LXV. SOME PHOSPHORUS COMPOUNDS OF MILK.

I. THE PRESENCE IN MILK OF ORGANIC ACID- SOLUBLE PHOSPHORUS COMPOUNDS.

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MILK, as the sole foodstuff of the majority of young, growing mammals, contains all the substances necessary for this growth—proteins, fats, carbohydrates, salts and certain as yet chemically unknown factors. The necessity for further knowledge concerning these unknown substances is evident and urgent, and the present communication deals with the earlier stages of an investigation of the organic acid-soluble phosphorus compounds which are to be found in protein-free milk filtrates.

My attention was first drawn to these substances during the course of an enquiry into the nature of the acid-soluble phosphorus compounds of blood [Kay and Robison, 1924, 1, 2], when it was found that blood corpuscles contained at least two such esters, both hydrolysed by acids. One was hydrolysed by extracts of bone and of other tissues, whilst the other was more refractory towards enzyme activity. An average of 30 % of the organic acid-soluble phosphorus of blood was present in the more readily hydrolysed form. The view that these esters play an important part in bone formation has been supported by much evidence brought forward by Robison and his co-workers, and their possible rôle in carbohydrate metabolism [Kay and Robison, 1924, 2] and in the acute acidosis which follows ammonium chloride ingestion [Kay, 1924] has also received attention. The likelihood that young animals might receive their organic phosphorus direct from the milk, though, as experiments shortly to be described will indicate, a somewhat remote one, led me at the time to search for similar substances in milk. It was then found that phosphorus compounds, very similar to those occurring in blood corpuscles, were present in a free state in fresh milk in important quantities.

Although during the last 30 years a certain amount of intermittent attention has been paid to the organic phosphorus compounds of milk other than caseinogen, little is known as to the actual chemical state of the phosphorus, and still less has light been thrown on to its possible biochemical significance. It has been accepted for some time, in spite of sporadic denials with regard to

the last two forms, that phosphorus occurs in organic combination in milk in at least three forms—as caseinogen, as lecithin, and also in protein-free, lecithin-free milk filtrates. The work of Osborne and Wakeman [1915, 1916] on the phosphatides of milk has established their presence and identity firmly, one having been shown to be lecithin, another being found to be a diaminomonophosphatide. But we still do not know how the phosphate group is united to the rest of the molecule in caseinogen, nor whether there is any biological necessity for the presence of this group in the molecule of the protein. Our knowledge of the acid-soluble organic phosphorus compounds is, up to the present, hardly more than nominal. Apart from having given names which are somewhat misleading to these compounds the earlier workers can only be said to have rather shakily established their presence.

It is not surprising that discussion should have arisen as to the bare fact of the existence in milk of compounds belonging to the last group when one reviews the earlier methods used for getting rid of the other milk constituents. Siegfried [1896], for example, succeeded in separating a phosphorus-containing, non-protein substance from milk by precipitating the caseinogen with acetic acid and removing the albumin and globulin by boiling the filtrate. He then precipitated the free phosphates with calcium chloride and ammonia, and boiled the filtrate with excess of ferric chloride and ammonia. The precipitate formed contained iron, phosphorus and nitrogen, and was of roughly constant composition. It was variously called “nucleon,” “carniferrin” or “phosphor-fleischsaure,” the last two because it was considered to be allied in composition to a substance obtained from muscle, using a similar technique. Wittmaack [1897] went even further. He precipitated the caseinogen from human milk by warming the diluted milk to 50° with dilute hydrochloric acid, then boiled the filtrate to coagulate the albumin and globulin, and examined the protein-free filtrate for “nucleon.” Sikes' [1906] criticism is not without justification, that this treatment hardly errs on the side of caution, and that any “phosphocarnic acid” found in the later stages might well be produced during this drastic process of protein precipitation. He instances Bayliss and Plimmer's [1905] finding that part of the caseinogen phosphorus may readily be split off in organic, non-protein form. Sikes asserted that no “phosphocarnic acid” was present in the milk he was investigating—human milk—when the protein was precipitated with tannic acid mixture in the cold, though he gave no experimental details to support his statement.

There is, however, little doubt that organic phosphorus compounds are present in the filtrates from cow's, goat's and human milk from which caseinogen and other proteins have been carefully removed, as the experiments outlined in this communication abundantly show. Methods of protein precipitation have been employed which are not open to the above criticism, and further, when more than one method has been applied to the same milk sample, the quantitative results have agreed well.

(It is of interest to note that “lecithin” in milk has passed through the

same stages of assertion [Nerking and Haensel, 1908], denial [Njegoram, 1913] and re-establishment [Osborne and Wakeman, 1915].)

Cow's, goat's and human milk have been investigated, and a readily hydrolysable organic phosphoric ester has been found present in all samples examined. For the normal milk of each species there is a fairly constant amount of this ester present, but the quantity differs from one species to another. It varies somewhat during the period of lactation, being greatest in goat's milk in the colostrum, and slowly diminishing in the later stages. (This aspect is still under investigation, and will not be dealt with here.) That these amounts are by no means negligible may be the more readily appreciated when it is pointed out that, in the case of human and of goat's milk, the readily hydrolysable phosphoric ester accounts on the average for between 10 % and 20 % of the total milk phosphorus, including that of caseinogen. In cow's milk the proportion of hydrolysable phosphorus is about 5 %.

EXPERIMENTAL.

In the earlier experiments fresh milk was obtained and kept as cool as possible until the proteins had been precipitated. The fact that the similar hydrolysable organic phosphorus of blood diminishes on standing, particularly if the blood be laked, owing to the activity of a phosphoric esterase in the corpuscles, suggested that a similar enzyme might also be present in milk, but it was found later that there was no appreciable diminution in the amount of readily hydrolysable phosphorus in milk after it had been allowed to stand for some hours at room temperature, or even in presence of antiseptic at 38°.

Methods of removal of proteins.

The first method used was to precipitate the caseinogen by bringing the diluted milk to the isoelectric point of this protein with dilute acetic acid, and then to throw down the albumin and globulin from the filtrate by adding sufficient trichloroacetic acid solution to make a final concentration of this acid of 2.5 %. The neutralised filtrate was used for the phosphorus determinations.

The second method, and the one usually employed in the later experiments, was to carry out the whole of the precipitation in one operation by the use of "dialysed iron." A perfectly clear, iron-free filtrate, giving no biuret reaction, and containing very nearly as much hydrolysable phosphoric ester as the filtrate prepared by the first method, could thus be obtained. The filtrate from the dialysed iron precipitation contained, however, much less free phosphate and was therefore more useful for determinations of increase of this ion. This diminution in free phosphate is the result of two causes: (1) caseinogen takes down with it the minimum of free phosphate if it is precipitated in the neighbourhood of its isoelectric point (p_H 4.7) [György, 1923], whilst the dialysed iron precipitate was well on the alkaline side of this, and (2) there was some formation of insoluble ferric phosphate.

Using the same sample of milk in each case and the phosphoric esterase

of bone as the hydrolytic agent the following results were obtained for the quantities of hydrolysable phosphoric ester present.

Table I. *Hydrolysable phosphorus in mg. per 100 cc. milk.*

	Proteins precipitated by acetic and trichloroacetic acids	Proteins precipitated by dialysed iron
Cow's milk	4.6 4.7 5.0	4.2 4.9 4.5
Average	4.77	4.53
Goat's milk	17.1	17.1
„ colostrum	25.4	23.0

From the above figures it appears possible that in addition to most of the inorganic, a small fraction of the hydrolysable organic phosphorus may have been carried down by the dialysed iron, but this, for present purposes, is insignificant.

In order to get complete precipitation of the proteins from milk with dialysed iron, certain precautions are advisable. It is better to use quantities of milk which are not too large, and the following volumes for normal goat's milk work well. 30 cc. milk are diluted to 120 cc. with distilled water. 45 cc. of 1.5 % dialysed iron (prepared by diluting B.P. *liquor ferri dialysati*) are run slowly from a separating funnel into the diluted milk, which is held preferably in a stout bottle to allow of continuous and fairly violent shaking. After the whole 45 cc. has been introduced, the bottle is shaken well and allowed to stand 10 minutes. The filtrate is usually perfectly clear. Rather less dialysed iron is required for 30 cc. cow's milk, usually from 35–40 cc. being sufficient¹.

The filtrate is now made faintly pink to phenolphthalein with a few drops of *N/10* NaOH, and allowed to stand. If a precipitate appears it is filtered off (it does not contain organic phosphorus). The filtrate is now ready for the action of enzymes or other agents.

The method employed throughout for the determination of free phosphate was that of Briggs. A comparison between the results obtained by this method, and those obtained by the Neumann method as adopted for micro-estimations by Robison [1923] using the same milk filtrate, is given below. The hydrolysing agent used was a 5 % extract of rat bone. The figures represent mg. free phosphorus per 50 cc. of milk filtrate.

Table II.

	At 0 hours		After 18 hours at 38°		After 40 hours at 38°	
	Briggs	Neumann	Briggs	Neumann	Briggs	Neumann
A. Cow's milk filtrate + enzyme	0.34	0.33	0.67	0.64	0.75	0.70
B. Cow's milk filtrate + boiled enzyme	0.34	0.32	—	—	0.34	0.30
C. Cow's milk filtrate + water	0.24	0.23	—	—	0.25	0.25

The amount of enzyme used in this experiment was only half that used in the later determinations reported in this paper, in which the hydrolysis was completed, or very nearly so, in 18 hours.

¹ It has been found that the commercial "B.P." dialysed iron varies considerably in precipitating power and in acidity. The figures given above are therefore no more than an approximate guide.

The discrepancy, which is not large, between the values found by the two methods is doubtless due to the fact that a small amount of protein from the enzyme solution was present in A and B and interfered with the complete precipitation of the ammonium phosphomolybdate in the micro-Neumann method. Traces of this compound remained in colloidal solution and were thus not estimated. It is quite clear, however, from these figures that there is no analytical fallacy in applying the Briggs method to such milk filtrates.

Amounts of hydrolysable phosphoric ester present.

The amount of this ester present was determined by adding 5 % extract of rat bone to the neutralised milk filtrate in sufficient quantity to complete the hydrolysis in 18 hours, determining the free phosphate at zero hour and at intervals. It is convenient to use the bone enzyme, as some of its capabilities are known from previous work with blood filtrates, but, as will be seen, extract of intestinal mucosa, or of kidney will, under similar conditions, effect the same hydrolysis. In certain cases the last two extracts have been found to hydrolyse slightly more than bone extract, so that the figures for enzyme-hydrolysable ester when bone extract is used are to be taken as minimal.

Method.

10 cc. of the milk filtrate is measured into each of a series of test tubes, and 1.0 cc. 5 % bone extract and 0.25 cc. chloroform are added. Boiled enzyme replaces the active enzyme in the control tubes. The whole series, carefully stoppered, is then placed in the incubator at 38°. At intervals, two tubes are taken, and 2 cc. 15 % trichloroacetic acid are added to each, to precipitate the enzyme proteins. Of the filtrate from each tube, 10 cc. are taken for a Briggs' phosphate determination. The duplicates usually agree exactly. The results of a typical experiment are given below.

Table III. *Goat's milk, precipitated by dialysed iron.*

	mg. phosphorus per 100 cc. milk			
	0 hours	6 hours	18 hours	24 hours
Free P	7.9	24.1	24.5	24.5
Hydrolysed P	—	16.2	16.6	16.6

It will be observed that most of the hydrolysis takes place in the first 6 hours.

Table IV gives the collected results of a number of determinations using the milk of three different species.

It is clear that goat's milk contains considerably more phosphoric ester than either cow's milk or human milk, and in most of the experiments yet to be described, goat's milk has been used as the source of the ester. The relative constancy of the values shown above for cow's milk is probably due to the fact that each sample contained the mixed milk of several cows. The figure

for "non-hydrolysable" phosphorus is the difference between the total acid-soluble phosphorus in the milk filtrate (determined by the usual incineration method) and the sum of the inorganic and hydrolysable phosphorus.

Table IV. *Mg. organic acid-soluble phosphorus per 100 cc. milk.*

Cow's milk		Human milk		Goat's milk		Goat's colostrum Hydrolysed
Hydrolysed by bone enzyme	Not hydrolysed	Hydrolysed	Not hydrolysed	Hydrolysed	Not hydrolysed	
4.6	2.9	3.9*	2.0	16.6	—	25.4†
4.7	1.0	3.1*	2.0	17.1†	—	23.0†
5.0	3.6	2.5*	1.4	17.1†	—	
4.2	—	4.0	—	12.0	3.2	
4.9	—	2.4	2.0	8.6†	4.4	
2.5	2.1	3.1	4.0	8.9†	—	
4.5	1.1	[<0.5	—]	16.5	1.5	
4.4	3.5	[1.2	2.9]	12.4	1.7	
		[1.0	2.3]	10.6	—	
		4.0	1.9			
		3.1	4.0			
		3.2	2.8			
		3.3	3.9			
Av. 4.35	2.3	3.25	2.5	13.3	2.7	

* These samples late in lactation period from different mothers.

† Same sample, using different methods of precipitating proteins.

Note.—The three human milk samples in brackets are not included in the averages as they are probably abnormal. The first of the three was obtained from a mother whose child had just died from intestinal trouble, the last two were from hospital cases of difficult labour.

A comparison of these average amounts with those obtained for "nucleon" or "phosphocarnic acid" phosphorus by previous workers is of interest, although, as stated earlier, some of their methods would hardly be expected to give reliable figures. A short calculation from the published figures of Siegfried [1896], Wittmaack [1897] and Ellenberger, Seeliger and Klimmer [1902] has been made.

Table V.

mg. P per 100 cc. milk.

	Nucleon or Phosphorfleischsaure P			Hydrolysable P (Present author)	Total organic acid-soluble P
	Siegfried	Wittmaack	E. S. & K.		
Cow's milk	3.9	3.4	4.7	4.4	6.7
Goat's milk	—	8.7	—	13.3	16.0
Human milk	8.7	8.5	9.5	3.3	5.8

Only in the case of cow's milk is there approximate agreement. It would seem that there is some, but not a very direct or quantitative correspondence between the substances which have been called "nucleon" and the organic, phosphorus-containing substances present in milk filtrates, and estimated by the methods described in this paper.

The absence from milk of a phosphoric esterase.

Different samples of milk from animals of the same species, particularly goat's milk, gave markedly different figures for the hydrolysable phosphorus.

It was at first thought that this might be due to the presence of an enzyme in the milk itself.

No such enzyme appears to be present. Several experiments have been made (1) allowing the milk to stand for some hours at laboratory temperature, (2) allowing the milk to stand at 38° for 24 hours in presence of antiseptic, and in no case were more than minimal quantities of organic acid-soluble phosphorus hydrolysed. The results of two experiments are summarised in Tables VI and VII.

Table VI. *Effect of standing at room temperature.*

	mg. P per 100 cc. milk.				
	Free P	P hydrolysed by bone enzyme at 38°		Total acid-soluble P	Non-hydrolysed acid-soluble P
		After 6 hours	After 18 hours		
Milk drawn into cold bottle and precipitated at once	7.9	16.5	16.5	25.9	1.5
Same milk stood 4 hours at 20°	7.9	16.2	16.6	25.6	1.1
Same milk stood 8 hours at 20°	7.8	16.2	16.2	25.6	1.6
Stood overnight at 0°	8.8	16.2	16.4	26.8	1.6

Table VII. *Effect of standing at 38°.*

	mg. P per 100 cc. milk.		
	P hydrolysed by bone enzyme at 38°		
	After 6 hours	After 24 hours	
Fresh milk precipitated at once ...	9.9	10.6	
Same milk after standing 6 hours at 38°	9.4	10.1	
Same milk after standing 24 hours at 38°	8.4	9.5	
Same milk boiled for 1 minute ...	9.9	10.5	
Boiled milk after standing 24 hours at 38°	8.8	9.9	

If a phosphoric esterase is present at all in fresh milk it is there in no more than traces. Boiling for 1 minute appears not to affect the organic acid-soluble phosphorus of fresh milk.

The rate of hydrolysis of the acid-soluble phosphorus compounds by bone enzyme and by acid.

The rate of hydrolysis by bone enzyme was first determined in order to find out when hydrolysis could be taken as completed. Later, filtrates from the three kinds of milk examined were compared. The results, together with the curve given by a blood filtrate under similar conditions of hydrolysis [Kay and Robison, 1924, 1], are shown in Fig. 1.

In each case, using bone enzyme, well over 80 % of the hydrolysis occurs in the first 6 hours. Using double quantities of enzyme, the reaction goes to completion in this time so that determinations of the amount of hydrolysable phosphorus in a given sample of milk can be made in a single day.

Fig. 1: A. Goat's milk filtrate + bone enzyme at 38°.
 B. Goat's milk filtrate + $N/4$ sulphuric acid at 100°.
 C. Rabbit's blood filtrate + bone enzyme at 38°.
 D. Cow's milk filtrate + bone enzyme at 38°. Fresh enzyme added at 24 hours.
 E. Human milk filtrate + bone enzyme at 38°.

Strength of enzyme: 1 cc. 5 % bone extract to each 20 cc. filtrate.

Action of various reagents on the phosphorus compounds.

The substrate is more stable to heat, acids and alkalies than its ready hydrolysis by bone enzyme would suggest. Results of experiments are summarised below.

Milk filtrate + antiseptic kept at 0° at p_H 8.2
 Filtrate at p_H 8.2 boiled for 2 minutes
 Prolonged boiling at p_H 8.2
 Autoclaving 1 hour at 120° (p_H 8.2)
 2N/3 sulphuric acid at 38°
 Autoclaving 1 hour with $N/10$ sulphuric acid at 120°
 Boiling $\frac{1}{2}$ hour $N/4$ sulphuric acid
 Boiling 48 hours $N/4$ sulphuric acid
 Heating with 2N sulphuric acid for 72 hours at 100°

$N/2$ sodium hydroxide at 38°
 $N/2$ sodium hydroxide at 100°

Less than 5 % change in 3 months
 Less than 3 % hydrolysis
 Very slow hydrolysis
 11 % hydrolysis
 No hydrolysis in 18 hours
 25 % hydrolysis

22 % hydrolysis
 80 % hydrolysis
 Hydrolysis not yet complete

Slight hydrolysis after 18 hours
 Marked hydrolysis in $\frac{1}{2}$ hour and 80 % hydrolysis after 18 hours

The proportion of organic phosphorus which remains unhydrolysed after prolonged boiling with dilute sulphuric acid varies with the milk sample, and agrees in general with the findings in the bone enzyme hydrolyses, that a portion of the organic phosphorus, varying irregularly from 10–40 % of the total is not hydrolysable.

Plimmer [1913] found that glycerophosphates and similar esters containing PO_4 in the same grouping were quite stable to *N* alkali at 95° . The instability of the hydrolysable phosphoric ester, at least towards alkali, would indicate that it is not of the glycerophosphate type.

Action of digestive enzymes and tissue extracts.

In order to obtain information as to the possibility of the absorption of these milk compounds unchanged from the alimentary tract, a few experiments have been carried out on the hydrolytic power of digestive enzymes *in vitro* at 38° . At the same time it was thought worth while to investigate the distribution in various tissues of a phosphoric esterase similar to that present in bone. The results are summarised below.

Ptyalin. Very active ptyalin gave absolutely no hydrolysis, either at p_{H} 7 or at p_{H} 8.4 in 24 hours.

Pepsin. Armour's pepsin, very active towards fibrin, had no action whatever on the organic phosphorus of two milk filtrates at the optimum p_{H} for this enzyme.

Trypsin. Active trypsin (Liquor Trypsin Co., Allen and Hanbury), rapidly hydrolysing fibrin and caseinogen, was entirely without action on the organic phosphorus, either at p_{H} 7 or p_{H} 8.4.

Pancreatic juice, obtained by cannula direct from the duct of an anaesthetised dog, but not activated, had a slight but definite hydrolysing action. The same juice, activated by adding a 10 % extract of duodenal mucosa, hydrolysed as much organic phosphorus as did the bone enzyme. The effect was traced to the intestinal extract itself, which was able to hydrolyse the same, or even a slightly greater amount of the organic phosphorus than the bone enzyme.

Extract of pancreas and of spleen. A 5 % pancreatic extract from an adult dog gave in 18 hours almost as much hydrolysis (7.8 mg. P per 100 cc. milk) at p_{H} 8.4 as did bone enzyme (9.0 mg. P), whilst 5 % spleen extract was almost inactive (0.9 mg. P).

Intestinal extract. A 5 % extract of duodenal mucosa gave the same amount of hydrolysis as standard bone extract:

Table VIII.

mg. P per 100 cc. milk. Initial p_{H} 8.4. Time 18 hours.

Experiment	Hydrolysed duodenal extract	Hydrolysed bone extract
A	16.4	16.5
B	16.6	16.6
C	12.6	12.0
D	9.0	8.8

Intestinal juice. The juice was obtained by drawing the contents of the small intestine of a starving dog through cotton wool. The clear fluid so obtained was diluted to give a 5 % solution. At p_H 8.4 the diluted juice of two adult dogs gave in each case the same amount of hydrolysis as with bone enzyme. A fortnight-old puppy's diluted juice gave 28 %, a 7 days old puppy's 66 % of the amount hydrolysed by bone enzyme in the same time.

Gastric mucosa. A 5 % extract of the gastric mucosa of a dog brought about, at p_H 8.4, 75 % of the hydrolysis effected by bone enzyme. It was thus somewhat less active than intestinal mucosa.

Kidney extract. 5 % extract was slightly more active than bone enzyme at p_H 8.2, hydrolysing, in one experiment, as much as 16 % more organic phosphorus.

Mammary gland. A 10 % extract of the mammary gland of a lactating goat hydrolysed at p_H 8.4, 10.1 mg. P per 100 cc. milk in 24 hours, whilst standard bone enzyme in the same time hydrolysed 12.9 mg. P. The same mammary extract, when added to a protein-free filtrate from rabbit's blood, hydrolysed 5.9 mg. P %, whilst bone enzyme in the same time hydrolysed 9.2 mg. P %.

Blood. Diluted blood (one part blood to four of water) gave in 48 hours a small but definite amount of hydrolysis.

Muscle extracts. Fresh rabbit muscle extract, made by grinding up the tissue with sand and 10 times its weight of chloroform water and filtering, brought about, at p_H 8.4, 15 % hydrolysis of the organic phosphorus after 11 hours, and 22 % hydrolysis after 48 hours. By this time the hydrolysis had virtually ceased.

If the hydrolysable phosphorus compounds had been esters of the hexose-diphosphate type, this concentration of muscle enzyme and substrate should have resulted in hydrolysis of the order of 52 % in 11 hours, and 82 % in 48 hours [Kay and Robison, 1924, 2, Tables I and II]. Thus there is indirect evidence that these compounds are probably not to any large extent esters of the yeast hexosediphosphate type. As in blood, so in milk there would appear to be at least three forms of organic acid-soluble phosphorus present, the portion hydrolysable by bone enzyme in each case containing more than one substance. Consideration of the rate of hydrolysis of the organic phosphorus of milk filtrate by boiling with $N/4$ sulphuric acid leads to a similar view, the velocity constant falling off rapidly, and then becoming constant at about 8 hours, remaining constant until the experiment is discontinued at 48 hours.

Collecting the foregoing results into one table:

Table IX.

	Activity		Activity
Ptyalin	0	Intestinal extract	+++
Pepsin	0	Spleen extract	+
Trypsin	0	Kidney	+++
Pancreatic juice	+	Muscle	++
Intestinal juice	+++	Mammary gland	+++
Gastric mucosa	+++	Blood	+
Pancreas extract	+++	Bone extract	+++

The effect of dialysis.

Raudnitz [1903], in criticising some of the "nucleon" findings mentioned earlier in the present paper, says that so long as it cannot be found in milk dialysates, the pre-existence of nucleon in milk is doubtful. The acid-soluble phosphorus compounds of milk may readily be separated from the milk colloids by dialysis at low temperatures in collodion sacs, and there would appear to be no reason to believe that they are not present in the same form in fresh milk. The results of a dialysis experiment are given below.

Table X.

mg. P per 100 cc. fresh goat's milk. Dialysis at 0°.

	In filtrate after pptn. of orig. milk with dialysed iron	Dialysate	
		6 hours	22 hours
Phosphate P	2.5*	11.4	12.8
Total organic acid-soluble P	13.0	11.2	12.0
Hydrolysable P	8.6	5.1	6.1
Non-hydrolysable P	4.4*	6.1	5.9

* The dialysed iron taken down much of the phosphate P and probably also some of the non-hydrolysable P.

It is clear that both types of organic phosphorus may be separated from goat's milk by dialysis. The experiment has been repeated using cow's milk, and the same result obtained.

DISCUSSION.

The presence of acid-soluble, organic phosphorus compounds in milk having been established, questions arise as to the origin and function of these compounds.

They must originate either (1) by diffusion from the red blood corpuscle in which very similar compounds are present, or (2) by synthesis in the mammary gland, or (3) by breakdown of the cells of the mammary gland with discharge of their nuclear materials and other contents into the milk, or (4) by the breakdown of some more complex organic compound after secretion of the milk. The last possibility seems to be excluded by experiments mentioned early in this paper, the third is open to the objection that since, at least in goat's and in human milk, the amount of the phosphorus of the acid-soluble compounds is a relatively large fraction (15–25 %) of the total phosphorus of the milk, their derivation from this source would indicate a far more rapid breakdown of glandular tissue than appears possible.

That these phosphorus compounds may leak from the blood into the milk, as for example, urea and creatine [Denis, Talbot and Minot, 1919] appear to do, seems at first unlikely, since the phosphoric esters of blood are carried *in vivo* almost entirely in the corpuscles. It is possible, however, that when the corpuscles are slowly squeezing through the smaller capillaries, and are in intimate contact over a large portion of their surface with the capillary wall, conditions may exist for a direct interchange of substances between them and

the cells of the capillaries¹. One would at least expect fundamental alteration in the surface relationships. As against the possibility of such diffusion from the blood is the fact that the bone-enzyme-hydrolysable phosphorus compound is preponderant in milk, whereas in blood it forms only one-third, or thereabouts, of the total phosphoric esters. This is, perhaps, not a very serious objection, since all recent work in this field goes to show that the phosphorus compounds in the tissues have a far more intimate kinetic relationship with one another than was previously realised, cf. the findings of Lawaczek [1924] and of Hansman, Martland and Robison [1924] working on the dynamics of phosphoric acid compounds in blood.

The remaining possibility, that the substance may be definitely manufactured and secreted by the cells of the mammary gland derives some little support from the fact that an enzyme is present in mammary glands capable, *in vitro*, of hydrolysis of part of the acid-soluble phosphorus compounds, and possibly associated, *in vivo*, with their synthesis.

As regards the further history and possible function of these compounds, from the experiments with digestive enzymes and extracts of alimentary mucosa just described, it would seem on the whole unlikely that the organic phosphorus compounds are absorbed unchanged from the milk. Nevertheless, they are not attacked by the digestive enzymes before their arrival in the intestine, and in view of their relatively rapid diffusibility, there is at least the possibility of their being absorbed unchanged.

Table XI.

Forms in which phosphorus occurs in milk.

1. Free phosphate.
2. Phosphate loosely associated with caseinogen and not diffusible [György, 1923].
3. Caseinogen phosphorus.
4. Lecithin.
5. Diaminomonophosphatide [Osborne and Wakeman, 1915, 1916].
6. Acid-soluble phosphoric ester, hydrolysed by muscle phosphatase.
7. Acid-soluble phosphoric ester hydrolysed by bone enzyme, etc.
8. Acid-soluble organic phosphorus compound unaffected by these enzymes and stable to acids.

To the present author it would appear not unlikely that the imperative necessity for meeting the phosphorus requirements of the growing suckling has led, in the mammary gland, to the conscription of every possible synthetic mechanism by which the phosphorus content of the milk can be increased. Moreover, assuming that complete hydrolysis actually does precede absorption, and remembering that phosphorus compounds occur in milk in at least seven other forms of combination beside that of caseinogen (see Table XI), there is the supplementary possibility that this series has been evolved in relation to

¹ This conception may perhaps also be extended to the hypothesis advanced by Robison and his co-workers, that the organic esters in the red corpuscles play an important part in bone formation. A serious difficulty of this hypothesis has been the fact that at most only very small amounts of these esters are present, under normal conditions, in the plasma.

certain solubility and absorptive difficulties in the intestine. Having differing hydrolysability and therefore, during digestion, maintaining for a longer or shorter period their un-ionised PO_4 , these compounds may be absorbed, with or without previous hydrolysis, in different forms, and at different levels in the small intestine, so that both calcium and phosphorus, each essential to the growing suckling, may be taken up most advantageously. This could be achieved if the concentration of PO_4''' ions were kept continuously low, so that the solubility product of $\text{Ca}_3(\text{PO}_4)_2$ should not be exceeded, and as little transformation as possible into this almost insoluble salt—and therefore the loss of both its irreplaceable constituents in the faeces—ensue.

The chemical identity of these substances is now being investigated, and from preliminary experiments it appears that one of the organic acid-soluble phosphorus compounds present in goat's milk filtrate, and hydrolysable by bone enzyme, is also capable of reducing alkaline copper solutions.

SUMMARY.

It has been found that at least two acid-soluble compounds containing phosphorus in organic combination are present in cow's, goat's and human milk, one compound being readily hydrolysable by extracts of various tissues and by dilute acids, and one being considerably more stable.

The more readily hydrolysable compound is present to the average extent of 4.4 mg. P per 100 cc. in cow's milk, 13.3 mg. in goat's milk, and 3.3 mg. in human milk. In the last two milks, the acid-soluble organic phosphorus compounds account for from 10–25 % of the total phosphorus of the milk.

Both the hydrolysable and the stable phosphorus compounds pass readily through a collodion dialysing membrane which holds back protein but allows methylene blue to diffuse slowly.

Though not acted on by pepsin or trypsin, and only slowly attacked by pancreatic juice, the hydrolysable ester is probably broken down by the intestinal juice before absorption. Extracts of many tissues are also able to hydrolyse this phosphoric ester.

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