CXXXIV. THE COLLOIDAL PHOSPHATE OF MILK.

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THE presence of insoluble, or rather colloidal, calcium phosphate in milk has been recognised for a long time; but despite numerous researches both into the type of phosphate present and the nature of its association with other colloids, it cannot yet be said that either of these matters has been definitely cleared up. To judge from the literature, opinion seems to be still divided, firstly, as to whether the phosphate is secondary or tertiary, or a mixture of the two, and secondly, as to whether it is in chemical combination with calcium caseinogenate or merely protected by it. It is hoped in the present paper to present some additional evidence on these points, which appears to favour the view that the phosphate is mainly tertiary and is present in intimate union with the caseinogenate.

Hammarsten [1879] supposed that calcium phosphate is held in solution in milk by calcium caseinogenate, and Lehmann [1894] regarded the two as united to form a double salt in which the phosphate is the tricalcium salt. Lindet's views [1912] appear to be similar. Söldner [1888] concludes that both di- and tri-calcium phosphate are present in milk, the former in somewhat the greater amount, and apparently rejects the hypothesis of a chemical union between these substances and caseinogen. Hoffman and Gortner [1923] and Porcher [1930] also consider the phosphate to be a mixture of the same two salts and their relation to the protein to be solely one of colloidal protection. Van Slyke and Bosworth [1914, 2], while accepting and apparently verifying, by their experiments on the centrifugal separation of milk, the merely protective rôle of the caseinogenate to calcium phosphate, consider the latter to be the di-calcium salt exclusively—an opinion apparently shared by Palmer [1924], Grimmer [1926], Wright [1928] and many others. On the other hand, György's experiments [1923] on the pancreatic digestion of milk and of artificially prepared caseinogen-calcium phosphate complexes led him back to the earlier view that some type of definitely chemical combination exists between calcium phosphate and the protein. Recent work by Kometiani [1931] on heated milk, which will be referred to more fully later, also supports this view.

In the course of some work in another connection [1932], the present writer noticed (*a*) that the formaldehyde titration of milk protein was much lower in the presence of colloidal calcium phosphate than in its absence, and (*b*) that the titratable acidity of milk was greatly diminished on addition of a sufficient quantity of a soluble oxalate. The first observation seemed to bear the interpretation, then stated, that the colloidal phosphate and calcium caseinogenate were chemically combined; and the second, which was not indeed new, but was susceptible of a different explanation [*cf.* Pyne and Ryan, 1932] from that given

by Van Slyke and Bosworth [1914, 1], suggested that the phosphate was largely tertiary.

The present paper contains a development of these observations and some others, which appear to confirm these views.

A. Relation of calcium phosphate to calcium caseinogenate.

In the previous paper [1932] it was shown that addition of a soluble oxalate (4 % of the saturated potassium salt) to milk increased its formaldehyde titration by about 11 % on the average. For some artificially prepared caseinogen-calcium phosphate complexes in which about one-half of the total calcium was combined with phosphoric acid, the percentage increase in formaldehyde titration was somewhat greater on oxalation, *viz.* about 16 %. (The oxalate is regarded as dissolving the union between the caseinogenate and the phosphate.)

Somewhat similar results have since been obtained with other complexes of the same type, *e.g.* a caseinogen-calcium carbonate, and also with a caseinogen-calcium phosphate complex to which gelatin had been subsequently added. On the other hand, where colloidal calcium phosphate was protected by gelatin, the addition of oxalate did not appreciably affect the formaldehyde titration, and this held even when calcium caseinogenate, subsequently added, was present in the mixture at the time of oxalation and titration. A few typical results are given in Table I.

The caseinogen-carbonate complex was prepared, following Porcher [1930], by bubbling carbon dioxide at ordinary temperature through an alkaline solution of calcium caseinogenate (3.7 % CaO) until decidedly turbid and then removing the excess carbon dioxide by a current of air; the gelatin-calcium phosphate colloid was obtained according to the directions of De Toni [1921].

Table I.

Solution	% total protein	Formaldehyde value ml. N alkali per 100 ml. solution	
		With oxalate	Without oxalate
Caseinogen-calcium phosphate complex	2.3	1.30	1.15
„ carbonate complex	2.0	1.14	0.98
Gelatin-calcium phosphate, Complex A	4.5	1.39	1.37
„ „ B	—	1.32	1.31
„ „ C	4.75	2.05	2.07
+ calcium caseinogenate (2.5 %)			

It is clear that protection of colloidal calcium phosphate (and carbonate) by calcium caseinogenate is quite different in type from protection by other proteins, *e.g.* gelatin. The marked difference is most simply explained by assuming that in the first case, where the properties of the protein are considerably modified by this “protection,” the union is more definitely chemical. The potentiometric titration of the complex described later in this paper also favours this view.

In this connection the experiments of Kometiani [1931] on heated milk are of interest. This author attributes some at least of the increased titratable acidity that results from heating to a partial separation of calcium phosphate from its caseinogenate complex, thereby increasing the number of free carboxyl groups in the protein and thus its buffer value and titratable acidity. In apparent confirmation of this view he finds that the formaldehyde titration of milk increases on heating, the increase reaching its highest value of about 15 % on heating milk for half-an-hour at 120°. It is remarkable that a difference of

much the same order is shown (*cf.* Table I) for the formaldehyde titration of calcium caseinogenate according to whether it is associated with calcium phosphate or not.

These findings favour then the views of György, *i.e.* that the relation of calcium phosphate to the caseinogenate is one of chemical union and not merely of colloidal protection. As to the type of union between them, its rapid dissolution under the action of oxalate suggests that the common bond is one or more calcium atoms, and that the complex is thus a double salt of caseinogenate and phosphate. The marked shift in the reaction of the complex towards alkalinity on addition of potassium oxalate, a matter which will be discussed more fully later, makes it probable that the phosphate liberated is largely K_3PO_4 arising from the tertiary calcium salt. The complex should on these views be essentially a double salt of calcium caseinogenate and tricalcium phosphate, and its primary interaction with oxalate might be represented schematically by:

where Ca' represents for convenience a half-atom of calcium. It is of course not intended to suggest that the phosphate complex should be a definite individual salt, but only a certain type of compound in which however the proportion of phosphate can be varied greatly according to the method of preparation. The evidence in favour of the presence of the elements of tricalcium phosphate in this complex will now be considered more fully.

B. Nature of the colloidal phosphate.

Ignoring the small amount of colloidal magnesium phosphate which is present in milk in somewhat the same form, no doubt, as the corresponding calcium phosphate, and regarding the caseinogen-calcium phosphate of milk as essentially a double salt of caseinogenate and phosphate, the exact nature of the latter, whether secondary, tertiary or a mixture of these remains to be determined. In a sense, it may appear immaterial which, for owing to the variation in the calcium content of the caseinogenate with p_H , a compound of, say, dicalcium phosphate and calcium caseinogenate, might be regarded from one point of view as identical with one of tricalcium phosphate and a more acid caseinogenate. For definiteness, however, the colloidal calcium phosphate ingredient of the complex must be considered to be that phosphate which can unite with calcium caseinogenate to form the complex without any considerable change in p_H . This is, in fact, the assumption made by Porcher in his experiments on the synthesis of the complex (see later).

Action of soluble oxalate on milk. The writer and Ryan [1932] have shown elsewhere that addition of a soluble oxalate to milk in sufficient amount (*e.g.* 4 % of a saturated solution of potassium oxalate) reduces the titratable acidity to phenolphthalein by about 1.20 ml. $N/100$ ml. milk. For whey, on similar treatment, the fall in acidity is about 0.30 ml. $N/100$ ml., so that the net alkalinity developed by the interaction of oxalate and the caseinogen-calcium phosphate complex of the milk amounts to about 0.90 ml. $N/100$ ml. The apparent source of most of this alkalinity is K_3PO_4 produced from tricalcium phosphate by the action of oxalate. For solutions of calcium caseinogenate alone develop only very slight alkalinity on addition of oxalate, and any colloidal dicalcium phosphate ($CaHPO_4$) that may be present in the complex will give rise to

K_2HPO_4 which is practically neutral to phenolphthalein¹. Assuming then that 0.90 ml. N represents approximately the amount of acid to convert the tricalcium phosphate of 100 ml. milk *via* K_3PO_4 into K_2HPO_4 , the tricalcium phosphate content of milk can be calculated to be about 0.14 g. $Ca_3P_2O_8$ /100 ml., containing 0.076 CaO. Söldner [1888] estimates the lime content of the colloidal phosphate to be 0.071 g./100 ml. milk and Porcher and Chevallier [1923] about 0.065 g. The figure obtained from oxalate titration, which represents the lime content of tricalcium phosphate only, does not differ greatly from these values, and the agreement suggests that much or all of the colloidal phosphate of milk is tertiary.

More direct evidence is obtained by comparison of the tricalcium phosphate (calculated from ordinary and oxalate titrations of milk and whey as above) and the total colloidal phosphate for the same sample obtained by analysis. The latter was taken as the difference between the acid-soluble inorganic phosphorus contents of milk and rennet whey determined by the Briggs colorimetric molybdate method and using the technique of Lange and Miethke [1933]. Table II gives some results.

Table II.

Sample	Net oxalate shift ml. $N/10$ per 100 ml. milk	Inorganic P as ml. $N/10 H_3PO_4$ per 100 ml.		
		Total	Soluble	Insoluble
A	8.8	20.0	11.7	8.3
B	8.8	19.9	10.9	9.0
C	12.2	21.7	8.7	13.0

The agreement between the values in the first and last columns is quite close. For strict comparison the former still require to be reduced by about 0.3 ml. $N/10$ —approximately the degree of alkalinity to phenolphthalein developed in 100 ml. 3 % calcium caseinogenate on addition of the amount of oxalate here used—a correction which does not materially affect the results. These show that the greater part of the colloidal phosphate, if not all, reacts to oxalate as tricalcium phosphate.

Preliminary experiments on caseinogen-calcium phosphate complexes.

These experiments were made primarily in order to see how far the effects of oxalate on milk could be reproduced on synthetic materials, and they are somewhat qualitative. More detailed examination of the phosphatic complexes is reserved for the next section. It was found that these complexes whether obtained by the action of phosphoric acid on alkaline calcium caseinogenate at about 0° or formed in similar conditions by the successive slow addition of calcium chloride and disodium phosphate solutions to one of nearly neutral caseinogenate (a process which in the absence of caseinogenate gives $CaHPO_4$), showed in all cases, in common with milk, the property of becoming much more alkaline on addition of oxalate, and thus appeared to contain some tricalcium phosphate.

The following results found for complexes prepared by the second method illustrate this point.

¹ The applicability of this oxalate method for the approximate determination of small amounts of tricalcium phosphate, and the correctness of these statements have been checked experimentally on solutions or suspensions of $CaHPO_4$ and $Ca_3(PO_4)_2$, including colloidal suspensions of the latter in gelatin. The last react with oxalate somewhat slowly.

Table III.

Complex	Total P added as ml. $M/10$ H_3PO_4 per 100 ml.	Total oxalate shift of complex ml. $M/10$ per 100 ml.	Oxalate shift of papain filtrate ml. $M/10$ per 100 ml.	Net oxalate shift ml. $M/10$ per 100 ml.
Complex A	11.1	8.2	1.4	6.8
„ B	10.0	7.4	1.5	5.7

An approximate titrimetric estimation of the soluble phosphate in the papain¹ filtrates gave 4.1 ml. $M/10$ and 4.6 ml. $M/10$ per 100 ml. respectively for A and B, so that the $CaHPO_4$ which might have been expected from the method of preparation is apparently replaced in these experiments by almost equal amounts of tricalcium and of soluble phosphates.

No doubt the reaction of the solution changed during the formation of these phosphates, and would need to have been followed to allow more quantitative interpretation of the results, but the conclusion that tricalcium rather than dicalcium phosphate is formed can hardly be avoided. The following experiment offers further confirmation of this view.

A solution of $M/5$ disodium phosphate of p_H 8.47 was added in successive small amounts to a 3 % calcium caseinogenate solution of about the same p_H , viz. 8.4, and the hydrogen ion concentration of the mixture was determined after each addition, using the glass electrode. If dicalcium phosphate is formed from the interaction of the two solutions as the inorganic colloid associates with the caseinogenate, the mixture should not differ greatly in p_H from its constituents. If however tricalcium phosphate is formed, from what may be regarded as the elements of $CaHPO_4$, the resulting mixture should become more acid, particularly in the earlier stages. The experimental results, shown diagrammatically in Fig. 1 are in complete accordance with the latter view. To demonstrate that the changes in p_H which occurred on addition of the phosphate to the caseinogenate were not due merely to partial interchange of bases in the latter, *etc.*, a similar series of potentiometric titrations was made on part of the same calcium caseinogenate solution treated with neutral $M/5$ potassium oxalate. These results, shown also in Fig. 1, are entirely different from those with phosphate.

Fig. 1.

Distribution of inorganic phosphorus in caseinogen-calcium phosphate complex.

Porcher and Chevallier [1923] concluded that the colloidal calcium phosphate was the tertiary salt, but Porcher later [1930] formed the opinion, from experiments on the artificial preparation of caseinogen-calcium phosphate complexes,

¹ These solutions contained too little soluble calcium to allow the use of rennet in preparing the serum and papain was employed instead, as by Kometiani [1931].

that the inorganic colloid was neither entirely secondary nor tertiary but a mixture of the two. The complexes were prepared by the addition of sufficient phosphoric acid to decidedly alkaline calcium caseinogenate to bring the whole to about p_H 7.0. On the assumption that the calcium belonging properly to the protein is approximately equal in the complex to that found in pure calcium caseinogenate of the same p_H , he found that 1 g. mol. H_3PO_4 was required in order to neutralise approximately 1.25 g. mol. of excess CaO , a result which corresponded to the formation of di- and tri-calcium phosphates in almost equal proportions. Porcher recognised that a small amount of monocalcium phosphate was formed during the neutralisation, and that dicalcium phosphate is itself slightly soluble, but does not seem to have considered quantitatively the bearing of these facts on the type of colloidal phosphate formed at the same time. It is clear that they may influence the interpretation of the titration results considerably. For, if soluble phosphate is present at a p_H of about 7, it will consist of a mixture of nearly equal amounts of monocalcium and dicalcium phosphates, for which CaO/H_3PO_4 in g. mols. = 0.75. It is only necessary for one-fourth of the total phosphate to be present in this form in order to account for an experimentally found ratio CaO/H_3PO_4 in g. mols. = 1.25, on the basis of the other three-quarters being entirely tertiary.

It was decided therefore to repeat the preparation of these complexes, determining at the same time the proportion of the added phosphate which remained in solution. For this purpose a calcium caseinogenate solution containing about 5 % of the protein and having p_H about 7.0 was prepared by trituration of "Hammarsten casein" with lime water, allowed to stand overnight in an ice-chest and then filtered through hardened paper. To 50 ml. of the filtrate were added 30–40 ml. saturated lime water, and then $M/20$ phosphoric acid was slowly run with constant rapid mechanical stirring into the ice-cooled mixture until it had attained approximately its original p_H of 7.0. After some hours the hydrogen ion concentrations of the phosphate complex and of the original filtered calcium caseinogenate (similarly diluted with water) were determined with the glass electrode. One or two further determinations were also made on the latter solution after the addition of small quantities of lime water, so as to be in a position to allow accurately for the calcium directly associated with caseinogen in the complex, in case the p_H values of complex and original caseinogenate should differ slightly.

The total inorganic calcium and phosphorus contents of the complex were, of course, known from the amounts of lime water and phosphoric acid used in its preparation, but the determination of the soluble phosphate required the removal of the caseinogen-calcium phosphate complex from solution without disturbing the equilibrium of the soluble constituents. Rennet was unsuitable for this purpose as the soluble calcium content was too low and could not be increased, *e.g.* by addition of a soluble calcium salt, without danger of upsetting the equilibrium of the system. The enzyme papain, used successfully by Kometiani [1931] for preparing the serum of heated milk, proved unsatisfactory in the present instance, since the filtrate obtained could not be rendered sufficiently free from nitrogenous matter by trichloroacetic acid and gave cloudy solutions with the Briggs molybdate reagent. Ultrafiltration was finally adopted for the preparation of the serum and the phosphate determined colorimetrically as already mentioned. The calcium associated with phosphate in solution was calculated from the latter for the particular p_H of the complex, using Sørensen's value for the phosphate buffers [*cf. e.g.* Clark, 1928]. The results are shown in Table IV. The figures in the last column entitled "corrected oxalate shift"

Table IV.

% protein	p_H	Inorganic P as ml. $M/10 \text{ H}_3\text{PO}_4$ per 100 ml.			Inorganic Ca as ml. $M/10 \text{ Ca(OH)}_2$ per 100 ml.			Ratio ml. $M/10 \text{ Ca(OH)}_2$ ml. $M/10 \text{ H}_3\text{PO}_4$		Cor- rected oxalate shift ml. $N/10$ per 100 ml.
		Total	Soluble	In- soluble	Total	Soluble (calc.)	In- soluble	Total	In- soluble	
2.37	6.97	7.40	—	—	9.15	—	—	1.24	—	5.0
2.05	6.91	5.90	1.48	4.42	7.29	1.15	6.14	1.24	1.39	4.0
2.11	6.87	6.60	1.28	5.32	8.51	0.99	7.52	1.28	1.41	4.5

represent the alkalinity developed in the complex on addition of the usual amount of oxalate, less an allowance of 0.5 ml. N to cover the sum of the alkalinities developed in 2 % calcium caseinogenate and the ultrafiltrate from the complex on similar treatment.

The ratio of total inorganic calcium to phosphorus is seen to agree closely with that found by Porcher, but, as expected, allowance for that fraction of these elements present in solution raises the ratio of the remainder which is available for the formation of colloidal phosphate to about 1.40. As the corresponding ratios for dicalcium and tricalcium phosphates are 1.0 and 1.5 respectively, it is clear that this result requires the bulk of the colloidal phosphate to be tertiary but does not exclude however the possibility that some dicalcium phosphate is also present. The values for the shift in reaction of the complex towards alkalinity on addition of oxalate, shown in the last column, are seen to be slightly lower than those for insoluble or colloidal phosphate and thus also support this possibility.

In spite of the suggestion from these results that the colloidal phosphates may be partly secondary, there are other considerations which favour the simpler view that the colloid is entirely tertiary and that the slight divergencies in these experimental results from what might have been expected in such circumstances are due to interfering causes. For instance, it has been assumed that the caseinogen-calcium phosphate complex and the corresponding caseinogenate free from colloidal phosphate will possess the same p_H values in solution. While some such assumption is necessary to prevent the problem becoming indeterminate, it is probably not correct to expect that the two corresponding substances will really have identical p_H values, although they will probably not differ greatly in this respect. Differences in the degree of hydrolysis, *etc.*, which occur, for instance, between the alkali and alkaline earth caseinogenates, are likely to operate here also. Thus, results which make a caseinogen-calcium phosphate complex appear to consist, somewhat improbably, of calcium caseinogenate associated with a mixture of phosphates of which some 80 % is tertiary and 20 % secondary may more properly be susceptible of the simpler interpretation that the complex consists of caseinogenate and tricalcium phosphate only, the union of which is accompanied by a slight change in reaction. Evidence on the point was sought by means of potentiometric titration.

*Potentiometric titration of calcium caseinogenate and
caseinogen-calcium phosphate complex.*

On the assumption that the amount of calcium associated directly with caseinogen in the complex is identical with that present in calcium caseinogenate of the same p_H , the titration curves of the two substances (freed of course from soluble phosphate) given with calcium hydroxide will be exactly similar. If

however this assumption is not strictly correct, as is suggested, the titration curves should differ somewhat. Table V gives the results of some experiments, one of which is also shown graphically in Fig. 2. Solutions of calcium caseinogenate and of various phosphatic complexes prepared from them were made and matured, as previously described, and then dialysed side by side in cellophane sausage skin dialysers for (mostly) 18 hours to remove soluble phosphate from the complex. A little mustard oil was used as a preservative. The solutions which contained some 2–3 % protein were then titrated potentiometrically with lime water, using the glass electrode and allowing an interval of 10 minutes for attainment of equilibrium between each addition of alkali and the succeeding measurement.

Table V.

Exp. 1				Exp. 2			
Dialysis (18 hrs.)				Dialysis (18 hrs.)			
Complex		Caseinogenate		Complex		Caseinogenate	
ml. N/10 Ca(OH) ₂ per g.	p _H	ml. N/10 Ca(OH) ₂ per g.	p _H	ml. N/10 Ca(OH) ₂ per g.	p _H	ml. N/10 Ca(OH) ₂ per g.	p _H
0.0	6.94	0.0	6.85	0.0	7.10	0.0	6.82
1.10	7.82	1.12	7.68	0.78	7.71	0.80	7.63
2.20	8.73	2.24	8.73	1.56	8.54	1.60	8.66
3.30	9.34	3.36	9.26	2.34	9.17	2.41	9.11
4.40	9.71	4.47	9.57	3.12	9.45	3.21	9.36

Exp. 3			
Dialysis (18 hrs.)			
Complex		Caseinogenate	
ml. N/10 Ca(OH) ₂ per g.	p _H	ml. N/10 Ca(OH) ₂ per g.	p _H
0.0	7.10	0.0	6.79
0.69	7.55	0.68	7.41
1.38	8.13	1.36	8.27
2.07	8.76	2.04	8.88
2.76	9.30	2.72	9.16
4.14	9.71	4.08	9.58

Dialysis (66 hrs.)			
Complex		Caseinogenate	
ml. N/10 Ca(OH) ₂ per g.	p _H	ml. N/10 Ca(OH) ₂ per g.	p _H
0.0	7.19	0.0	6.79
0.73	7.82	0.72	7.28
1.46	8.67	1.44	8.11
2.18	9.24	2.17	8.69
2.91	9.48	2.89	9.09
4.36	9.90	4.33	9.57

All the results show that the two titration curves are not identical in shape, the buffer value of the complex being somewhat greater than that of the caseinogenate in the earlier stages of the titration, *e.g.* from about p_H 7.0 to p_H 8.5, and lower thereafter. The type of difference found in the earlier stages

Fig. 2.

was somewhat unexpected and was perhaps partly due to the presence of small amounts of soluble phosphate which had escaped diffusion, for it was less evident on longer dialysis (*cf.* curves for 18 and 66 hours' dialysis, Fig. 2). Conclusions

drawn from the results of prolonged dialysis must be accepted with some reserve however since it is difficult completely to eliminate bacterial changes which may alter the shape of the titration curves.

It is clear from the present results however that the titration curves of the caseinogenate and its calcium phosphate complex are not identical, and that is the main point, for it indicates that the quantity of calcium associated with the protein in the complex may differ somewhat from that attached to the isolated protein at the same p_H . This finding favours then the more natural view that the complex consists of calcium caseinogenate and tricalcium phosphate, uniting probably with some slight change in reaction, rather than that which, on a strict interpretation of the synthetic experiments, would make it appear to consist of calcium caseinogenate united with a perhaps variable mixture of phosphate (chiefly tertiary) and possessing a p_H identical with that of the parent caseinogenate.

It is hoped to return to a more quantitative consideration of these and similar titration curves on a future occasion. It may be remarked here however that the differences between them provide additional evidence of the existence of a chemical union between calcium caseinogenate and calcium phosphate.

SUMMARY.

1. Further experiments on the effect of colloidal calcium phosphate (and carbonate) on the formaldehyde titration of calcium caseinogenate are described, and are interpreted as showing the existence of some type of chemical union between the calcium caseinogenate and the colloidal calcium phosphate of milk.

2. The effect of oxalate on the titratable acidity of milk and caseinogen-calcium phosphate complexes is examined. It appears to indicate that the inorganic colloid constituent is mainly if not entirely tertiary phosphate, a conclusion supported also by the results of experiments on the preparation and electrometric titration of the complexes.

3. The results, taken together, suggest that the caseinogen-calcium phosphate of milk has the nature of a double salt of calcium caseinogenate and tricalcium phosphate.

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