C. SOME PHOSPHORUS COMPOUNDS OF MILK.

II. THE LIBERATION OF PHOSPHORUS FROM CASEINOGEN BY ENZYMES AND OTHER AGENTS.

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OF all the naturally-occurring proteins, caseinogen has perhaps been most studied, but there is remarkably little information available in the literature as to the mode of chemical attachment, and none as to the biological significance, of the group which gives caseinogen its place in protein classification as a member of the small group of phosphoproteins. That the phosphoric acid group in these proteins is not in the nature of an inorganic impurity, but is definitely a portion of the molecule, is now generally accepted, and rests on a secure foundation. The experiments described in this paper were undertaken with a view to eliciting further information as to the mode of combination of phosphorus in caseinogen, with an ultimate intention of throwing light on the *raison d'être* of the phosphoprotein in the natural foodstuff of the young animal. The results recorded here must be regarded as merely preliminary steps toward the solution of these problems.

With regard to the significance of the phosphoric group present in organic combination in caseinogen there are three possibilities. Either this group (*a*) has some definite part to play in the economy of the suckling, or (*b*) is there because of certain synthetic necessities of the mammary gland, or (*c*) is present adventitiously, and, in the milk, could be replaced equally well by inorganic phosphoric acid as far as the needs either of the mother or of the growing suckling are concerned. Although there is no direct evidence at present, there are nevertheless a number of reasons why the third possibility would appear unlikely. Thus the natural food of the young of all animals contains similar substances. The caseinogen of mammalian milk has its analogues in the vitellin of the egg-yolk which nourishes the growing chick, in the ichthulin which performs a similar function in the roe of fishes and in a phosphoprotein occurring in frogs' eggs.

If these compounds had no significance in virtue of their *organically* bound phosphorus, it is difficult to understand why they should be produced by organs as widely different as the mammary gland of the cow and the ovary of the herring, for the same purpose, namely, that of forming a quantitatively large part of the only foodstuff of the young animal. Did they not subserve some particular function, one would expect that the phosphorus would be supplied in inorganic form.

Assuming that the organically bound phosphorus of the food is entirely converted into inorganic phosphates either in the lumen or in the walls of the digestive tract, and is therefore absorbed as such, it would appear that any advantage derived from the organic phosphorus by the young animal is connected with the maintenance of phosphoric acid in un-ionised form for a shorter or longer period. This would allow of absorption of phosphorus at different levels in the intestine and prevent its immediate conversion (in presence of Ca ions and at the p_H of the intestinal contents) into insoluble calcium phosphate no longer capable of being taken up at all. Some experiments brought forward in this communication would indicate that, although phosphorus in organic combination is easily removed from the caseinogen molecule by digestive enzymes, the further hydrolysis to inorganic phosphate does not proceed quite so easily as might have been anticipated.

HISTORICAL.

The presence of phosphorus as an integral part of the caseinogen molecule was clearly demonstrated by Hammarsten [1872, 1877, 1883], who in 1872 proved that the phosphorus content of caseinogen, derived from the milk of any one species, and separated from all mineral impurities, was a constant quantity characteristic for the species.

Action of pepsin. Lubävin [1871] had early noticed that when gastric juice was allowed to act upon caseinogen, a greyish deposit was gradually formed during the course of the experiment and collected at the bottom of the flask. It contained phosphorus in a proportion varying with the amount of enzyme used, the duration of the digestion, and other factors. These results were fully confirmed by Klinkenberg [1882] and Milroy [1896].

The analogy between this precipitate and the insoluble "nuclein" produced from nucleoprotein when acted upon by pepsin had early been recognised, and to avoid confusion the term "paranuclein" had been suggested for the former by Kossel [1891] and "pseudonuclein" by Hammarsten [1893]. Kossel [1886] had definitely proved that the similarity was one of physical features only, for this substance contained no carbohydrate group or purine bases, whereas the true nucleins invariably showed evidence of these bodies on hydrolysis.

Up to the year 1893, when Salkowski commenced his experiments, it was believed that the whole of the caseinogen phosphorus was to be found in the paranuclein, and in Hoppe-Seyler and Thierfelder's *Handbuch* it is so stated. Szontagh [1893], on the other hand, maintained that paranuclein yielded to

prolonged peptic digestion, undergoing re-solution with the separation of some phosphorus as phosphoric acid. This latter fact Salkowski and Hahn [1895] were unable to confirm, although they amply proved that if sufficient pepsin be present practically the whole of the precipitate may go into solution. The re-solution of the phosphorus was also noted by Krehl and Matthes [1895], Alexander [1898] and by Willdenow [1893], although v. Moraczewski [1895] was of the opinion that a small fraction always remains undissolved.

The great variability in the phosphorus content of paranuclein led Robertson [1907] seriously to question its individuality. Values were to be found in the literature ranging from 0·88 to 6·86 % of phosphorus and as a result of his own work Robertson concluded that from the paranuclein first formed a soluble substance containing organically combined phosphorus was split off, leaving a residue of lower phosphorus content (*ca.* 1·8 %).

Salkowski [1899] found that prior to the precipitation of paranuclein the first action of pepsin was so to transform caseinogen that it was no longer precipitable by acetic acid; the separation of the paranuclein then began gradually.

Attempts to isolate individual phosphorus-containing substances from peptic digests of caseinogen have been made by Salkowski [1901], Reh [1907] and Dietrich [1909], but in no case can it be said that a pure product has been obtained.

Action of trypsin. The ability of trypsin to liberate phosphorus during the digestion of phosphoproteins has received much less attention. Sebelien [1895] was the first to conduct experiments of this nature and as a result he stated that in this case no paranuclein was formed, the whole of the caseinogen, except for a negligible residue, going into solution. He did not, however, investigate the form in which the soluble phosphorus was present.

This was done by Biffi [1898] who found that only a fraction (27 %) of the soluble phosphorus could be precipitated by magnesia mixture. The figure was somewhat variable, being influenced by the concentration of enzyme and duration of digestion. Plimmer and Bayliss [1906] confirmed the results of Biffi, and found, on the average, 35 % of the phosphorus present as phosphoric acid after tryptic digestion. These authors also studied the rate at which the phosphorus of caseinogen became soluble (*i.e.* not precipitated by tannic acid) under the influence of trypsin, and found that it was very rapid, the production of phosphoric acid occurring at a considerably slower speed.

Plimmer and Bayliss made no direct attempt to isolate the soluble organic phosphorus-containing substance although they investigated the action of various agents upon it. Thus 1 % sodium hydroxide was found to split off 50 % of its phosphorus as phosphoric acid in 40 hours at 38°. In later experiments Plimmer [1913] found that the whole of the phosphorus could be converted into phosphoric acid under these conditions. Intestinal extracts were also found to have a hydrolytic action upon the organic phosphorus compounds of the digest.

Action of sodium hydroxide. The action of 1 % sodium hydroxide upon caseinogen at 38° for 24 hours was found by Plimmer and Bayliss to result in the liberation of the whole of the phosphorus of the protein as free phosphate, precipitable by magnesium citrate mixture.

So far as we can discover the literature since this date contains no new fact of importance concerning this aspect of the breakdown of caseinogen, and there is in particular no mention of the action of phosphatases on the partially hydrolysed intermediate compounds derived from caseinogen.

EXPERIMENTAL.

The methods employed for the determination of inorganic phosphate in the experiments described in this paper were either the well-known colorimetric method of Briggs, or, where this was inapplicable, precipitation of magnesium ammonium phosphate with magnesium citrate mixture and weighing as pyrophosphate. In certain protein digests, in which, after precipitation with trichloroacetic acid, a cloudy suspension is produced when acid molybdate is added, it is not always possible to carry out the Briggs technique. A satisfactory precipitant for the interfering substances was sought without success. It was found that some of the commoner protein precipitants inhibit colour formation in a marked manner [Rimington, 1924]. Provided, however, that the amount of turbidity produced by the acid molybdate is minute, it is possible to centrifuge the precipitate off without appreciable loss of phosphorus. With a very turbid reaction mixture this procedure will not give accurate results, and it is better, in all cases where turbidity develops with molybdate, to use citrate mixture. In the experiments in which 1 % caustic soda and caseinogen were left in contact at 37° it was found quite impossible to use the Briggs technique, and the rather less accurate magnesium citrate method was employed.

Action of pepsin on caseinogen.

A 1 % solution of Armour's pepsin yielded on filtration a satisfactory enzyme preparation. Hammarsten's "casein" was dissolved in chloroform water (to which 0·8 cc. *N* soda per g. of caseinogen had been added) to form a 0·47 % solution, and portions of this, adjusted to a p_H of 1·2 were incubated in stoppered test tubes at 37° in presence of chloroform. From time to time duplicate tubes were removed and the inorganic phosphorus determined. Our results indicate that no measurable amount of free phosphate is liberated within 9 days, although at the end of this time all the caseinogen has been converted into a form not precipitated by trichloroacetic acid. In this, our findings confirm those of previous workers [Salkowski and Hahn, 1895; Plimmer and Bayliss, 1906].

Bone phosphatase prepared by the method of Kay and Robison [1924] had no hydrolytic effect on the soluble phosphorus compounds in this digest at p_H 8·6. Paranuclein, prepared by the action of 0·25 g. of Armour's pepsin on

1 litre of 2 % caseinogen solution in hydrochloric acid at a p_H of about 1·2, was found to contain 1·4 % of phosphorus. It was dissolved in dilute caustic soda, and the p_H adjusted to 8·6. Bone phosphatase again displayed no hydrolytic activity.

Action of trypsin on caseinogen.

Various aspects of the action of trypsin on caseinogen have been studied, and a phosphorus-containing peptone has been separated from the intermediate products of tryptic digestion.

In order to ascertain to what extent the phosphorus of caseinogen was hydrolysed to inorganic phosphate by trypsin, 200 g. of caseinogen and 200 cc. of pancreatic extract (Allen and Hanbury) in a total volume of 2 litres were allowed to digest at 37° and the acid-soluble phosphorus and free phosphoric acid were determined at intervals in aliquot portions. The following figures show that after over 2 months hydrolysis is not complete:

Days	Soluble P in 10 cc. (mg.)	Free P in 10 cc. (mg.)	% of soluble P as inorganic P
0	0·8	0·8	—
14	6·02	2·47	41·1
73	6·03	3·44	57·1

Total P 6·05 mg. in 100 cc.

The rate at which the acid-soluble phosphorus increases in the earlier stages was determined in another experiment, in which the amino-nitrogen was also followed by the method of Van Slyke. In this experiment 40 g. of caseinogen at p_H 8·4 in a total volume of 2 litres were digested by 100 cc. of pancreatic extract. The results are shown in Fig. 1.

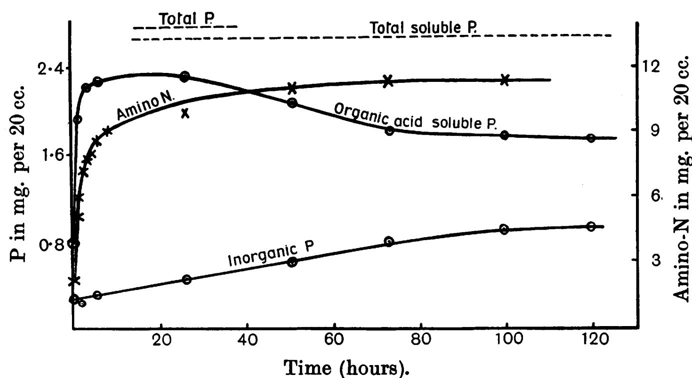


Fig. 1. Action of trypsin upon caseinogen.

During the first 3–5 hours the phosphorus, still in organic combination, is removed from the protein at a rate comparable with that at which amino-nitrogen is produced. After this period, the amino-nitrogen continues to increase whilst the organic phosphorus more slowly approaches a maximum and then begins to diminish. Production of free phosphoric acid takes place

throughout, but at a considerably slower rate, and after several days the organic acid-soluble phosphorus is evidently suffering slow hydrolysis.

With a relatively higher concentration of enzyme it is possible to remove all the phosphorus of caseinogen in an inorganic form. For this purpose pancreatic juice activated by a trace of enterokinase was used. Controls were set up in which the same very low concentration of enterokinase was present as in the pancreatic juice. The substrate was a 0·5 % solution of caseinogen in borate buffer at p_H 8·4, and in presence of chloroform. Fig. 2 shows the results obtained.

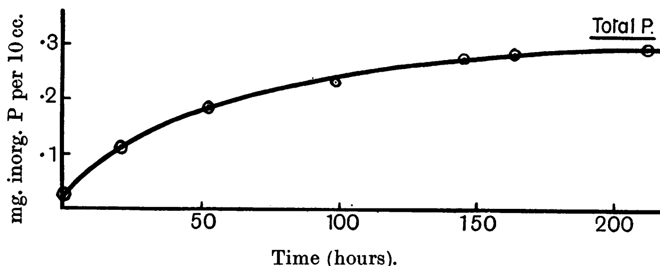


Fig. 2. Action of pancreatic juice on caseinogen at p_H 8·4.

According to Plimmer and Kaya [1909] pancreatic juice itself contains phosphoprotein and nucleoprotein. A phosphorus determination on a sample of pancreatic juice revealed a content of only 0·008 mg. P per cc., which was negligible in comparison with the quantity of phosphorus derivable from the caseinogen.

Hence, in modification of what has been formerly surmised, the organic phosphorus compounds liberated from caseinogen by trypsin are not entirely stable towards this enzyme, and may be almost completely decomposed if the enzyme concentration is sufficiently great and the digestion prolonged.

We could find no account in the literature of any serious attempt to isolate the organic phosphorus compound or compounds produced during tryptic hydrolysis of caseinogen, and it appeared to us not unlikely that some hint as to the mode of combination of the phosphoric acid group in the protein molecule might be obtained from the study of this compound. We have isolated a diffusible substance which contains over 60 % of the organic phosphorus of the parent caseinogen, has strongly acidic properties, forms insoluble salts with the heavy metals, but a soluble barium salt, and has all the reactions and properties of a peptone. It contains 10·5 % N and 3·8 % P[1]. It is hoped to publish, in the near future, the results of an investigation of the properties and composition of this substance, which we call, for convenience, "phosphopeptone."

Action of enzymes on phosphopeptone. The action of *trypsin* upon isolated phosphopeptone is to liberate the whole of the phosphorus as inorganic phosphate in 6 days.

[1] *Note added Aug. 4th.* On further purification the P content rises to 4 %.

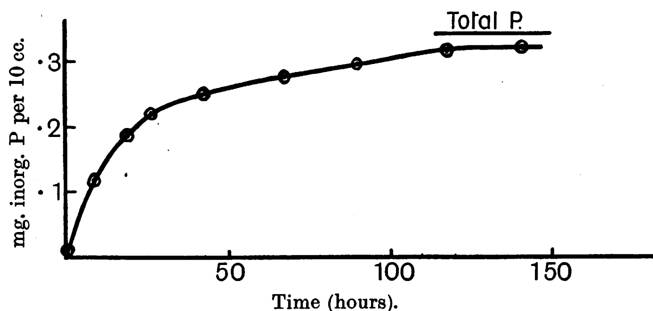
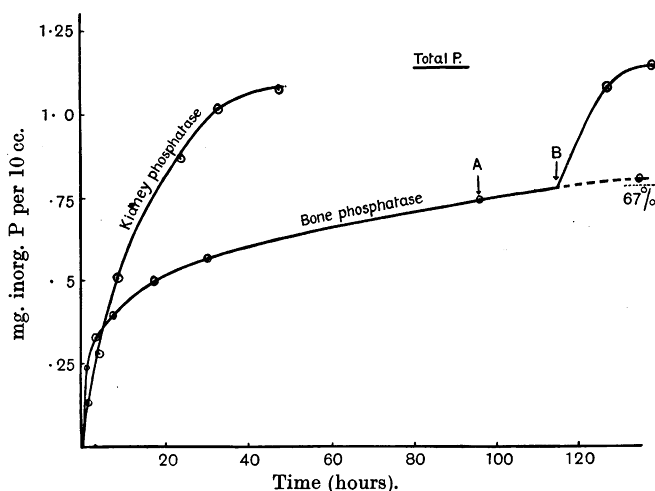


Fig. 3. Action of trypsin on phosphopeptone.

After 9 days at 37° an active *pepsin* preparation had liberated no inorganic phosphoric acid from the phosphopeptone.

Bone phosphatase (rat or rabbit), which is quite unable to liberate phosphoric acid from caseinogen, has a marked action on the phosphopeptone. Two-thirds of the phosphorus of this substance is readily split off, after which any further hydrolysis appears to take place with extreme slowness.



At A a further quantity of bone phosphatase was added.
At B kidney phosphatase (rabbit) was added.

Fig. 4. Action of bone and kidney phosphatase on phosphopeptone.

Kidney phosphatase, obtained from the pig's kidney by maceration with 19 times the weight of chloroform water, displays exactly the same behaviour, but quite a different effect is produced by the phosphatase of the kidney of the rabbit. With this preparation 100 % of the phosphorus of phosphopeptone is liberated as phosphoric acid and the time-curve illustrating the hydrolysis is dissimilar from that afforded by bone phosphatase. In Fig. 4 is shown the course of the latter reaction and, for comparison, the curve obtained when kidney phosphatase (rabbit) is allowed to act upon the same phosphopeptone

solution. At the points indicated fresh enzyme was added and the additional 33 % hydrolysis due to kidney enzyme is seen to take place in a manner closely resembling that observed when this alone is present from the start.

It would appear, therefore, that the phosphatase of bone and rabbit kidney are not identical in nature [see also Kay, 1926] and that phosphopeptone either comprises two different substances, or has two different linkages by which phosphorus is held in organic combination.

Diluted intestinal juice, obtained by washing out the small intestine of a rabbit with 0·9 % sodium chloride and filtering through cotton wool, brings about a fairly rapid conversion of the organic phosphorus both of caseinogen and of phosphopeptone into inorganic phosphate. With caseinogen the process is almost complete in 98 hours, with phosphopeptone complete in 48 hours.

Extract of intestinal mucosa (5 % aqueous) brings about a slow liberation of inorganic phosphorus from caseinogen, but rapid hydrolysis of phosphopeptone, 60 % of the phosphorus of the latter compound appearing as inorganic phosphate in 10 hours. The hydrolysis of the remaining 40 % is a slow process, being almost completed in 5 days. It may therefore be concluded that the organic phosphorus of the phosphopeptone is present in two forms, one fairly easily attacked by phosphatases, the other relatively resistant. All the organic phosphorus of caseinogen is very resistant to phosphatase activity. Thus one of the effects of trypsin upon caseinogen is to render its phosphorus far more readily hydrolysable.

None of the phosphatase preparations—bone, kidney or intestinal mucosa—is capable of removing phosphoric acid from paranuclein (prepared by the action of pepsin upon the same caseinogen) in more than minimal quantities, thus demonstrating another marked difference between the linkages attacked by the two proteolytic enzymes.

Action of alkali on caseinogen.

In investigating the action of alkali upon caseinogen we have adopted the following procedure. A specimen of caseinogen is prepared from separated milk by the usual method, employing acetic acid as precipitant and reprecipitating from a solution of the crude product in dilute sodium hydroxide. Care is taken that the protein shall on no occasion be exposed to strong alkali, never more than the minimum quantity necessary to dissolve the caseinogen (0·8 cc. N sodium hydroxide per g. caseinogen) being added to the water in which solution is to be effected.

The product after washing with alcohol is extracted by ether for 6 hours, then dried in a vacuum desiccator. The white powder is then easily soluble in alkali of the above-mentioned strength and contains traces only of ash. A 2 % solution of this caseinogen is made and at the same time a solution of sodium hydroxide; both are warmed to 37° and then equal volumes mixed so that the final solution has a concentration of alkali of 1 %. A sample is taken at once for the zero reading, toluene added and the reaction mixture placed in

a water-bath at 37°. Phosphoric acid is determined in aliquot portions at intervals by magnesium citrate mixture, after removing most of the protein by precipitation with dilute sulphuric acid. The whole of the caseinogen phosphorus becomes liberated as phosphoric acid within 24 hours. By determining the total amino-nitrogen of the solution at intervals, an endeavour was made to find out whether a corresponding increase in free NH_2 groups accompanied the liberation of inorganic phosphorus. A large increase in substances titratable by Sørensen's formaldehyde method did actually occur, but there was a strong smell of ammonia in the reaction flask. In further experiments concurrent determinations of ammonia and amino-groups (Sørensen) were made. Ammonia was determined by drawing a fairly rapid current of air through the reaction mixture at 37° (with precautions to avoid frothing) and absorbing the ammonia in standard acid which was titrated at hourly intervals (Fig. 5). The liberation of ammonia was found to take place in the early stages in a linear manner and is sufficient to account for the increase in the formaldehyde titration figures, within experimental error. At the end of 30 hours, by which time the liberation of phosphorus is complete, ammonia formation is still proceeding with only slightly diminished velocity. It is therefore unlikely that the two processes are directly related.

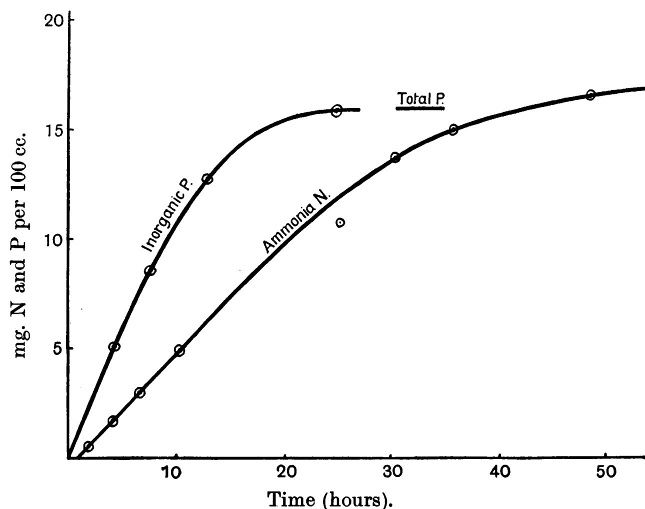


Fig. 5. Action of 1% sodium hydroxide on caseinogen.

During the action of 1 % caustic soda on phosphopeptone, there is also a rapid liberation of phosphoric acid, accompanied by a gradual increase in amino-nitrogen (or ammonia) as determined by Folin's colorimetric method. In the early stages the nitrogen and phosphorus are liberated in the proportion of about 3 P : 1 N, but amino-nitrogen is still being liberated after all the phosphorus has been set free. During the action of bone phosphatase on phosphopeptone there is no increase in amino-nitrogen as measured by

formaldehyde titration. The evidence on the whole does not favour the view that a linkage exists in the caseinogen molecule by which phosphorus and nitrogen are directly associated.

Dephosphorised caseinogen.

Plimmer and Bayliss [1906], and Maynard [1919], remarked upon the opalescence of the solution obtained when 1 % sodium hydroxide acted upon caseinogen, and stated that upon acidification a substance was precipitated which resembled the original caseinogen in its solubility in alkali and insolubility in dilute acetic acid.

This substance may best be prepared by adding acetic acid (33 %) drop by drop to a 36-hour digest of 1 % caseinogen in 1 % soda until maximum precipitation is obtained. The yellowish gummy material separating out is filtered off through muslin, washed, redissolved in sodium hydroxide and reprecipitated; again washed and dried as far as possible on a Buchner funnel (using muslin); it is then transferred to a mortar and ground up with absolute alcohol. At first gummy, it becomes more friable on grinding. The gummy change we were unable to avoid by using alcohol of gradually increasing strength during the dehydration. The final product is a fine white powder, very similar to caseinogen in appearance and properties but slightly hygroscopic. It gives all the protein colour reactions given by caseinogen, but contains traces only of phosphorus and we have called it, for the time being, "dephosphorised caseinogen." The yield from three different specimens of caseinogen is about 40 %. There is a variation in composition according to the previous history of the caseinogen used, but fairly constant figures are obtained from different digests of the same caseinogen. Thus from "Glaxo physiological casein" two different preparations of dephosphorised caseinogen gave the following figures:

	Sample 1	Sample 2
Moisture lost at 107° (% of wet weight)	7·68	5·55
Total nitrogen (Kjeldahl, % of dry weight)	13·17	13·14
Total phosphorus (Neumann, % of dry weight)	0·019	0·019

The caseinogen used for preparation of "sample 2" had been extracted in a Soxhlet for 6 hours with ether.

The low content of nitrogen is a remarkable feature of dephosphorised caseinogen, the accepted figure for the nitrogen of caseinogen itself being 15·65 %.

The explanation of this difference is almost certainly the liberation of ammonia which occurs during the reaction. Schutzenberger [1875, 1, 2] early showed that during the alkaline hydrolysis of caseinogen quite a large evolution of ammonia occurs and Griggs [1921], in a study of the hydrolysis of caseinogen by alkali under pressure at a temperature of 150°, has further found that the amide-nitrogen continues to increase slowly in the later stages probably at the expense of the amino-nitrogen. Sakaguchi [1925], using less drastic conditions, finds that sodium hydroxide not only attacks amide linkages (such

as were shown to be present by Luck [1924]), but also hydrolyses the arginine group in the caseinogen to ornithine. His residual product was found to possess only 0·54 % of its nitrogen in the form of ammonia-nitrogen instead of the 10·3 % of the total nitrogen which is present as ammonia-nitrogen in caseinogen. The composition of dephosphorised caseinogen is being investigated and also that of the albumoses which remain behind in the digest after acidification, but are precipitated by saturation with ammonium sulphate.

Discussion.

The fact that neither bone nor kidney phosphatase has any appreciable action upon the phosphorus of caseinogen, whilst a rapid separation is brought about by these agents of phosphoric acid from phosphopeptone—the substance containing organically bound phosphorus which is produced by the action of trypsin upon caseinogen—suggests that in the latter, linkages are present comparable to those found in hexosemono- and hexosedi-phosphoric acids, α- and β-glycerophosphates, yeast nucleic acid, etc., compounds all of which readily lose their phosphorus as phosphoric acid under the action of phosphatase preparations. The linkages present in caseinogen cannot, however, be of this type. The phosphorus group must be modified in some way, or shielded from the attack of the enzyme.

Both pepsin and trypsin are able to remove the phosphorus of caseinogen, still in organic combination, from the remainder of the protein, but the substances so produced are quite different in their properties, thus affording additional evidence that caseinogen is attacked in completely different ways by the two proteolytic enzymes. The phosphorus-containing substance in peptic digests is not hydrolysed with liberation of phosphoric acid by phosphatase preparations, neither does it yield phosphoric acid under the prolonged action of pepsin. On the contrary, that present in tryptic digests is attacked by both bone and kidney phosphatase, and it is also hydrolysed, more slowly, by trypsin itself, until eventually all the phosphorus appears as phosphoric acid.

The nature of the difference existing between the organic phosphorus as it occurs in caseinogen and as it is present in phosphopeptone after hydrolysis of the protein by trypsin is not yet clear, although the structural modification produced by the enzyme is probably not radical in nature. Thus both compounds display similar behaviour towards alkali (1 % caustic soda at 37° removing the whole of the phosphorus from both caseinogen and phosphopeptone as phosphoric acid within a few hours), towards 1 % acid at 37° and towards pepsin (neither reagent being able to bring about the liberation of phosphoric acid from either caseinogen or phosphopeptone). An additional fact brought to light as a result of the study of the action of phosphatases is that the phosphatases present in rabbit kidney and rat bone respectively have not similar actions upon phosphopeptone, the former removing all the phosphorus of this substance as phosphoric acid, whilst the rat bone preparation

effects only about 66 % hydrolysis. A dissimilarity of the two phosphatases is also indicated by their differing activity towards the phosphoric esters in the blood [Kay, 1926]. It would appear therefore that the phosphopeptone fraction is either a mixture of two or more substances containing organic phosphorus linked in different ways, or that such different linkages exist together in the same molecule. Of necessity these considerations must apply with equal force to caseinogen itself[1] from which the phosphopeptone is derived.

It would appear unlikely that the diffusible phosphoric esters of milk [Kay, 1925] are derived from caseinogen or are directly related to paranuclein or phosphopeptone. The former are hydrolysed to some 60–80 % by bone and kidney phosphatases, but paranuclein is unaffected in presence of these enzymes. Moreover, 2 % sodium hydroxide only liberates traces of inorganic phosphate from the milk esters in 18 hours at 38°, whilst all the organic phosphorus of the phosphopeptone is liberated by 1 % sodium hydroxide in 24 hours.

The frequency of occurrence of phosphoproteins in the material provided by the maternal organism as food for the growing animal has been pointed out earlier. We have not been able to discover in the literature any definite experiments in which very young animals have been maintained on a diet free from organic phosphorus. The relative difficulty with which the organic phosphorus of caseinogen is converted into inorganic phosphate by the alimentary enzymes and the ready diffusibility of some at least of the intermediate phosphopeptones seem to us to support the view that the possibility of uptake of organic phosphorus by the young animal cannot be completely dismissed. The observation of Keller [1900] that in infants there is a rise in the organic phosphorus of the urine after milk feeding is of significance here.

Summary.

Action of pepsin.

On caseinogen. No inorganic P produced in 9 days. Paranuclein obtained, containing a large proportion of the original caseinogen P.

On phosphopeptone. No inorganic P liberated in 71 hours.

Action of trypsin (activated pancreatic juice).

On caseinogen. Complete hydrolysis of the organic to inorganic P when the enzyme is in sufficient concentration; goes through intermediate stage of phosphopeptone; process slow.

On phosphopeptone. Separation of P is more rapid than with caseinogen; half of the organic P is liberated in 20 hours, the remainder is then hydrolysed much more slowly; process almost complete in 6 days.

On paranuclein. Slow hydrolysis.

[1] This is, of course, quite apart from the combination of inorganic calcium phosphate with caseinogen ("acidic caseinogen") described by Lindet [1912, 1916] and by Mellanby [1915].

Action of bone or kidney phosphatase.

On caseinogen. No hydrolysis with bone phosphatase. Very slight hydrolysis with kidney phosphatase.

On phosphopeptone. With each of the two preparations of phosphopeptone used, only 67 % of the organic P was hydrolysed to inorganic P by bone phosphatase (rat). Kidney phosphatase (rabbit) produced 100 % hydrolysis.

On paranuclein. No hydrolysis.

Action of 1 % *sodium hydroxide at* 37°.

On caseinogen. Liberation of 100 % of the organic P as inorganic P in 24 hours. Considerable evolution of ammonia also occurs. Acetic acid precipitates "dephosphorised caseinogen."

On phosphopeptone. 100 % of the organic P liberated as inorganic P in 24 hours.

On paranuclein. 100 % of the organic P liberated as inorganic P in 24 hours.

Action of 1 % *sulphuric acid at* 37°.

No inorganic P liberated from either caseinogen or phosphopeptone after 48 hours.

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