CLXXV. DIALYSIS OF MILK.

II. SOME FACTORS AFFECTING THE DISTRIBUTION OF CALCIUM AND PHOSPHORUS.

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IN a previous paper [Lampitt and Bushill, 1933] reference was made to the unsatisfactory state of knowledge regarding the distribution of the phosphorus of milk as indicated by dialysis, and certain results bearing on the subject obtained by a well-defined technique were recorded. Figures available in the literature for the amount of calcium dialysable from milk are equally unsatisfactory as will be seen from Table I where it is demonstrated that various workers have found as little as 13 % and as much as 50 % of the total calcium in a dialysable form. Much has been written by experimenters on the results obtained by them¹ but it is apparent that before any conclusions can be drawn from dialysis studies, consideration must be given to the factors which may be responsible for the lack of concordance indicated.

As stated in the previous paper, it was considered that certain advantages would accrue from the use of "dried milk" for dialysis experiments, since, by

Table I.

Reference	Method of preparation of serum		Serum-Ca as % of total Ca in milk
	Dialysis	Filtration	
Bell [1925]	—	Chamberland filter	23·8
Grosser [1913]	—	"	23·4
Magee and Harvey [1926]	Against cold running water, using collodion thimbles	—	26·4
Mattick [1929]	25 ml. milk (parchment capsules). 150 ml. 3 % NaCl. Time 18 hrs. Temp. not stated	—	24–30
Piettre and Achard [1931]	Against water. Temp. not stated	—	13–25
"	Against glycerol. Temp. not stated	—	29–33
Rona and Michaelis [1909]	25 ml. water (sac). 1000 ml. milk. Time 2 days. Preservative toluene	—	40–50
Rossi [1930]	20 ml. whole milk (collodion sac). 25 ml. water. Time 84 hrs. Temp. "ice-chamber"	—	40
Van Slyke and Bosworth [1915]	—	Pasteur-Chamberland filter	22–46
Van Slyke and Bosworth [1916]	—	"	27
Wardlaw [1914]	25 ml. water (collodion sac). 1000 ml. milk. Time 2 days. Preservative toluene	—	30–40
Wha [1924]	10 ml. milk (collodion or parchment sac). 5 ml. water. Time 24 hrs. Temp. not stated. Preservative toluene	—	50

¹ A summary of the available knowledge has been published by Allen [1931].

this means, repetitive experiments could be carried out enabling the technique to be standardised and simplified. Ultimately such technique could form the basis of similar dialysis studies on fresh milk.

In parenthesis it may be remarked that many of the methods used by previous workers for the determination of calcium and of phosphorus in milk and in serum have been found not to be absolutely reliable.

As a means of standardising technique, the use of dried milk still appears to be sound in principle provided that the milk powder used is a standard product and does not differ to any large extent in its chemical and physical attributes from the original milk from which it was produced. The dried milk¹ used in all this work has been of such a character; it represents mixed bulk separated milk chiefly from Red Devon and from Shorthorn herds and "morning" and "evening" milk taken indiscriminately and at various seasons of the year. Justification for the claim that such a product represents "average" milk, certainly as concerns the constituents studied herein, is afforded by the fact that the phosphorus and calcium contents, calculated on non-fatty solids, of a considerable number of different samples fall within a comparatively narrow range.

This is illustrated by the following results:

Total P	...	...	0.98-1.14 % (mostly 0.98-1.07 %)
Total Ca	...	...	1.31-1.44 %

It is difficult to compare these figures with those of other workers owing to the unknown composition of the milks used by them, but assuming a total non-fatty solids content of 9 %, the following figures are typical:

Total P.	Van Slyke and Bosworth [1915, 1916]	...	0.95-1.40
	Jordan <i>et al.</i> [1906]	...	0.85-1.20
	Lenstrup [1926]	...	1.00-1.10
Total Ca.	Van Slyke and Bosworth [1916]	...	1.36-2.43
	Mattick [1929]	...	1.29-1.49

The dried milk in question has a solubility greater than 99.8 % when tested by the method previously described [Lampitt and Hughes, 1924; Lampitt and Bushill, 1931], and moreover, as far as can be determined by ordinary analysis, its "solution" is practically indistinguishable from the milk from which it was made. However, the results obtained from the work on phosphorus indicated that possibly the heat treatment to which the product had been subjected had effected a change in the ratio of dialysable to non-dialysable phosphorus; other workers have reported a similar effect both with phosphorus and calcium. Some preliminary work on this point is reported herein, and two other factors have also been studied, namely the effect of the "acidity" and of the dilution of the milk. It is not necessary to emphasise the importance of the effect of "acidity" on milk, particularly in respect of those constituents containing calcium; this factor has been studied by dialysis of milk after inoculation with an acid-producing organism.

The difference between the results obtained with the "continuous" and with the "static" methods of dialysis, which have already been reported, may be attributed to the shifting of the phosphorus equilibrium by the infinite dilution produced by the first method. Consequently in the work now reported the "static" method only has been employed, so that the results obtained represent most nearly the state of the constituents in the liquid dialysed².

¹ The milk powder used in this work was prepared by the Milkal Process.

² In these experiments pasteurised milk has been almost exclusively employed, so that they are not affected by the presence of milk-phosphatase [Kay and Graham, 1933].

EXPERIMENTAL.

(a) *Method of dialysis.*

This was essentially the same as previously detailed [1933]. A collodion sac containing 25 ml. of distilled water is suspended in 900 ml. of the milk under investigation and the whole maintained at 0–5° for a period of 2 days. Tests have shown that by that time the dialysable constituents have come to equilibrium with the liquid in the sac in agreement with the results of Wardlaw [1914], and with the aid of an analysis of the contents of the sac the percentage of the various constituents may be calculated on the non-fatty solids of the original milk. In the results given later the amounts of these dialysable constituents are also given as percentages of the total amount of each constituent originally present in the milk itself.

(b) *Methods of analysis.*

Calcium. The method employed for the determination of calcium was a combination of the oxalate precipitation of Washburn and Shearer [1932] with a titration of the calcium oxide obtained therefrom as described by Fiske and Logan [1931]. The precipitation is carried out at p_H 3 to avoid precipitation of phosphate with the calcium oxalate.

The sample is reduced to ash in a platinum dish, the ash dissolved in $N/2$ HCl, washed into a beaker and the volume made approximately to 100 ml. Thymol blue is added and more $N/2$ HCl, if required, to give a pink solution, then 5 ml. of 6.4 % oxalic acid solution are added, and the solution is heated to boiling. N NH_4OH is then added, drop by drop, with stirring until the solution is definitely yellow (p_H 3). After heating on a water-bath for 3 hours the solution is cooled, the precipitate collected on a No. 41 Whatman filter-paper and washed with a solution prepared as follows: 5 ml. of 6.4 % oxalic acid solution are diluted to 100 ml. and then adjusted by the addition of NH_4OH to p_H 3—yellow to thymol blue. The filter-paper and washed precipitate are reduced to an ash in a platinum dish by heating with a Bunsen burner followed by an hour's treatment at 1000° in a gas muffle. The CaO so produced is dissolved in 5 ml. of $N/2$ HCl, washed into a conical flask with cold boiled water and the excess acid titrated (micro-burette) with $N/2$ NaOH, using methyl red as indicator. With a titration of 3 ml. the error is less than 2 %.

Phosphorus. The total phosphorus and the inorganic phosphorus were determined by the method given in the earlier paper [1933] (the colorimetric method of Kuttner and Lichtenstein [1930] being employed), the error of the P determination being less than 2 %.

Acidity and p_H . The titratable acidity was determined by titration with $N/10$ NaOH (phenolphthalein as indicator) and the result calculated as lactic acid %. It is of course realised that this is an absolutely arbitrary method of expression, but it is simple and convenient as indicating change during the development of acid. The p_H was determined by means of the glass electrode described by Kerridge [1926] employing the potentiometric valve circuit of Morton and Best [1933].

(c) *The dilution effect.*

Dialyses were carried out on separated milk, on the powder (brought previously to a total solids content of approximately 9 %) obtained by spray-drying the same milk, and on these milks or "solutions" diluted with twice their volume of water. The results obtained from the analysis of the dialysates are shown in Tables II and III.

Table II.

Figures represent percentages.

	Pasteurised milk			Solution of powder (1)		
	Dialysate			Dialysate		
	Original milk	Undiluted milk	Diluted milk	Original milk	Undiluted milk	Diluted milk
Calculated on non-fatty solids:						
Ca	1.34	0.41	0.58	1.36	0.44	—
Inorganic P	0.63	0.40	0.46	0.66	0.38	0.44
Organic P (by diff.)	0.35	0.10	0.10	0.33	0.08	0.06
Calculated on total present in milk:						
Ca	100	31	43	100	33	—
Inorganic P	65	40	47	67	39	44
Organic P (by diff.)	35	10	10	33	8	6

Table III.

Figures represent percentages.

	Powder (2)			Powder (3)			Powder (4)		
	Dialysate			Dialysate			Dialysate		
	Original milk	Undiluted milk	Diluted milk	Original milk	Undiluted milk	Diluted milk	Original milk	Undiluted milk	Diluted milk
Calculated on non-fatty solids:									
Ca	1.43	0.36	0.43	1.31	0.38	0.49	1.35	0.43	0.52
Inorganic P	0.71	0.35	0.41	0.69	0.35	0.41	0.68	0.35	0.42
Organic P (by diff.)	0.35	0.10	—	0.30	0.10	0.10	0.26	0.10	—
Calculated on total present in milk:									
Ca	100	25	30	100	29	37	100	32	39
Inorganic P	67	33	39	69	35	41	73	37	45
Organic P (by diff.)	33	10	—	31	10	10	27	11	—

(d) The effect of change in acidity due to bacterial action.

A solution of spray-dried milk was inoculated with *Streptococcus lactis* and incubated at 30°. At different times after the inoculation, samples of the milk were removed, the titratable acidity and the p_H determined, and a volume

Table IV. The effect of change in acidity due to bacterial action.

Sample	p_H		Acidity calculated as % of lactic acid	Dialysable Ca and P					
	Inocu- lated milk	Dialy- sate		Calculated on non- fatty solids of original milk			Calculated on total present in milk		
				Ca	Inorg. P		Ca %	Inorg. P %	Org. P (by diff.) %
					Org. P (by diff.)	P			
<i>a</i>	6.55	6.55	0.14	0.46	0.38	0.07	32	34	7
<i>b</i>	6.05	6.15	0.24	0.57	0.43	0.11	40	39	10
<i>c</i>	5.75	5.95	0.29	0.70	0.50	0.09	49	45	8

Original milk solution.

p_H	...	...	...	...	...	...	6.55
Acidity (calculated as lactic acid)	...	...	...	...	...	...	0.12 %
Total Ca (calculated on non-fatty solids)	...	...	...	...	...	...	1.44 %
Total P (calculated on non-fatty solids)	...	...	...	...	...	...	1.12 %
Inorg. P (calculated on non-fatty solids)	...	...	...	...	...	...	0.74 %
Org. P (calculated on non-fatty solids)	...	...	...	...	...	...	0.38 %

dialysed in the standard manner at 0–5°; it had previously been shown that under such conditions no appreciable change in acidity occurs during the period of dialysis. The results of a typical experiment are given in Table IV.

(e) *The effect of heat.*

This effect is deduced from the figures given in Tables II and V. The dried milk (Table II) was a commercial sample obtained by drying the pasteurised milk the dialysis figures of which are given.

DISCUSSION.

The effect of dilution.

Reference to Tables II and III shows that dilution of one part of “reconstructed” pasteurised separated milk with two of water causes an increase of 5–8 % in the dialysable inorganic P and in the dialysable Ca and of double that amount in the dialysable Ca in non-dried pasteurised milk. The difference between the effects of dilution on the dried milk on the one hand and on the pasteurised milk on the other, indicates the presence of some heat or dehydration effect which demands further investigation. The point to be emphasised, however, is that the distribution of the constituents studied is affected by the method of dialysis in so far as the ratio of milk to water in the liquid dialysed is concerned.

It is not suggested that dilution of milk with water has necessarily the same effect as the dilution of the dialysable constituents with water which occurs during dialysis, but the results obtained may be regarded as indicative of the dilution effect which takes place particularly when a small volume of milk is dialysed against a large volume of water. At equilibrium, when the conditions of “static” dialysis as described herein are satisfied, the dialysable constituents both in the sac and in the volume of milk are diluted in the ratio 1000 to 1028, whereas, in such experiments as those recorded by Mattick [1929], the dilution ratio would be 1000 to 7000. It can be concluded, therefore, that considerable differences in the dialysable constituents would be obtained by various workers depending upon the ratio of milk to water used in the experiments. Where a small volume of milk is dialysed against a large volume of water, the results recorded will refer to highly diluted dialysable constituents with possible reaction on the potentially dialysable bodies. Where a large volume of milk is dialysed against a small volume of water, the resultant figures will refer to a practically undiluted milk. The extremes are obtainable by “continuous” dialysis and by “static” dialysis. Reference to the previous paper on the phosphorus distribution shows that whereas 40 % of the total P of a freshly separated non-pasteurised milk was dialysable as inorganic P under “static” conditions, 54 % was dialysable when the “continuous” method was employed. Under “static” conditions limited dilution of pasteurised milk (1 part milk: 2 parts water) causes, according to Table II, an increase in dialysable inorganic P from 40 to 47 %.

Although the present object is merely to indicate the factors which may in part be responsible for the great variation in figures recorded by the numerous workers who have been interested in the problem, it may be remarked that such a dilution effect would be anticipated, but has apparently not been previously considered as a factor of importance. In particular the studies of Holt *et al.* [1925] and of Hastings *et al.* [1926] suggest that dilution would have considerable effect on a system which contains various phosphates in a state of supersaturation.

The dialysable organic P shows a marked difference from the inorganic P in that it is not affected by dilution (see Tables II and III). This is in agreement with results reported in the earlier paper where it was shown that at infinite dilution ("continuous" dialysis method) the dialysable organic P agreed with that obtained by minimum dilution ("static" dialysis method).

This effect is what would be expected from phosphorus organically combined and serves as a confirmation that the difference between the total and the inorganic P figures, determined as indicated, is indeed organically bound P.

Effect of change in acidity.

The results, Table IV, show an increase of 11 % in inorganic P and of 17 % in Ca in the dialysable fraction during a change in p_H in the milk from 6.55 to 5.75. Even the comparatively small change in p_H from 6.55 to 6.05 causes increases of 5 % and of 8 % in the dialysable inorganic P and in the Ca respectively.

This would be expected from a consideration of the action of lactic acid on the inorganic constituents of milk. Van Slyke and Bosworth [1915] deduced from a study of milk inoculated with *B. lactis acidii* and *B. lactis aerogenes* under somewhat similar conditions, that the whole of the CaHPO_4 (which they consider to be a normal inorganic constituent of milk) passes into solution in $13\frac{1}{2}$ hours. The normal bacterial flora of milk does not, however, consist only of lactic acid-producing organisms, but it is obvious that organisms of this type are of first importance, and it follows that acidity during dialysis must be controlled if reliable figures for the dialysable constituents are to be obtained. The time and temperature of dialysis described above have been shown to satisfy the necessary conditions, and to ensure that acidity changes due to the activity of bacteria, *S. lactis* in particular, are reduced to a minimum.

It may be of interest to note that, according to Table IV, there appears to be an increase in the difference between the p_H of the milk and of the dialysate of that milk as the acidity increases.

The titratable acidity and p_H are dependent to some extent on the dilution as is shown in Table VI which gives the results of such determinations on milk powder "solutions" already examined in Tables II and III.

Table V.

	Fresh milk		Sterilised milk	
	Original milk	Dialysate	Original milk	Dialysate
Calculated on non-fatty solids:				
Ca	1.44	0.52	1.44	0.46
Inorganic P	0.77	0.43	0.78	0.39
Organic P (by diff.)	0.24	0.11	0.23	0.10
Calculated on total present in milk:				
Ca (%)	100	36	100	32
Inorganic P (%)	77	43	77	39
Organic P (by diff.) (%)	23	10	23	10

It will be observed (Table VI) that in all three cases dilution has caused an increase in p_H and a corresponding decrease in titratable acidity when due allowance is made for the dilution. It has previously been shown, Tables II, III and IV, that both dilution and development of acidity cause an increase in dialysable Ca and in inorganic P. It appears, therefore, by comparison with Table VI that dilution of milk, which is accompanied by an increase in p_H , causes an increase in the dialysable Ca and in inorganic P notwithstanding the

Table VI. *Change in p_H and acidity on dilution.*

	Powder (2)		Powder (3)		Powder (1)	
	Undiluted milk	Diluted milk	Undiluted milk	Diluted milk	Undiluted milk	Diluted milk
p_H	6.45	6.70	6.40	6.65	6.50	6.75
Titrateable acidity calculated as lactic acid (%)	0.13	0.035	0.15	—	0.15	0.037
Titrateable acidity calculated as lactic acid on undiluted milk (%)	0.13	0.11	0.15	—	0.15	0.11

fact that such an effect is also produced by a decrease in p_H (development of acidity).

It can therefore be inferred that were the p_H maintained constant during the dilution, the effect on the dialysable Ca and P would be even greater than that observed.

It is evident that the acidity effect is an important one and should not only be controlled but also defined when considering the dialysable constituents of milk. For such a definition p_H appears to be the more suitable means since titrateable acidity is affected by factors which may differ in different milks, *i.e.* their buffer capacity.

Effect of heat.

From the time when the original statement was made that heating of milk caused a precipitation of calcium citrate, various workers have studied the effect of heat on milk. Many of the studies have been undertaken from the practical standpoint, for any such changes are of obvious importance in the "processing" of condensed milk. Whilst the original statement, as far as can be found, has not been confirmed in respect of the actual composition of the body precipitated, the reported results, on the whole, indicate that heat causes a decrease in the soluble calcium salt content of the milk.

Table V offers some support for such a conclusion. It is shown that the dialysable constituents of milk suffer a decrease of 4 % in inorganic P and a similar decrease in Ca when the milk is heated to 100°. (In this particular case the milk was placed in a "steamer" for 1 hour.)

It is doubtful whether the differences recorded in Table II between the pasteurised milk and the powder made from it are significant, but it is difficult to ignore the evidence (the effect of dilution of the pasteurised milk and of the "solution" of the dried milk) that some change had occurred. It appears that whilst heat-treatment reduces the amount of dialysable Ca and P of milk, spray-drying affects the physical state of the normally non-dialysable Ca by rendering it less susceptible to dissolution by dilution.

SUMMARY.

1. Evidence has first been given that the milks and dried milks employed in this investigation do represent "average" milk.

2. A combination of the methods described by other authors for the determination of calcium has been chosen as suitable for this work. Details of the method are given. Phosphorus has been determined by a method described in a previous paper.

3. Dilution of milk causes a decrease in acidity, and, as has been shown by "static" dialysis, results in an increase in the proportion of dialysable calcium and of inorganic phosphorus.

4. Increase in acidity by bacterial action materially increases the proportion of dialysable calcium and of inorganic phosphorus.

5. It follows from conclusions 3 and 4 that a decrease in p_H (by bacterial action) and an increase in p_H (by dilution) both result in an increase in dialysable calcium and inorganic phosphorus.

6. The necessity of suitable acidity control and of appreciation of the effect of dilution has been emphasised since they are factors rendering comparison of the results of many workers impossible.

7. Neither change in acidity nor dilution of the milk appears to affect the dialysable organic phosphorus of the milk.

8. Using the "static" method of dialysis and with controlled conditions, the range, calculating the various ingredients on the total present in separated "average" milk, is as follows:

Dialysable Ca	...	...	...	25-33 %
Dialysable P—total	...	...	...	43-51 %
Dialysable P—inorganic	...	...	...	33-41 %

These include results from fresh, pasteurised and dried separated "average" milks.

9. The effect of dilution on the calcium is much less in the case of spray-dried milk than in that of pasteurised milk. This appears to indicate either a heat or a dehydration effect.

10. The effect of heat is much less defined, but it appears that the conclusion that heat decreases the amount of soluble phosphorus and of soluble calcium is substantiated.

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