pointed out further, that paranuclein does not arise directly from caseinogen; but that during digestion,

the opalescent caseinogen solution is converted into a clear albumose solution, from which latter the paranuclein precipitates.

It was shown by Giertz (1899) that pseudonuclein was readily soluble in baryta, differing thus from the true nucleins, and that it was decomposed by the baryta yielding acid-albumins, albumoses, and phosphoric acid. He confirmed, however, that pseudonuclein was not itself a simple compound of protein and phosphoric acid, and that the synthetic preparation obtained by Liebermann (1888) from albumin and phosphoric acid, was not a real pseudonuclein*.

Plimmer and Bayliss (1905 - 1906) undertook a comprehensive investigation of the digestion of caseinogen by pepsin, trypsin, papain, and 1% NaOH, using tannic acid as precipitant. Though exceptionally interesting in itself, their work does not add much new information concerning paranuclein, though they show directly that it is digested by trypsin, and confirm this point once and for all.

To explain the wide variations in P content found

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*A summary of the early literature may be obtained from Gustav Mann's "The Chemistry of the Proteids".

by the earlier workers, Brailsford Robertson (1907), in work in which he claimed to have synthesized paranuclein, expressed the opinion, from a review of the previous work, that paranuclein must be a mixture of two or more substances. In confirmation of this view, he offered the somewhat slender evidence that he could find little superficial difference between a paranuclein preparation containing 4% P, and the product obtained after treating the same preparation with lime water, which contained only 1% P. His synthetic paranuclein was obtained by digesting caseinogen with pepsin, heating to destroy the enzyme, then concentrating to a syrup, and adding to the latter a concentrated pepsin solution, (10%). Neither the concentrate nor the pepsin solution yielded a precipitate with acetic acid, showing that neither caseinogen nor paranuclein was present, yet after two hours' standing, there began to settle out a white flocculent material indistinguishable from paranuclein prepared according to the customary method.

Some time after, Rimington and Kay (1926) published the results of their investigations into the digestion of caseinogen, including a collateral study of paranuclein. They found that paranuclein contained about 2% P, and that it was unstable to 1% NaOH; and

confirmed its resistance to pepsin, a resistance shown also to bone and kidney phosphatases.

In 1932, Holter, Linderstrøm-Lang and Funder endeavoured to settle the question whether paranuclein is a single substance. Using several samples of caseinogen with different P content, they found nevertheless that the paranucleins obtained had all a $^N/_P$ ratio between 8.3 and 8.7, a divergence considered to be within the limits of experimental error, and suggesting the identity of the material obtained, "... dasz es sich bei allen Caseinfraktionen um ein gleiches oder mindestens ähnliches Phosphorpepton handelt,". They confirmed that on further slow digestion, the $^N/_P$ ratio falls. It may be remarked that their determinations were based on estimation of the supernatant liquor, after centrifuging.

Using the technique of precipitation by trichloroacetic acid, Stirling and Wishart (1932) investigated the organic P produced on peptic digestion of caseinogen, and found a fairly constant $^N/_P$ ratio for the acid-insoluble material of 12.8. This work, however, is difficult to correlate with the preceding, since, as will be shown below, the acid-insoluble residues and paranuclein are not identical.

Breese-Jones and Gersdorff (1934) investigated

various fractions produced in the digestion of caseinogen by pepsin; these were (a) paranuclein, (b) the precipitate obtained by adjusting the pH of the supernatant liquor to 6, and (c) the residual liquor. They found that paranuclein as prepared by them, a method which involved some half-dozen washings, had a $^{N}/_{P}$ ratio of 6; it showed several interesting differences from the original caseinogen in amino-acid composition, chiefly the absence of cystine. The second fraction, (b), had a $^{N}/_{P}$ ratio of about 12, the third fraction, or residual liquor, a $^{N}/_{P}$ ratio of about 30.

Certain work on the other P derivatives of caseinogen which have from time to time been obtained, is also of interest. Salkowski (1901) prepared a "paranucleic acid" by precipitation of the digest liquor with ferric ammonium sulphate. This substance had, when purified, a $^{N}/_{P}$ ratio of 3.1, and was precipitated by tannic and phosphotungstic acids, though but slightly by trichloroacetic acid. It gave the biuret and xanthoproteic reactions, but was negative to the other colour tests. Dietrich (1909), following up some work by Reh, prepared several P-containing peptides from peptic digestion of caseinogen, with $^{N}/_{P}$ ratios varying from 0.45 to 1.75. A well-defined basic phosphopeptone was isolated by Rimington (1927) after tryptic digestion of caseinogen. This material, which had a $^{N}/_{P}$ ratio of

1.4, gave no precipitate with phosphotungstic or trichloroacetic acids, gave a rose colour in the biuret reaction, a positive result with ninhydrin, and a negative result in the xanthoproteic and other colour tests. Posternak (1927) isolated a phosphopeptone of $^N/_P$ ratio 2, but it differs from that obtained by Rimington in amino-acid content. Subsequently, Utkin (1936) has investigated the rate of splitting of soluble P from caseinogen by pepsin, and describes two different phases; the first is the liberation of 19 - 20% of the material as a compound of $^N/_P$ ratio 7, recalling paranuclein; the second is characterized by liberation of material of $^N/_P$ ratio 3.5, which Utkin compares with Salkowski's paranucleic acid.

It is thus apparent that there is no unanimity of opinion whether paranuclein is a single substance, or by what salient characteristics it may be recognised. The constant nature of the material isolated by Linderstrøm-Lang et al. does not explain the widely different compositions obtained by the other workers under different conditions, nor the early results of Lubavin. There is, moreover, no indication what role paranuclein may play in the degradation of caseinogen in relation to the various other materials which have been obtained.

The work to be described below was begun in the hope that a study of the degradation of paranuclein by 1% NaOH, and by the proteolytic enzymes, might yield information as to its composition, and the part it plays in the breakdown of caseinogen by pepsin. It may be well to state here that the term "paranuclein" will be used with specific reference to the material deposited during the digestion of caseinogen with pepsin.

The Time-Course Digestion Curves of Paranuclein.

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 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 only was used; later it was found that the residual material, which contained about half of the caseinogen, was readily dissolved by pepsin similarly to the supernatant liquor; and finally, a more dilute solution of 10 g. caseinogen to 400 ml. water was made up directly, and used, it being found that any undissolved caseinogen disappeared during digestion long before the paranuclein began to precipitate.

2 g. pepsin (B.D.H.), dissolved in 100 ml. water, were added to the above solution, and the mixture incubated at 37.5°. Simple measurements showed that the point of maximum precipitation occurred at the end of one hour. This was coincident with the point of definite flocculation of the gelatinous precipitate, incidentally

the end-point used by Linderstrøm-Lang et al., and was subsequently used as the end-point of the reaction in all experiments described. The precipitate was now centrifuged off, washed once with water, recentrifuged immediately, and dried in vacuo. The yield is roughly 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, and immersed in the thermostat for several minutes. At a noted time, sufficient N.NaOH was added to give a final concentration of 1%, and the paranuclein at once dissolved. Thereafter, at noted intervals, 5 ml. samples were removed and added to 5 ml. portions of 10% trichloroacetic acid. The precipitate was filtered off, and the filtrate analysed.

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 (Dyfco), and 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 intervals, and precipitated with an equal volume of trichloroacetic acid. To maintain the pH at an

optimal value for tryptic action, measured amounts of $^{N}/_{10}$ NaOH were added from time to time to maintain the pink colour with phenolphthalein; although allowing of a certain fluctuation of pH value, it was felt that the variations could not be large, and that any error introduced thereby was preferable to the use of a buffer, since salt effects might complicate the reaction, particularly when the only suitable buffer involved the use of borate. It has been my previous experience that borate may inhibit certain enzymes, i.e. arginase and urease, as found by Hunter and Morrell (1933).

Pepsin: Hydrolysis of paranuclein by pepsin proved, as was to be expected, extremely slow. With extremely high pepsin concentrations, a measurable hydrolysis did occur, but the N and P values of the controls were many times that of the paranuclein, and experimental error was thereby greatly increased. Accordingly, only one experiment is recorded, the data for which are believed to be reliable.

5 g. pepsin were dissolved in 100 ml. N/10 HCl; to 50 ml. of this solution was added 1 g. paranuclein, the remaining 50 ml. served as control. Samples were taken for precipitation only twice, (a) immediately after the mixing of the reagents, and (b) after twenty hours' incubation.

<u>Papain</u>: Considerable uncertainty appears to exist in the literature with regard to the optimum pH for this enzyme. Plimmer and Bayliss (1905) used it in slightly acid or neutral medium on caseinogen. Willstätter and Grassmann (1924; 1926, 1, 2) found an optimum pH for various proteins corresponding 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 indicated a maximum activity at pH 6.7, and showed that the enzyme at this pH possessed sufficient activity to avoid the use of an activator. One experiment with the fully activated form of the enzyme was, however, carried out for comparison. The papain was used in the form of an extract obtained by allowing 1 g. papain powder to stand overnight at 0° with 10 ml. water, centrifuging, and decanting. The liquor obtained was diluted to give final concentrations of 1, 0.2, and 0.1% papain as required. The experiment with activated papain was analogous to that with the 0.1% papain, except that the enzyme had been activated by cysteine hydrochloride as described by Purr (1935).

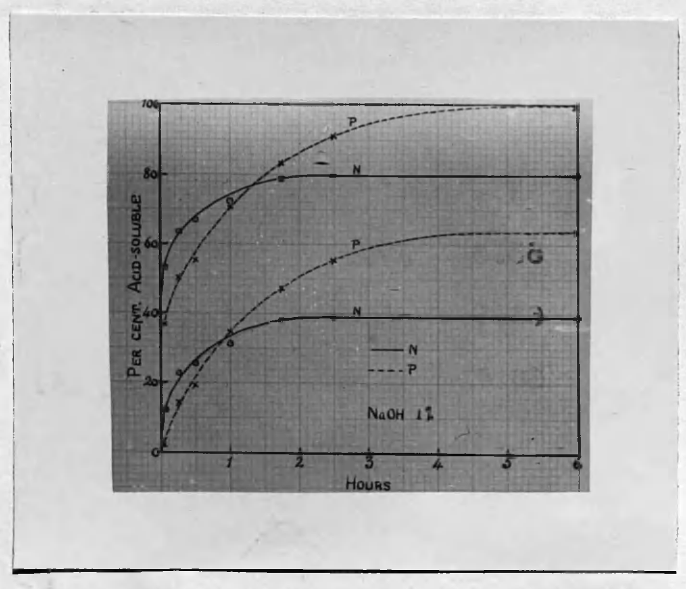
The final procedure adopted was as follows. Some paranuclein (about 0.5 g.) was suspended in 30 ml.

water, and 15 ml. citrate buffer pH 6.7 added. After five minutes immersion in the thermostat, 5 ml. enzyme extract were added, and samples removed for precipitation as in the previous experiments.

All incubations were performed at 37.5°, and the trichloroacetic acid used was invariably 10%. The N determinations were made by micro-Kjeldahl, and P by the colorimetric method of Fiske-Subbarow. In every case, the values for the enzyme present have been subtracted. All the enzyme preparations contained P as well as N, but while pepsin-P was entirely soluble in trichloroacetic acid, papain-P was only partly so, and trypsin-P was entirely precipitated. In all cases, the N was only partly soluble in trichloroacetic acid.

The blank experiments described, two in NaOH at pH 8 and one in citrate at pH 6.7, were carried out in an identical manner with the complete experiments, except for the absence of enzyme.

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



Results.

Digestion with 1% NaOH.

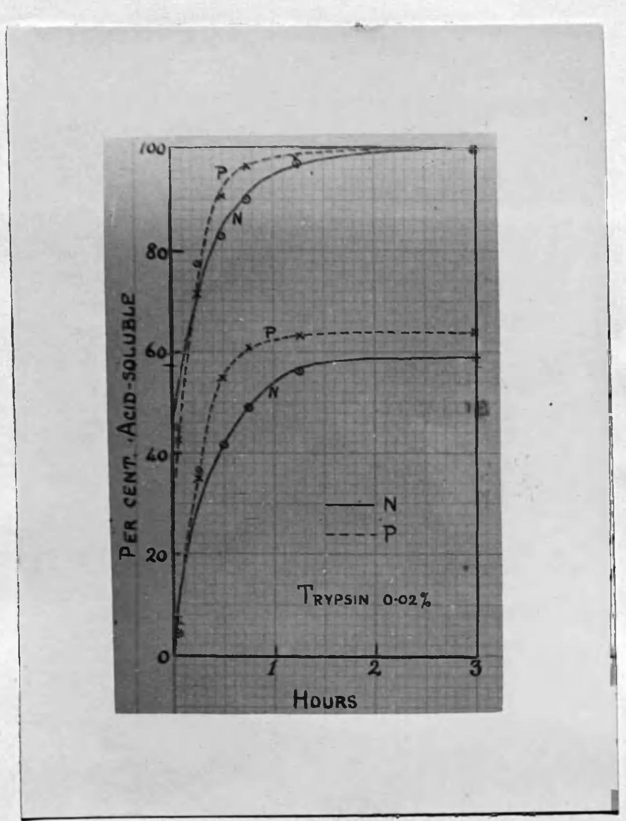
The results of the three experiments with 1% NaOH are given in Table I. Experiment 1 is illustrated opposite graphically in Fig. I, upper pair of curves.

Table I.

Exp.	1		2		3	
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		% acid-soluble	
Time	N	P	N	P	N	P
2 min.	53.3	37.5	43.9	30.3	47.8	30.3
15	63.9	50.0	51.7	34.1	56.9	41.3
30	66.7	55.6	56.1	41.3	59.7	51.4
1 hour	72.3	70.5	59.5	49.0	-	-
1.75	79.1	83.4	64.8	63.5	-	-
2.5	80.0	91.6	-	-	69.3	62.3
4.5	-	-	76.8	78.3	-	-
*7	80.0	99.9	79.3	83.4	75.5	75.4
24	81.0	101.6	-	-	77.6	79.6
72	82.8	103.2	-	-	83.4	87.6

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

Fig. II. The action of 0.02% trypsin. N' and P' represent the hydrolysis of the originally acid-insoluble material.



Digestion with Trypsin.

The results of five experiments with trypsin are given below in Table II and Experiment 6 is represented in Fig.II opposite (upper pair of curves).

Table II.

Exp. ...	4		5		6		7		8	
Final Trypsin concentration	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	
	% acid-soluble		% acid-soluble		% acid-soluble		% acid-soluble		% acid-soluble	
Time	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

Digestion by Pepsin.

The results of the single experiment with pepsin are given in Table III.

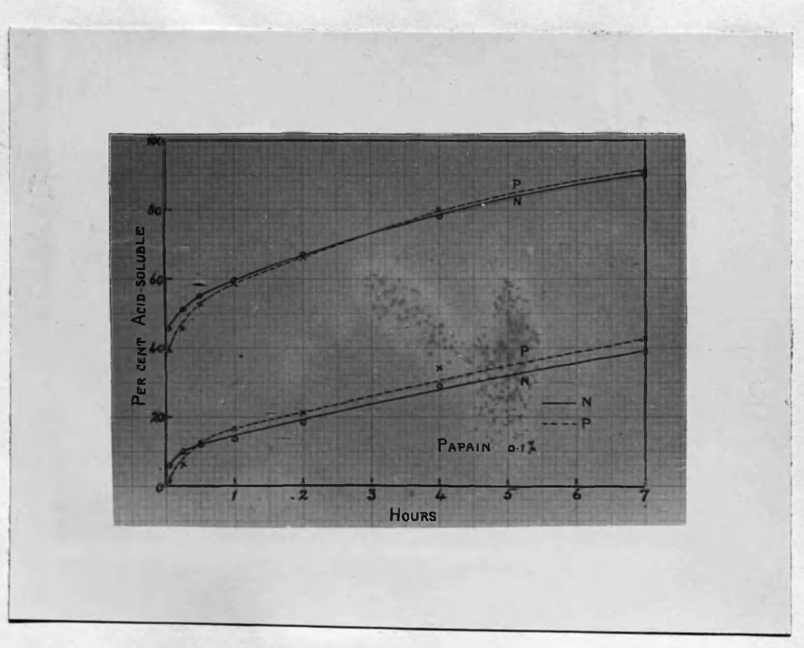
Table III.

Exp. 9.

Total N/5 ml. 13.4 mg. Total P/5 ml. 1.74 mg. N/P, 7.7

Time	% acid-sol. N.	% acid-sol. P.
2 min.	19.4	20.2
20 hr.	77.6	71.3

Fig. III. Action of 0.1% papain.
N' and P' represent the hydrolysis of the
originally acid-insoluble material.



Digestion by Papain.

The results of the experiments with papain are given in Table IV, and Experiment 12 is represented in Fig. III opposite (upper pair of curves). Experiment 13 is analogous to Experiment 12, but before incubation the papain was activated by cysteine hydrochloride.

Table IV.

Exp. ...	10		11		12		13	
Final papain concentration	1.0%		0.2%		0.1%		0.1%	
Total N/5 ml.	6.54 mg.		6.18 mg.		6.47 mg.		6.05 mg.	
Total P/5 ml.	0.905 mg.		0.806 mg.		0.860 mg.		0.811 mg.	
N/P ...	7.2		7.7		7.5		7.5	
	% acid-soluble		% acid-soluble		% acid-soluble		% acid-soluble	
Time	N	P	N	P	N	P	N	P
1 min.	45.4	41.8	45.0	39.0	45.8	39.2	49.6	51.1
15	86.2	75.4	56.1	46.2	51.3	45.8	76.0	82.6
30	94.2	85.3	70.7	62.4	55.0	52.7	84.3	93.0
45	95.3	86.5	-	-	-	-	-	-
1 hr.	96.2	89.1	81.2	74.8	59.7	59.1	87.1	97.9
2	98.9	90.4	86.2	79.3	66.3	66.0	90.9	99.6
3	101.5	93.5	91.3	83.8	-	-	-	-
4	-	-	98.1	86.9	78.2	80.0	96.4	101.9
7	-	-	-	-	90.0	90.7	100.8	101.6

On looking at these curves, and excepting the pepsin experiment, one is struck by two things, first, that the N and P curves cross, and second, that the curves when produced back to zero time cut the ordinate at quite high values of N and P, roughly half of the N and rather less of the P. This suggested the totally unlooked-for result that a portion of the paranuclein was already soluble in trichloroacetic acid, before the enzyme or catalyst had acted at all.

Accordingly, a set of blank experiments was carried out in which the liberation of acid-soluble N and P was observed (a) in NaOH at pH 8.5, analogous to the trypsin experiments, and (b) in citrate buffer at pH 6.7. These results are shown in Table V. The average of Experiments 14 and 15 is represented with Experiment 16 in Fig. V (p.26); and it is these average values depicted which were subsequently used for subtraction (vide infra).

Fig. IV. The action of water at pH 8.0 and 6.7.

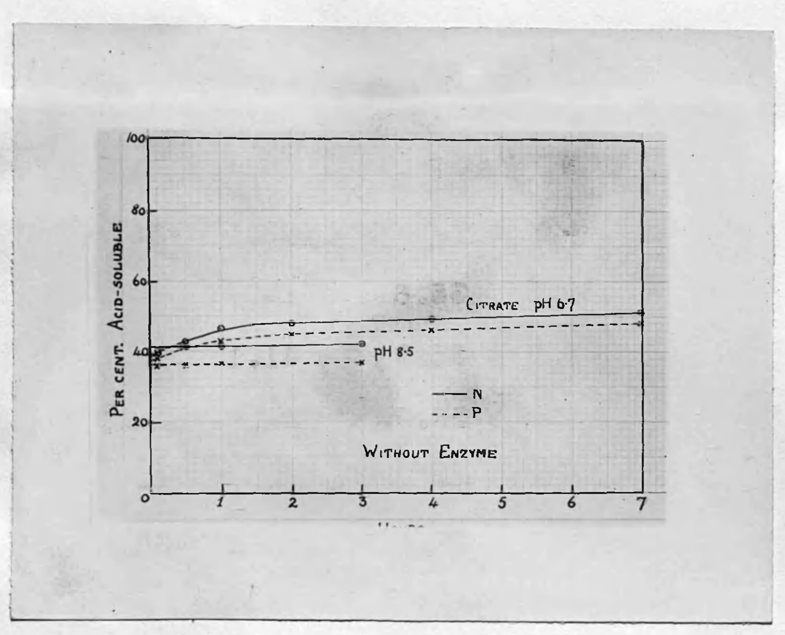


Table V.

	pH 8				pH 6.7 (citrate)	
Exp. ...	14		15		16	
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			
	% acid-soluble		% acid-soluble		% acid-soluble	
Time	N	P	N	P	N	P
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

It will be seen that at pH 8, the very first analysis showed that 40% of the N and 36% of the P were already acid-soluble, and these values did not vary appreciably throughout the experiment, which lasted 3 hours. At pH 6.7, similar quantities of N and P were

soluble at the beginning, but the values increased slowly during the course of the incubation. Clearly, therefore, a solution of paranuclein at pH 6.7 or 8 contains some 41% N and 36% P in a preformed acid-soluble condition; further, these two fractions are stable to pH 8, but in the presence of citrate buffer at pH 6.7, the insoluble form undergoes slight hydrolysis, for which we can suggest only the explanation that it is due to the citrate ion.

It thus appears justifiable to subtract these values for preformed acid-soluble N and P from the original time-course curves, since obviously precipitation by trichloroacetic acid is going to indicate only the rate of solution of the insoluble fraction. The result of this subtraction is shown graphically in the lower pair of curves in each graphical illustration.

The curves now start from zero, there being good agreement between the control and the digestion experiments. Moreover, the crossing of the N and P curves is almost entirely eliminated. The slight degree of crossing which remains with papain might be due to experimental error, though the crossing left in the 1% NaOH curves is larger than could be explained this way. The action of pepsin and trypsin is clearly distinct, in that with pepsin, soluble N is liberated more rapidly than P, with trypsin the reverse is true; this difference had previously

been found by Stirling and Wishart, with caseinogen as substrate. The action of papain once more appears to be intermediate in type between pepsin and trypsin, the N and P being liberated at fairly equal rates; activated papain, however, while liberating both N and P more rapidly than the untreated preparation, shows an interesting acceleration of the rate of P liberation as compared with that of N. The curve becoming asymptotic at 80% N, the hydrolysis of 1% NaOH reveals the existence of a nucleus containing some 20% of the original N, but no P, which is resistant to further attack by the alkali.

The Study of the Components of Paranuclein.

The most interesting point which emerged from the above study of the time-course digestion curves of paranuclein is, of course, the indication that paranuclein is not a single substance, since it is possible for trichloroacetic acid to separate it into two fractions.

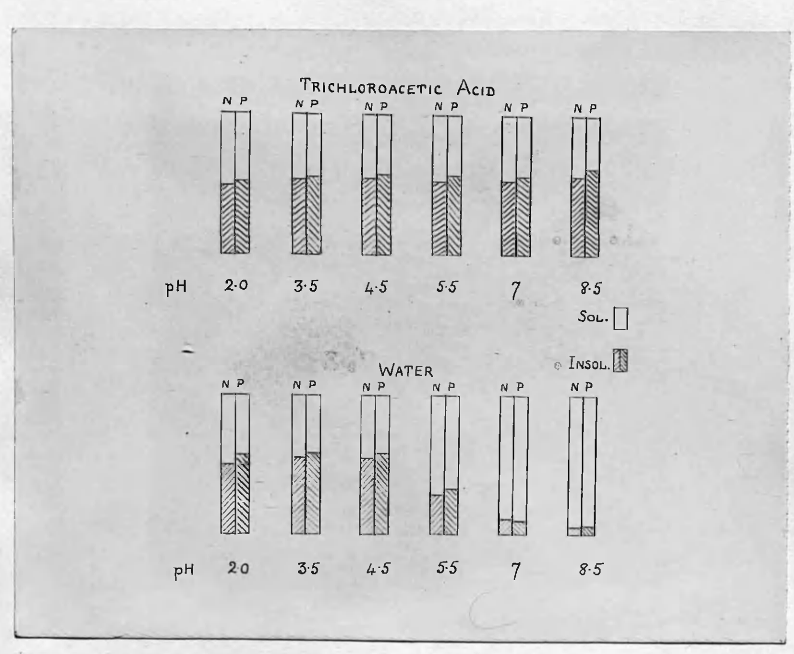
Attention was therefore devoted to a study of paranuclein from various viewpoints, in an endeavour to show how these two components are related quantitatively, and whether there exists any simple chemical difference between them.

A. The Solubility of Paranuclein under Different Conditions.

Up to this point, the solubility of paranuclein only in trichloroacetic acid has been considered. In Experiment 17, the solubility of paranuclein in water at different pH values, in the absence of trichloroacetic acid, was determined. Experiment 18 was carried out analogous to Experiment 17, except that in each case, the solution was precipitated by trichloroacetic acid, and filtered.

Procedure:- Some paranuclein was suspended in water and immersed in the thermostat for 30 mins. The pH of this suspension was 4.2. Samples of 5 cc. were then brought

Fig. V. The solubility of paranuclein at different pH values.



to different pH values by the addition of appropriate quantities of N/10 HCl or NaOH, the pH values being determined by indicators. After standing a further 30 minutes in the thermostat, the samples were filtered, in the case of Experiment 17, or treated with 5 cc. trichloroacetic acid, in the case of Experiment 18.

The results are given below in Table VI, and illustrated in Fig. V.

Table VI.

Exp.	17		18	
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		Acid-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 table shows, separation of paranuclein into two fractions can be effected by mere solution in water. At a pH of 4.2 and below, there exists a water-soluble fraction identical quantitatively with that obtained on acid-precipitation. Above this pH, the solubility of the second fraction in water increases, until at pH 8.5, the paranuclein is almost entirely dissolved. There is evidence of slight hydrolysis at pH 2.

The question then arises, why is paranuclein precipitated during peptic digestion, when a part is subsequently found to be water-soluble?

It was proved that the process of drying in vacuo is not responsible for this phenomenon, since paranuclein redissolved immediately after precipitation shows the same effect.

The original digestion mixture from which paranuclein was prepared, differed from Experiment 17 only in the presence of undigested caseinogen, other digestion products of caseinogen, and pepsin. The influence of these materials on the solubility of paranuclein was tested by precipitation of paranuclein with trichloroacetic acid in the presence of (a) caseinogen and (b) pepsin, the concentration of each being, in the final solution, 1%. With the caseinogen, there was no lowering of acid-soluble

material; and a caseinogen control gave no acid-soluble material. With pepsin present, there occurred a fall in the amount of acid-soluble N to 25% in one experiment, and 33.6% in another, the P values being affected similarly. It may be recalled here, that in the time-course experiment with pepsin, Experiment 9, in which the pepsin concentration was 5%, the initial values for acid-soluble material were roughly 20%. An experiment was also carried out analogous to the above, in which no trichloroacetic acid was added, but the paranuclein suspension merely filtered in the presence of pepsin. The reduction of soluble material was not quite so great, but was nevertheless present, the values for soluble N and P being 37% and 30% respectively.

The above results suggest that the presence of pepsin is an important factor in the precipitation of paranuclein, quite apart from its formation during digestion.

B. The Precipitation of Paranuclein under Different Conditions.

As was mentioned in the Introduction, it was stated by Salkowski (1899) and by many others, that the quantity of paranuclein which precipitates is dependent on the conditions of digestion. This being the case, it is probable

that the two fractions present in paranuclein will not always be formed in the same relative amount, and further, that the variation, if present, would indicate something of their character. Accordingly, three experiments were carried out in which caseinogen was digested at five different pH values, and the paranucleins obtained centrifuged and analysed, in the usual manner. In addition, however, the supernatant liquor after digestion and centrifuging was also estimated for N and P, to give indirect measurement of the paranuclein formed. In two experiments, also, a 5 ml. sample of the supernatant liquor referred to was precipitated by trichloroacetic acid, and the filtrate estimated.

Procedure:- A solution of 10 g. caseinogen (Merck, nach Hammarsten, N/P = 15) was made up to 400 ml. in the usual manner (this new caseinogen used requiring 1.6 ml. HCl per g. to give a pH of 2). To it, at a noted time, were added 100 ml. 2% pepsin; two 3 ml. samples were removed for analysis, the remainder was divided into five portions. These were then adjusted to different pH values by the addition of N.HCl or NaOH, and a suitable indicator, and incubated until precipitation and flocculation occurred. At pH values 3 and 4, these criteria were somewhat arbitrary, since part of the original caseinogen precipitated

initially with rise in pH; this material underwent subsequent digestion in the normal manner, as will be seen from the results. The paranucleins precipitating were then centrifuged off and analysed, after one washing. The supernatant liquors were also analysed, and in Experiments 20 and 21, 5 ml. samples were precipitated with an equal volume of trichloroacetic acid, filtered, and the filtrate analysed for N and P.

The results are shown below in Table VII. For comparison, the three experiments are grouped together. The top line of each group represents Experiment 19, the middle line Experiment 20, and the bottom line, Experiment 21.

Table VII.

pH	Paranuclein (indirect)		Supernatant Liquor		Acid-soluble fraction of sup. liq.		Acid-insol. fraction of sup. liq.	
	%N	%P	%N	%P	%N	%P	%N	%P
1	22.2	36.7	77.8	63.3	-	-	-	-
	17.4	32.1	82.6	67.9	61.1	36.5	21.5	31.4
	20.5	25.6	79.5	74.4	68.8	36.4	10.6	36.9
2	15.1	29.9	84.9	70.1	-	-	-	-
	15.3	26.1	84.7	73.9	63.0	34.5	21.7	39.4
	12.0	22.5	88.0	77.5	71.2	35.9	16.8	41.7
2.5	13.8	27.6	86.2	72.4	-	-	-	-
	14.1	23.8	85.9	76.2	61.6	35.1	24.3	41.1
	19.8	27.9	80.2	72.1	70.1	36.5	10.1	35.9
3.0	24.2	38.2	75.8	61.8	-	-	-	-
	29.0	40.4	79.0	59.6	44.0	26.0	27.0	33.6
	32.7	39.7	67.3	60.3	61.3	30.5	6.0	29.8
4.0	49.8	59.0	50.2	41.0	-	-	-	-
	40.6	48.1	59.4	51.9	30.7	22.1	28.7	29.8
	51.8	71.3	48.2	28.7	40.4	11.8	7.7	16.9

The N/P ratios of the above fractions are

represented below in Table VIII.

Table VIII.

N/P caseinogens, 15.2, 14.6, 15.2 respectively.

pH	Paranuclein (indirect)	Supernatant Liquor	Acid-sol. fraction of sup. liq.	Acid-insol. fraction of sup. liq.
1	8.4	19.0	-	-
	7.9	17.6	36.5	9.9
	12.2	16.5	28.3	4.4
2	7.6	18.4	-	-
	8.5	16.8	34.5	8.0
	8.1	17.3	30.2	6.2
2.5	7.6	18.0	-	-
	8.6	16.4	35.1	8.6
	10.8	16.9	29.2	4.3
3	9.6	18.6	-	-
	10.5	17.4	26.0	11.7
	12.5	17.0	30.6	3.1
4	13.1	18.6	-	-
	12.3	16.7	22.1	14.1
	11.1	25.6	52.3	6.9

The values obtained on the paranucleins by

direct measurement are given below in Table IX, including the values for acid-soluble N and P determined in the usual way.

Table IX.

pH	N/P paranuclein	Acid-sol. fraction			Acid-insol. fraction		
		%N	%P	N/P	%N	%P	N/P
1	7.3	37.9	23.6	11.6	62.1	76.4	5.9
	7.2	48.9	34.0	10.4	51.1	66.0	5.6
	8.3	58.2	35.2	13.7	41.8	64.8	5.3
2	6.8	49.5	32.3	9.8	50.5	67.7	5.2
	7.5	48.8	30.8	11.9	51.2	69.2	5.6
	8.4	56.4	34.8	13.6	43.6	65.2	5.6
2.5	6.2	37.5	26.5	8.7	62.5	73.5	5.2
	6.5	44.5	27.5	10.4	55.5	72.5	5.0
	7.6	48.2	29.1	12.6	51.8	70.9	5.6
3	6.5	36.1	24.2	8.6	63.9	75.8	5.6
	8.6	35.9	29.4	10.5	64.1	70.6	7.8
	8.5	44.9	31.0	12.1	55.5	69.0	6.8
4	8.8	24.2	18.5	11.6	75.8	81.5	8.2
	9.1	30.5	26.4	10.4	69.5	31.5	8.1
	9.5	28.1	24.2	11.1	71.9	75.8	9.0

It will be observed that there is a striking increase in the amount of paranuclein precipitated when

the pH diverges from the optimum value for digestion particularly at pH 4, where as much as 71% P may appear in the precipitate; and that this increase is associated with, in general, a rise of N/P ratio. That the increased amount of paranuclein, and its higher N/P ratio, are not due to adsorbed caseinogen, is shown by the N/P ratios of the acid-insoluble fractions of the paranuclein, which have a fairly constant low value throughout. Analysis of the paranucleins themselves, however, does not lead to any very conclusive results, it appears rather that the material may vary widely in composition without superficial change. (Comment may be made in passing to the fact that paranuclein prepared at pH 2.5 from this fresh sample of caseinogen, differed slightly from the original preparations, having an acid-soluble fraction amounting to, on an average, 43%N and 27.7% P). A certain constancy is obtained with the N/P ratios of the acid-insoluble material (Table IX), if the values for pH 4 be excepted, but the absolute amounts formed show considerable variation. Correlating the % acid-insoluble N in the paranuclein with the %N from caseinogen precipitated in the paranuclein as a whole, (Table VII), we find for the first of the three experiments that the absolute amount of N in the acid-insoluble compound may vary from 62.1 x 22.2 = 13.8%

at pH 1, to 50.5 x 15.1 = 7.6% at pH 2, and 63.9 x 24.2 = 16.5% at pH 3.

Two points in the work described above may, with interest, be correlated with the work of Breese-Jones and Gersdorff. In the first place, precipitation of the supernatant liquor of the caseinogen digest after removal of paranuclein again causes a separation into two fractions of very distinct N/P ratio. The acid-precipitate has a low N/P ratio somewhat similar to paranuclein, recalling the fraction which had a N/P ratio of 12 obtained by Breese-Jones and Gersdorff by adjusting the pH to 6. The residual liquor has a N/P ratio of roughly 30, again in agreement with the value obtained by the American workers.

In the second place, the ratio of approximately 5.6 obtained for the acid-insoluble fraction of the paranuclein recalls the ratio obtained by Breese-Jones and Gersdorff for their paranuclein. When it is remembered that they washed their precipitate some half-dozen times before analysis, it seems a reasonable deduction that the fraction we have found to be acid- and water-soluble, had been removed, and the discrepancy between the N/P ratio of their preparations and those of Linderstrøm-Lang is explained.

This was confirmed by an experiment in which a

paranuclein precipitate was divided into several fractions, which were subjected to different treatment with regard to washing.

Procedure:- To a solution of 10 g. caseinogen in 400 ml. water, was added 100 ml. 2% pepsin, and the mixture incubated in the usual way till flocculation. It was then centrifuged in four portions, the precipitates being treated as follows:-

A) Dried immediately;

B) washed once with water, recentrifuged, and dried;

C) washed once with alcohol, recentrifuged, and dried;

D) washed once with water, and once with alcohol, then dried.

The results of the analyses are given below in Table X.

Table X.

Preparation.	N/P ratio.	% acid-sol. N.	% acid-sol. P.	N/P acid-sol. fraction.	N/P acid-insol. fraction.
A	7.8	40.0	23.0	13.5	6.1
B	6.9	29.3	20.0	10.2	6.1
C	6.7	24.2	18.4	8.9	6.2
D	6.5	16.7	17.1	6.4	6.6

C. <u>Precipitation of Paranuclein by Tannic and Phosphotungstic Acids.</u>

The use of trichloroacetic acid as protein precipitant was first advocated by Northrop (1924). It was conclusively shown, however, by Stirling and Wishart (1932) that trichloroacetic acid precipitated much more than the undigested caseinogen. Since tannic acid and phosphotungstic acids are also well-known protein precipitants, it was thought of interest to see how they compared with trichloroacetic acid in their action on paranuclein.

Procedure:- A 1% solution of paranuclein was made up, with a minimum amount of N/10 NaOH to dissolve the paranuclein. To 5 ml. samples were added (a) 5 ml. 10% tannic acid and (b) 5 ml. 10% phosphotungstic acid. The precipitates obtained were filtered off, and the filtrates analysed. In the case of phosphotungstic acid, it was, of course, impossible to estimate the relatively small amount of paranuclein P compared with that of the reagent.

It was found that tannic acid closely resembled trichloroacetic acid in its action, the values of soluble N and P being 43.2% and 31.3% respectively. The phosphotungstic acid differed markedly, leaving only 14% N in

solution.

The results of this experiment suggest that the soluble fraction of paranuclein may not itself be an individual but that it too may be a mixture. This is an aspect which has not at present, however, been further investigated. The results are also of interest, in that they suggest that the action of different precipitants may be a valuable guide to the stage of degradation to which the protein has been digested, since presumably the different action of the reagents used above is an indication of the molecular complexity of the material with which they react.

D. Dialysis of Paranuclein.

The experiments reported above with trichloroacetic, tannic and phosphotungstic acids suggest that the two fractions of paranuclein differ in molecular size. An attempt to demonstrate this further was made by dialysis of a solution of paranuclein, using as semi-permeable membranes cellophane and collodion.

Procedure:- A solution of paranuclein in a minimum of alkali was made up to roughly 1% concentration. 10 ml. samples of this solution were then placed in the dialysers, which were immersed in distilled water to an equal level

of water inside and out. After what was considered to be a suitable lapse of time, varying from 7 to 24 hours, the dialysers were removed, and the N/P ratio estimated in the internal and external liquids. In the first four experiments recorded below, in Table XI, cellophane was used, in the last two, collodion; in both cases, there appeared to be considerable variation in the amount of material allowed to pass through the membrane, entirely unrelated to the time of dialysis, and the experiments have been considered only from the N/P ratios obtained.

Table XI.

Membrane	N/P paranuclein	N/P inner liquid	N/P outer liquid
Cellophane	5.9	5.7	6.6
Cellophane	5.9	5.5	11.5
Cellophane	7.6	7.3	8.0
Cellophane	7.6	7.4	20.4
Collodion	7.0	5.5	10.5
Collodion	7.0	6.1	11.3

The experiments are of a somewhat crude nature, but it is clearly evident that dialysis of paranuclein causes a partial separation of fractions of different N/P ratio. Unfortunately, from this type of experiment, no estimate can be made of the molecular size of the migrating particles; but the higher N/P ratio of the external

liquid is in agreement with the higher N/P ratio of the acid-soluble fraction, that which we would expect to have a smaller molecule.

E. Amino-Acid Composition of Paranuclein.

The amino-acid composition of the two fractions of paranuclein was determined by the usual protein colour tests (Table XII). In addition, the fractions were investigated with regard to their power of combination with iodine, according to the method of Blum and Strauss (1920). Procedure:- 2 gms. paranuclein were suspended in water and filtered. The filtrate and the insoluble residue were then rendered faintly alkaline by addition of sodium carbonate solution, the insoluble residue of course dissolving up. The two solutions were then treated with a solution of iodine in potassium iodide at 37° until the colour of the solution remained yellow, showing that no further absorption was occurring. The reaction mixtures were then cooled, and the excess of iodine present removed by addition of a few ml. sodium thiosulphate solution. The weakly yellow solutions were filtered through cotton-wool, and acidified slowly with acetic acid until the iodinated protein began to precipitate. The mixtures were now treated with several ml. 10% NaOH to remove any

iodic acid formed, and completely reprecipitated with acetic acid. The precipitates were removed, well washed with water, and then with 80% acetone containing a few drops of acetic acid. The precipitates were once more washed with water until no iodine test was given by the filtrate, and suspended in chloroform. After removal of the chloroform, they were suspended in boiling absolute alcohol, then thoroughly washed with absolute alcohol, acetone, and finally ether, and dried in vacuo.

The material obtained from the insoluble fraction of paranuclein was a pale yellow powder, the yield being about 10%. The product obtained from the soluble fraction was brownish, and the yield was very poor, only some 15 mg. being obtained. Analysis for N and I was carried out by Dr. Weiler, of the Micro-analytical Laboratory, Oxford.

Table XII.

	Residue	Filtrate
Biuret	Violet	Violet
Xanthoproteic	+	+
Labile S	+	-
Millon	+	Very faint
Glyoxylic	+	Very faint
Kapeller-Adler	-	+
Nitrogen %	11.73	8.43
Iodine %	10.30	26.19

The results suggest a deficiency in the soluble material of cysteine, tyrosine and tryptophan.

The point at which iodine enters the protein molecule is the subject of a detailed discussion by Blum and Strauss. In brief, however, substitution appears to occur in the rings of tyrosine, tryptophane and histidine; it would thus seem probable, in view of the deficiency of the soluble material in the two first-named amino-acids, that its high iodine value is to be explained by substitution in histidine. The presence of histidine in the soluble fraction was confirmed by the Kapeller-Adler test (1933).

DISCUSSION.

The essential point which emerges from all the experiments above detailed is, of course, the disclosure that paranuclein is not a single, but a mixture of at least two, compounds, which differ from each other in solubility in trichloroacetic acid, in N/P ratio, and in amino-acid composition. Referring again to the early literature, one is considerably struck by the manner in which the results obtained by Lubavin have been confirmed; and one may comment in passing, that in view of all the work carried out by subsequent workers in an endeavour to decide whether paranuclein was a single substance, it is surprising that the simple experiments which he described were never either quoted or repeated. In fact, so little notice has been taken of his work in the subsequent literature that the present author was unaware of its existence until the above experiments had been completed.

The presence in paranuclein of a soluble fraction affords a simple explanation of the discrepancy between the results obtained by Linderstrøm-Lang et al., and Breese-Jones and Gersdorff. There appears to be no doubt that under constant conditions of digestion there is formed a paranuclein of fairly constant composition; but that

too thorough a purification by washing will in reality remove an integral part of the material.

However, while it may be accepted that in paranuclein there are these two fractions, this in itself gives rise to another problem; for the presence of a fraction soluble in water at pH 2 is very difficult to reconcile with its initial precipitation as part of the paranuclein. Various hypotheses may be offered to explain this phenomenon. First, the precipitation of the soluble fraction may be simply a question of low solubility in the presence of pepsin. Second, the soluble material may be precipitated originally in combination with pepsin, a complex broken subsequently by dilution, when the paranuclein is redissolved. Third, the soluble and insoluble fractions when originally precipitated may be in combination, a union readily disrupted on re-solution. Fourth, the paranuclein is an unstable synthetic by-product of the original reaction.

Which of these various hypotheses is the true solution, cannot from my experiments be decided. Reference to the literature offers no conclusive evidence. In support of the first hypothesis, that of a solubility constant, it may be recalled that v. Moraczewski states that when a solution of paranuclein is precipitated by

acetic acid in presence of ovalbumin, the amount of the precipitate is doubled; and that it is probable that paranuclein, after separation from caseinogen, requires a certain concentration of protein for its precipitation. In the experiments I performed in which caseinogen and pepsin were added to a paranuclein suspension, only pepsin and not the caseinogen, was responsible for a lowering of solubility; it is possible, however, that the pepsin is fulfilling the role only of providing the necessary protein concentration. The second hypothesis assumes the formation of a complex between enzyme and digestion-product, but this is not outwith precedent. The third hypothesis involves the assumption of an unstable link between two amphoteric products of digestion, a link readily hydrolysed by water and capable of being reforged by a high pepsin concentration; but this salt-like union, if such is the case, certainly cannot exist in the caseinogen molecule, it must arise in the course of digestion. The last hypothesis, that paranuclein might be a synthetic by-product, is suggested by the ready synthesis by Brailsford Robertson of a product which he could not distinguish from a paranuclein prepared in the normal manner.

To all except perhaps the third hypothesis, it may be objected that the amount of paranuclein formed ought to be increased by a higher concentration of pepsin,

whereas the best yield is obtained with low enzyme concentration. The variations in amount and composition of the paranuclein which may be obtained, suggest that it is merely a loosely-bound mixture of those digestion products which the concentration of enzyme present is incapable of handling, and precipitating for reasons as yet unknown. It is noticeable all through that the more insoluble material invariably has a lower N/P ratio than the soluble, as if a concentration of P was prejudicial to rapid digestion by pepsin, and this observation receives confirmation from the experiments on synthetic phosphorylated protein to be described later. At any rate, there cannot exist in the caseinogen molecule, a preformed nucleus which becomes paranuclein; although the formation of digestion products of much higher N/P ratio than caseinogen itself shows that the P in caseinogen cannot be evenly distributed throughout the molecule, a conclusion previously recorded by Stirling and Wishart.

With regard to the nature of the two fractions which compose paranuclein, it is clear that the original time-course curves give information concerning only the acid-insoluble fraction. The deduction of the somewhat arbitrary values for initially acid-soluble material eliminates the original crossing of the N and P curves, and we obtain in the corrected curves a strong resemblance

to the findings of Stirling and Wishart on caseinogen. To recapitulate, there is a marked difference between the mode of attack of pepsin and trypsin, the former liberating N more rapidly than P, the latter attacking the P-rich material more readily. Papain is intermediate, liberating N at the same rate as P; the preferential acceleration of P liberation by the activated enzyme appears to confirm the possibility recently suggested by Bergmann and co-workers that papain is not a single enzyme. An insight into the actual structure of paranuclein is offered only by the hydrolysis of 1% alkali, in which there is left a resistant portion of the molecule containing 20% of the original N, and no P. The existence of this nucleus suggests that in paranuclein the P must be linked nearer the ends of the molecule.

The acid-insoluble fraction appears to be fairly complex in character. It gives all the protein colour reactions, including that of loosely-bound S, in contradiction to the findings of Breese-Jones and Gersdorff; it is very slowly, or not at all, diffusible, and appears to contain in large proportion those forms of amino-acid union which are either resistant to peptic digestion, or protected by the presence of other interfering groups.

The soluble fraction of paranuclein appears to

be a much simpler molecule. It is diffusible with reasonable speed through cellophane and collodion, though graded experiments have not yet been attempted. It gives a violet colour in the buiret reaction, and gives also the xanthoproteic reaction, but no longer contains S, and gives Millon's and the glyoxylic reactions only faintly. It shows a high power of combination with iodine, and the deduction that this must be due to histidine, since tyrosine and tryptophan are deficient, receives confirmation with the Kapeller-Adler test.

How the fractions may be connected with the other degradation products of caseinogen which have from time to time been isolated, cannot as yet be decided. There is no evidence that paranuclein is formed during tryptic digestion, but does not become visible owing to the alkalinity of the medium; on the contrary, the work of Szontagh, and Stirling and Wishart, appears to be against this view, particularly since the modes of attack of pepsin and trypsin are quite distinct. The other lower degradation products which have been obtained by Dietrich, Posternak, Rimington, etc., cannot be correlated with paranuclein until they have been prepared either

from solutions of paranuclein itself, or solutions from which the paranuclein has been removed.

The biological significance of paranuclein is still doubtful. It is probably produced by peptic digestion of milk in the stomach, yet this cannot be positively affirmed since the caseinogen will have been converted first to casein, and time has not yet been available to confirm the formation of paranuclein from casein. At any rate, even if produced in the stomach, paranuclein will be rapidly attacked by trypsin in the alkaline medium of the intestine, and there is no reason to suppose that valuable P will be lost by this vicissitude of digestion.

PART II.

The Paranuclein Derivatives of Other Phosphoproteins.

Introduction.

So far, interest has been centred entirely on the paranuclein derived from the phosphoprotein caseinogen. This production of paranuclein is not, however, unique to caseinogen, but with one recorded exception, that of human milk, it appears to be a constant characteristic of phosphoproteins in general.

The phosphoproteins occur naturally as components of all those foodstuffs especially designed for the nourishment of the embryo or the suckling, i.e. the yolk of eggs and the milk of mammals. In the latter case, we refer of course to caseinogen; in the former, phosphoprotein has been prepared from the eggs of many fishes, in which case it is usually termed ichthulin, and from the eggs of birds or fowl when it has been termed vitellin. It has also been obtained from other eggs, e.g., those of the frog, in which instance it is termed batrachiolin.

These phosphoproteins are not, of course, all identical. Vitellin is distinguished by possession of an appreciable percentage of iron, a characteristic shown by only one other phosphoprotein, the ichthulin from carp eggs. The ichthulins from different fish show variations among themselves, as probably do the various vitellins. This will be more fully discussed later from the standpoint

of the various paranucleins produced.

Let us consider first what is known about the paranuclein obtained from vitellin. It was observed by Miescher (1870) that an insoluble material separated on peptic digestion of egg-yolk. This material was recorded again by Bunge (1885); he observed that when vitellin was digested with pepsin at room temperature, there was little visible change, but that when the digestion was carried out at 37°, there appeared a white precipitate. This was purified by a thorough process of solution and reprecipitation, including solution in aqueous ammonia, reprecipitation with alcohol, filtration, and subsequent suspension in alcohol acidified strongly with HCl. It yielded, on analysis, on an average, 14.74% N, 5.2% P, (N/P = 2.8), and 0.29 Fe, representing almost all of the iron originally present in vitellin. No estimate is given, however, of how much of the vitellin N and P the precipitate contained. Although realising the possibility that this material was not a chemical individual, Bunge decided it was nevertheless worthy of a name, and called it "haematogen", to indicate that (in his opinion) it was a precursor in the embryo of haemoglobin.

The following year, Kossel (1886) demonstrated that paranuclein from caseinogen, and haematogen were not true nucleins, since they liberated no purine bases on hydrolysis.

However, while it was admitted subsequently that the pseudonucleins, as a class, were distinguishable from the true nucleins, attempts were made to prepare from them, or their parent proteins, lower degradation products comparable with the nucleic acids; and we find recorded in the literature a large number of "paranucleic acids".

The first of these paranucleic acids appears in a paper by Altmann (1889). Classifying nucleic acids as organic phosphorus compounds, which are precipitated from the original nucleins, and are distinguished from them by a higher P content and certain properties of solubility, he states that the general method of preparation is dependent on the nature of the mother substance. It consists essentially in a removal from the mother substance of protein, by the action of alkali or pepsin, and precipitation of the nucleic acid by acidification. This general principle was applied to the preparation of the nucleic acids of yeast, thymus, egg-yolk, and salmon sperm. Whereas, however, the preparation from yeast, thymus and sperm involved the use of alkali, the preparation from egg-yolk was as follows. The dried egg-yolk was dissolved according to Bunge's method in HCl, treated with pepsin, and digested at 50°. The vitellin dissolved up to give

a clear solution from which Bunge's precipitate, as Altmann calls it, slowly settled out. The precipitate was separated, redissolved with the aid of a little ammonia, acidified with acetic acid, filtered, fully precipitated with strongly acidified alcohol, then washed with absolute alcohol and finally ether.

Though somewhat less pure, the properties of the powder obtained were stated to be the same as those of the nucleic acids from yeast and thymus. Unfortunately, more detailed evidence is not given. Altmann's preparation gave 7.9% P on analysis; no mention is made of the N values, and again no estimate is given of what proportion of the vitellin P the precipitate represents.

The distinction between Altmann's preparation, and Bunge's, appears to consist in the partial precipitation of the ammoniacal solution by acetic acid, and filtration, before complete precipitation by alcohol. Presumably, the acetic acid was believed to precipitate off protein, leaving the "nucleic acid" behind. Certainly, the percentage of P in the final product is 7.9, whereas that obtained by Bunge was 5.2; this, however, can hardly be considered as proof that mere solution in aqueous ammonia has disrupted haematogen into a protein and a nucleic acid. It would be necessary also to show that the material precipitated by the acetic acid was purely protein,

and quite distinct from the so-called "nucleic acid". On investigating this aspect, we find to the contrary, that if the acetic acid precipitate is redissolved in 2% NaOH and reprecipitated as in the original method, a second sample of "nucleic acid" is obtained. No analyses of this second material for P are given, nor of the material again left behind; but it is surely obvious, that though Altmann had succeeded in fractionating haematogen, his conclusion that the fractions were two entirely distinct types of compound is due to preconception rather than proof. To the subsequent reviewer, the importance of Altmann's work lies in the disclosure of a strong resemblance between his results and those of Lubavin; since solution of paranuclein from caseinogen, in sodium carbonate, and of haematogen from vitellin, in weak ammonia, in both cases leads to the fractionation of the material into portions of differing solubility and P content.

Some years later, Milroy (1896 - 1897) published a paper on the protein combinations of the nucleic and thymus acids and their relation to the nucleins and paranucleins. Relevant here is the latter part. For this, he used haematogen, and investigated on it the action of pepsin and trypsin. Pepsin, he found, liberated organic P, trypsin dissolved part of the haematogen, but left an

insoluble residue. Following Altmann's method, he endeavoured to precipitate the protein out of the haematogen, and obtained a preparation giving on analysis 7.7% P, and N/P ratio 1.7, which material he found to be definitely not similar to thymus nucleic acid.

Giertz, in 1899, when investigating the action of baryta on paranuclein from caseinogen, investigated also the effect of baryta on haematogen. He found the latter much more sensitive to alkali than the paranuclein, and presumed an inherent difference between caseinogen and vitellin.

The following year, Levene and Alsberg (1900 - 1901) published the results of a study "der sogenannten Paranucleinsäure". Although apparently realising that this nomenclature suggested a relationship which did not exist, they continued its use, and coined the term "avivitellinsäure" for the paranucleic acid of vitellin. Further, without offering any proof that it resulted in the production of the same material, they state that vitellinic acid was obtained by a method previously used by one of them, involving essentially the hydrolysis of vitellin by 25% ammonia, precipitation of unchanged protein by picric acid, and finally of vitellinic acid from the filtrate by alcohol. The precipitate, after one reprecipitation and drying with alcohol, contained 9.69% P, a higher value than that obtained by Altmann or Milroy. After studying the solubility

reactions of the material in acid and alkali, Levene and Alsberg showed that the protein present was not an impurity, as Altmann had thought, but an integral part of the molecule. The authors finally compare their product with Bunge's haematogen with regard only to iron content, confirming that the iron is in stable combination.

The study of haematogen was undertaken next by Hugounenq and Morel. Their investigations were designed chiefly to elucidate further its relationship to haemoglobin in the developing egg; in pursuit of this, they show by amino acid analyses that it strongly resembled globin in composition.

The investigations on haematogen were continued by Posternak (1927), who subjected haematogen to tryptic digestion in an endeavour to obtain information with regard to the P of vitellin. From the tryptic digest, he isolated polypeptides of low N/P ratio, which he called ovotyrines α, β_1, β_2, γ; these Lipmann and Levene (1932) consider related to vitellinic acid.

The above investigations were all carried out before the separation from egg-yolk of the second protein "livetin", by Kay and Marshall (1928).

The further study of vitellinic acid is recorded by Lipmann and Levene (1932), and Levene and Schormüller; but their results, which are concerned with the isolation of a serine phosphoric acid, are not relevant here.

Blackwood and Wishart (1934) investigated the time-course production of haematogen from vitellin, samples of the digestion mixture being filtered at suitable intervals. Their results show a very constant N/P ratio for haematogen of 4.3; this value may fall, however, as low as 2.7 on prolonged digestion. The haematogen precipitate contained roughly 20% of the original vitellin N, and 77% of the vitellin P.

We may now consider briefly the various paranucleins from fish phosphoproteins which have been described in the literature.

Ichthulin was prepared first as early as 1854 by Valenciennes and Frémy. The observation that on digestion with pepsin it yielded a paranuclein is, however, due to Walter (1891). The latter used vitellin from carp eggs, which resembles vitellin from hen's eggs in that it contains iron. The paranuclein was obtained by dissolving the ichthulin in 0.1% HCl, adding stomach juice, and digesting at body temperature for 16 - 18 hours. The resulting dirty yellow product was centrifuged, washed with alcohol, then ether, and dried. The yield was a maximum of 4% of the ichthulin. It was soluble in weak alkali, but was completely precipitated by acetic acid; further addition of alcohol and HCl produced no further precipitate. The paranuclein gave on analysis,

N = 14.66%, P = 2.42%, thus having an N/P ratio of 6.1. The material also contained 0.25% Fe. Variations in composition between individual preparations cast doubt on their identity, but the author points out that this variation is in agreement with the variations found by Lubavin for paranuclein from caseinogen, and that there seems to him no doubt that the two products are closely similar.

Walter, however, obtained from his paranuclein a strongly reducing reaction; further, from the nuclein after hydrolysis by mineral acid, he managed to isolate a colourless powdery residue which reduced alkaline copper solutions, and gave a crystalline derivative with phenylhydrazine.

Salmon roe was investigated by Noel Paton (1898) in the course of an investigation on the life history of the salmon. On digesting the phosphoprotein of the roe with pepsin, there was produced a paranuclein containing 3.7% P and 0.064% Fe.

The study of ichthulin was continued by Levene (1901) who obtained the phosphoprotein from cod roe. Levene, however, preparing the paranuclein by the method he had used previously for vitellinic acid, i.e. by the action of 25% ammonia on the protein, found his product distinct from Walter's in composition and in its absence

of reducing power. The material, which contained some 10% P, was extremely similar to vitellinic acid, and Levene concluded that the ichthulin from cod roe was much more similar to vitellin from hen's egg than to the ichthulin from carp roe.

This conclusion appears to be based on rather slender evidence; let us summarise the position in Table I.

Table I.

	Vitellin	Carp Ichthulin	Cod Ichthulin	Salmon Ichthulin
Peptic digestion	(a) Haematogen (Bunge)	(a) Paranuclein (Walter) Strongly reducing	-	Paranuclein (Paton) non-reducing.
	(b) Nucleic acid (Altmann)	(b) No nucleic acid		
Strong Ammonia	Vitellinic Acid (Levene)	-	Vitellinic Acid (Levene)	-

Levene's contention, it will be seen, is entirely dependent on the production from the protein by strong ammonia of the same material as is precipitated by pepsin, or at any rate, of the same nucleic acid as was obtained by Altmann by fractional precipitation of haematogen. This alternative method of preparation has, however,

already been questioned above when the preparation of vitellinic acid was first discussed. The resemblance between vitellinic acid and "nucleic acid" was based solely on general similarity of composition; yet the digestion of protein must obviously lead to the production of a number of low degradation products which, though different, will necessarily show a general resemblance. Lacking thus conclusive proof on which to base a comparison, it appears premature to consider Levene's work except as far as his own preparations allow.

The only result which is in marked contrast to the others is the reducing effect observed by Walter with his preparation from carp eggs. This, however, may perhaps be explained by the work of Hammarsten (1908), who found with perch phosphoprotein that a reducing effect was always obtained until the material was purified from alcohol-soluble contaminants.

McCrudden (1921) investigated barbel and pike roe, and compared his products in detail with those described above.

The preparation of a phosphoprotein batrachiolin has been described for frog eggs by McClendon (1909).

The experiments recorded below with haematogen are the first of a series in which it was hoped to extend the comparison between paranucleins from caseinogen and other phosphoproteins. Unfortunately, owing to lack of material, the analysis of haematogen alone is recorded.

Experimental Procedure and Results.

Preparation of Vitellin:

This was carried out according to the method used by Blackwood and Wishart. The product obtained from one such preparation had a N/P ratio of 16.8, and was sufficient for the experiments described below.

Certain preliminary work indicated that vitellin was more difficult to digest than caseinogen, confirming the findings of Blackwood and Wishart. The method given below was that finally adopted after several trials.

Preparation of Haematogen:

6 g. vitellin are rubbed into a paste with water; 2.5 ml. N.HCl are then added gradually, and the mixture made up to 400 ml. The suspension obtained is incubated with 100 ml. 2% pepsin solution; the undissolved material gradually dissolves up, and later a cloudy gelatinous precipitate appears which settles out eventually in flakes. At this point, the mixture is centrifuged, the precipitate washed with water, recentrifuged, and dried in vacuo. The yield is approximately 20%.

The haematogen contains some 20% of the original vitellin N and 70% of the original P. The N/P ratio is fairly constant between 4.3 and 4.7.

Solubility of Haematogen in Water and Trichloroacetic Acid.

A weighed amount of haematogen, ca. 0.35 g., was suspended in 25 ml. water, and allowed to extract at 37.5^{o}, the pH being approximately 4.2. At the end of 30 minutes, a 10 ml. sample of the suspension was filtered, and the filtrate analysed for N and P.

To another 10 ml. sample, alkali was added to dissolve the haematogen, and the volume was made up to 20 ml. Part of this was estimated for total N and P; a 5 ml. sample was also precipitated with 5 ml. trichloroacetic acid, and filtered.

The results of experiment 1, and a subsequent duplicate, experiment 2, are given below in Tables IIa and IIb.

Table IIa.

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	%N	%P
Water-soluble	22.1	3.9	30.1	3.2
Acid-soluble	29.8	18.2	24.2	14.8

Table IIb.

Experiment	1	2
N/P haematogen	4.5	4.4
" water-soluble	25.0	41.2
" acid-soluble	7.7	7.2
" acid-insoluble	3.8	3.9

The results of amino-acid colour tests are given below in Table III.

Table III.

	Haematogen.	Water-soluble fraction.	Acid-soluble fraction.
Biuret	+	+	+
Xanthoproteic	+	+	-
Millon	+	faint	-
Glyoxylic	+	faint	-
Loose S	+	faint	faint.

Discussion.

It is clear from the results above recorded that haematogen shows distinct points of difference from, and resemblance to, the paranuclein from caseinogen. Considering the latter first, it may be remarked at once that haematogen also contains an acid-soluble and an acid-insoluble fraction; this was only to be expected from the work on paranuclein, and the early results of Bunge and Altmann. The acid-soluble portion contains some 26% of the total N, and some 20% of the total P; the results among duplicates are unfortunately not in very good agreement, probably due to the wide experimental error involved by the small quantities of P present. The acid-soluble fraction appears, like that from paranuclein, to be deficient in the aromatic amino acids, tyrosine and tryptophane, though it shows also an absence of cystine. As also with paranuclein, the acid-insoluble fraction has a still lower N/P ratio than the original material.

Haematogen, however, is sharply distinguished from paranuclein in two respects. First, the N/P ratio of all the fractions is lower than that of the corresponding fractions of paranuclein: thus we find a N/P ratio for the whole of 4.5 as against 8; for the acid-soluble

fraction, of 3.9 as against 6; and for the acid-insoluble fraction, a ratio of 7.5 compared with ratios varying from 8 to 12. Second, the water-soluble fraction and the acid-soluble fraction of haematogen are quite different from each other, whereas with paranuclein there was no reason to suppose that they were not actually as well as quantitatively identical. The water-soluble fraction of haematogen, containing some 25% of the original N and some 4% of the original P, and having the relatively high N/P ratio in the two experiments described of 25 and 41 respectively, introduces an entirely new type of fraction. This material could obviously not represent simply a part of the acid-soluble fraction, since in experiment 2 it contains a higher proportion of the original N; instead, it is necessary to postulate the existence in haematogen of three entirely distinct fractions, (a) an acid-soluble portion of N/P ratio 7.5, (b) a water-soluble portion of N/P ca. 30, part of which may or may not form a part of fraction (a), and (c) an acid-insoluble fraction of N/P 4.

Haematogen thus appears to be a fairly complex mixture. Why it should precipitate at all in the course of digestion becomes an even more debatable point than the precipitation of paranuclein itself. Even more than

with the latter, one receives the impression that the haematogen does not represent an intermediate stage in degradation, but rather that it consists of certain fragments which are more resistant to peptic attack, and for some reason as yet unknown come out of solution together.

PART III.

The Paranuclein from Synthetically Phosphorylated Proteins.

INTRODUCTION.

The preparation of a synthetically phosphorylated protein was first recorded by Neuberg and Pollak (1910), who acted on an aqueous solution of lactalbumin with phosphorus oxychloride in chloroform, in the presence of calcium carbonate or magnesium oxide. The product obtained showed a strong resemblance to caseinogen. In 1914, Neuberg and Oertel phosphorylated also serum globulin, and caseinogen, as well as certain amino acids, silk fibroin and Witte's peptone.

With regard to the phosphorylation of amino acids, they confirm the previous findings of Langheld (1910, 1911) that the union of P and N is extremely unstable, and readily hydrolysed by both acid and alkali to its component parts. The synthetic phosphoproteins were, however, comparatively stable substances, behaving as true phosphoproteins, inorganic P being liberated only after long standing in acid or alkali. These phosphorylated proteins had each a comparatively constant N/P ratio, 7.1 in the case of globulin, 8.0 for caseinogen. Neuberg and Oertel found that the digestibility of these proteins by enzymes was in no way impaired; they record, however, that although the phosphoric acid in phosphorylated

globulin was very slowly split off by pepsin, at a rate comparable with natural caseinogen, the newly introduced phosphorus in phosphorylated caseinogen was more readily removed than the original.

Rimington (1927) phosphorylated a number of proteins by means of phosphorus oxychloride in carbon tetrachloride, purifying the product by reprecipitation till the N/P ratio of the filtrate was constant. He confirmed the observation that it was possible to phosphorylate caseinogen further; moreover, dephosphorised caseinogen (by the action of ammonia) could be rephosphorylated to give a material closely similar to the original caseinogen, and coagulable by rennin. Globulin was also phosphorylated by Rimington to give a fairly typical phosphoprotein, differing from caseinogen in the fact that bone phosphatase was able to liberate all the P, whereas with caseinogen only 2/3 was liberated. In all cases, Rimington makes no mention of the production of a paranuclein.

In the results detailed below, the work of Rimington was repeated to determine whether phosphorylation affected the production of paranuclein, in the hope that some information with regard to the essential phosphorus linkage might be obtained.

EXPERIMENTAL.

Preparation of the Phosphoprotein.

Procedure: This was essentially that used by Rimington.

Some 10 g. of the protein to be phosphorylated were dissolved in 100 - 150 ml. N.NaOH and cooled in ice. (When globulin and albumin were used, the protein was first denatured by alcohol.). A solution of 20 g. $POCl_3$ in 80 ml. CCl_4 was then added dropwise, with constant stirring, the pH being maintained at a pink colour with phenolphthalein by the addition of suitable amounts of 20% NaOH. The temperature throughout was kept below 5°. At the end of the reaction, the mixture was allowed to warm to room temperature, more water being added if necessary to dissolve the salts formed. The aqueous layer was then separated and precipitated by addition of HCl to pH 5.

The precipitated protein was filtered off and redissolved and reprecipitated some half-dozen times. Since in the first two experiments the N/P ratio of the filtrate refused to become constant, but steadily fell, due presumably to gradual hydrolysis of P, and since some material was lost on each occasion, reprecipitation was stopped after the sixth time. The proteins obtained were then washed thoroughly with alcohol and ether and

dried in vacuo. The yield is about 60% for caseinogen, less with serum globulin, and only some 30% for egg albumin.

Digestion with Pepsin.

Method: For digestion experiments, a small quantity of protein, perhaps 1 g., was suspended in 70 ml. water, and HCl added to pH 2. To this suspension was added 17 ml. of 2% pepsin, and the mixture incubated. If the protein had not all dissolved in 30 - 40 minutes, as was unfortunately usually the case, the suspension was quickly filtered and digestion continued with the filtrate until signs of flocculation appeared. The precipitate was then centrifuged off in the usual manner and redissolved in dilute alkali for estimation.

Results.

The results of the digestion experiments are given below in Table I. It may be stated in addition that in every case the synthetic phosphoprotein was much more insoluble than the original. For instance, with phosphorylated caseinogen, the total protein which can be dissolved is only about half the concentration used in the normal preparation of paranuclein, and the time required for flocculation is fully twice as long.

Table I.

Phosphorylated Protein	N/P	% total N in ppt.	% total P in ppt.	N/P ppt.	N/P supernatant liquor	N/P paranuclein (directly)
Caseinogen	7.1	6.1	29.2	2.2	10.5	4.4
Glubulin I	6.5	33.3	6.3	34.4	4.7	3.8
Globulin II	5.2	10.5	6.4	8.5	4.9	5.1
Albumin I	5.9	-	-	-	-	5.3
Albumin II	6.7	-	-	-	-	6.8

It will be seen that there is not any very good duplication in the results. Owing to the small concentration initially of protein, the final yield of paranuclein is so small that considerable experimental error is involved. There is a distinct visible difference, however, in the yields of paranuclein, and incorporating this in the results, it may be remarked that the yield of paranuclein from phosphorylated caseinogen is comparable with that from the natural product, the yield from phosphoglobulin considerably lower, and the yield from phosphoalbumin too small to permit of the indirect estimation, (from the supernatant liquor), consisting as it does merely of a small deposit sufficient for one direct analysis.

In general, phosphorylation has led to the introduction of phosphorus in a fairly evenly distributed

manner throughout the molecule. This is shown by the fact that the N/P ratio of both caseinogen and its paranuclein falls after phosphorylation, leaving the same relative difference between the two; while with phosphorylated globulin and albumin, no separation into degradation products of markedly different N/P ratio can be obtained, the paranuclein having a ratio closely similar to the phosphoprotein itself.

Discussion.

The mode of attachment of phosphorus in the protein molecule has for long been a subject of argument. Without entering into the details of the various theories which have appeared in the literature, it is apposite to mention here briefly the modern viewpoint, arising from the work of Rimington (1927), Posternak (1927), Levene et al., and Lipmann (1933). It appears that phosphorus is present in the protein as an organic mono- or di- ester of phosphoric acid with the hydroxy-amino acids. Largely, it appears to be attached to serine; but undoubtedly certain other hydroxy-amino acids are involved, e.g., hydroxyglutamic acid and hydroxyproline, perhaps also tyrosine. It is not known whether phosphoric acid is attached to the enol forms of diketopiperazines. The work of Neuberg et al., and Langheld, precludes the attachment in the phosphoproteins of phosphorus to nitrogen, since the latter union is very unstable to acid.

The appearance of products of widely different N/P ratio on digestion of natural caseinogen indicates that the phosphorus is not evenly distributed throughout the molecule. If we admit the attachment of phosphorus

only to hydroxy groups, this uneven distribution of phosphorus does not necessarily imply, however, an uneven distribution of the hydroxy-amino acids; on the contrary, since it is possible to introduce considerably more phosphorus into the molecule, it is clear that the initial uneven distribution may be due to either of two causes. First, it may be due to an uneven distribution of the various types of hydroxy-amino acids, certain of which may be more readily phosphorylated than others; second, it may be due to a pronounced selectivity on the part of the mammary gland, independent of the hydroxy-amino acids involved. An analogous situation necessarily arises with both vitellin and ichthulin.

The introduction of phosphorus into the protein molecule definitely increases its resistance to pepsin, in contradiction to the findings of Neuberg and Oertel. This fact, which one could have deduced from the resistance of paranuclein itself to digestion is directly proved by the slowness of digestion of the phosphorylated proteins. The formation of paranuclein, however, appears to be directly proportional to the difference in concentration of phosphoric acid in it as compared with the other degradation products. It would seem as though digestion of the natural phosphoprotein by pepsin results in an accumulation of most of the resistant groups into

one resistant fraction, while more rapid degradation continues with the remainder. In the case of phosphorylated caseinogen a similar separation is still possible, since the introduction of the later phosphorus does not affect the uneven distribution initially present; with phosphorylated globulin and albumin, however, all the phosphorus is too evenly distributed to allow of such a fractionation, and digestion must continue at a slower rate, all fractions being almost equally resistant.

The above theory is in full accord with the hypothesis put forward previously (pp. 52, 72) to explain the production of paranuclein. To recapitulate, in conclusion, paranuclein appears to be essentially an accumulation of those products of digestion containing a high proportion of phosphorus which the enzyme is temporarily unable to attack, and which precipitate together for reasons not completely understood, but probably connected with the insolubility of a highly polar complex in the acid digestion medium.

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