CCXXX. DIALYSIS OF MILK

III. SALT EQUILIBRIUM WITH SPECIAL REFERENCE TO CALCIUM, MAGNESIUM AND PHOSPHORUS

By LESLIE HERBERT LAMPITT, JOHN HERBERT BUSHILL AND DOROTHY FRANCES FILMER

From the Lyons Laboratories, London, W. 14

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THE general object of this series of investigations is the study of the constituents of cow's milk by means of dialysis. It has already been shown that standard conditions must be observed during the dialysis and that, of the factors investigated, acidity of the milk is of distinct importance [Lampitt & Bushill, 1934]. As an example, it has been shown that an increase of titratable acidity (calculated as lactic acid) from 0.14 to 0.39 % results in an increase of the amount of dialysable Ca from 32 to 49 %, and of dialysable inorganic P from 34 to 45 %. (The amounts are calculated on the amount of Ca and P present respectively in the milk.) It is therefore impossible to compare the results of one worker with those of another, for, even where the titratable acidity of the milk has been recorded, no data exist by which a correction can be applied to the figures given for the various dialysable constituents. Consideration of the problem shows that comparison may possibly be achieved by one of two procedures, namely (1) the use of a factor, obtained experimentally, by which an "acidity" correction could be applied to determined values or (2) the adjustment of the milk, previous to dialysis, to a predetermined standard titratable acidity or pH. The first suggestion implies that all milks react similarly on acidification in respect of the dialysable constituents, while the second implies that the "acid effect" is reversible. This reversibility would of necessity be such that a sour milk, brought back by alkali to its original pH, yields, on dialysis, results similar to those obtainable previous to the souring. This second procedure has been studied in some detail in respect of Ca, Mg and P.

It will be noted that Mg has been added to the list of constituents previously studied. In comparison with the amount of data available relating to Ca and P that concerned with Mg is very small. Van Slyke & Bosworth [1916] have shown that the total Mg content of milk varies far more than does that of Ca, and as the result of a series of determinations they report that the Mg in the serum from milk may vary from 54 to 91 % of the total amount present. The presence of Mg, either as a colloidal phosphate or in other combination, complicates any conclusions to be drawn from studies of the inorganic constituents.¹

During the course of the investigation several points of interest have been studied. The keeping of milk for considerable periods, with the addition of a "preservative" which would not react with the dialysable constituents or modify the course of dialysis, has yielded interesting results regarding changes, at 0–5° in the presence of chloroform, attributable to bacteria and to enzymes.

The effects of lactic and sodium ions in certain concentrations on the dialysable constituents have been studied and points of interest regarding the instability of the equilibrium of the salts of milk have been indicated.

¹ For example Ling [1936] attributes to Mg certain discrepancies in the results obtained by him using a method for the determination of Ca_3PO_4 in milk.

It would be possible to draw general conclusions from the mass of results obtained in these dialysis studies (results reported in these communications are only examples selected from a number of similar experiments) and to formulate a scheme for the actions which take place on agitating, on acidifying and on neutralizing acidified milk. The authors, however, prefer to await the conclusion of work in hand concerned with the other inorganic constituents and with the various types of nitrogenous bodies present. The authors believe that the statement of Allen [1931] made 6 years ago can be applied to a large proportion of the work published during the intervening years "... a great deal (of the data) has been compiled with too little regard to the possible experimental error". In this series of communications the reliability of every method of analysis used has been tested and the experimental error is known.

SECTION I. GENERAL PROCEDURE

(a) *Materials used*

Fresh, raw milk, separated at room temperature by means of a Sharples super-centrifuge, and "solutions" of separated milk powder have been used in this work. The general procedure has been to carry out tests initially on separated milk powder "solution", whereby repetitive experiments have been possible thus allowing the technique to be standardized before applying it to fresh, raw, separated milk.¹ (Results of the analyses of the milk-powder "solutions" and of the milks used are given in Table X.)

(b) *Method of varying the acidity*

Increasing acidity of the milk has been obtained by "natural" souring and also by additions of lactic acid. Reduction of acidity has been obtained simply by adding NaOH solution, a test having indicated that lactate and Na ions, present under the conditions of the experiment, have no influence on the Ca, Mg and P equilibrium (see para. (f)).

(c) *Method of dialysis*

In order to reduce to a minimum the dilution effect, the importance of which has already been demonstrated [Lampitt & Bushill, 1934], the "static" method of dialysis previously described [1933] has been employed. In this method 25 ml. of distilled water are dialysed in a collodion thimble against 900 ml. of milk for 2 days at 0–5° after which period, as previously shown, the system reaches equilibrium. The dialysable constituents must of necessity become diluted during the course of any dialysis, a factor which itself affects the salt equilibrium. The "static" dialysis method employed in this and previous investigations results in a minimum of dilution (normally from 900 to 925 ml.) and a suitable correction is applied to the figures obtained by analysis of the dialysate in order to calculate the corresponding concentrations in the milk itself. The effect of this dilution on the salt equilibrium has been found to be negligible.

(d) *Methods of determination of Ca, Mg and P*

The determination of P [1934] was carried out on one sample; Ca and Mg were determined on a second. For the determination of these metals 20 ml. of

¹ Both milk and milk powder (made by the Milkal process) have been obtained by the courtesy of Messrs United Dairies.

milk or 30–40 ml. of dialysate are required. The sample is reduced to an ash which is subsequently dissolved in HCl. From this solution Ca is precipitated as oxalate at pH 3 [1934]. The resulting filtrate and washings are reduced to an ash in the presence of 2 ml. 10 *N* H₂SO₄ (which prevents decrepitation) and the ash dissolved in 20 ml. *N*/10 HCl. 1 ml. of this solution is used for determination of Mg [Bushill *et al.* 1937]. The experimental error of the methods of determination of total P, inorganic P, Ca and Mg has been found to be of the order of 2%.

(e) *Acidification with lactic acid*

It was considered possible that additions of lactic acid to milk might, to some extent, result in localized acid effects, a condition which would not occur during gradual acidification by “natural” souring. It was desirable therefore to obtain some indication as to whether the final salt equilibrium would be appreciably influenced by such localized effects. In separate experiments lactic acid was added (1) as a weak solution (to minimize localized effects) and (2) as a strong solution in total amounts equivalent to those in (1) (to accentuate localized effects).

The details of these solutions given in Table I are self-explanatory: milk powder solution (sample *a*) was used.

Table I. *The addition of increasing amounts of lactic acid to milk*

Method I. Using dilute lactic acid solution				Method II. Using strong lactic acid solution			
Milk powder (approx. 18%) solution ml.		Lactic acid (approx. 0.5%) solution ml.	Water ml.	Milk powder (approx. 9%) solution ml.		Lactic acid (approx. 10%) solution ml.	Water ml.
500	+	(0	+ 500)	1000	+	(0	+ 20)
500	+	(100	+ 400)	1000	+	(5	+ 15)
500	+	(200	+ 300)	1000	+	(10	+ 10)
500	+	(300	+ 200)	1000	+	(15	+ 5)
500	+	(400	+ 100)	1000	+	(20	+ 0)

Each solution was dialysed in the usual manner and the dialysable Ca, total P, inorganic P and organic P (by diff.) determined. The results (see curves *A* and *B*, Figs. 1 and 2) show the effect of increasing titratable acidity¹ on these constituents.

The similarity of these two curves indicates that, in so far as the dialysable Ca and P are concerned, there is no particular virtue in mixing, with agitation, dilute lactic acid with a strong solution (18%) of milk powder rather than a strong solution of the acid with milk of 9% total solids content. The value of this result lies in the fact that only the second method could be used in the case of fresh milk without introducing undesirable dilution effects.

(f) *The effect of lactate and of Na ions*

Raw, separated milk (sample *c*) was shaken with and without the addition of sodium lactate, the amount of that salt being equivalent to the acid necessary to raise the titratable acidity to approximately 0.30%.

¹ *Titratable acidity*, in the course of this paper, is expressed as % lactic acid and has been determined by titration of 50 ml. of the solution with *N*/10 NaOH (2.5 ml. 1% phenolphthalein as indicator).

Fig. 1.

Fig. 2.

Figs. 1 and 2

Milk-powder solution

Sample (a):

Curve A. Effect of increments of added lactic acid as a weak solution (cf. Method I, Table I).

Curve B. Effect of increments of added lactic acid as a strong solution (cf. Method II, Table I).

Sample (b):

Curve C. Effect of increments of added lactic acid as a weak solution (cf. Method I, Table I).

Curve D. Effect of increments of added alkali (NaOH) to acidified solution (approx. 0.36 % titratable acidity) 20 hr. after acidification (cf. Table III).

Curve E. Effect of increments of added alkali (NaOH) to acidified solution (approx. 0.36 % titratable acidity) 24 hr. after acidification (cf. Table III).

Curve F. Effect of increments of added alkali (NaOH) to acidified solution (approx. 0.36 % titratable acidity) immediately after acidification (cf. Table III).

Raw, separated milk

Sample (h):

Curve G. Effect of acidification by "natural" souring followed by increments of added alkali (NaOH) (see Table IX, B).

The figures given in Table II demonstrate that the effect of the sodium lactate was negligible in respect of the dialysable constituents studied.

Table II

Time of shaking ...	0 days					14 days				
	Milk Titratable acidity %	Dialysate* (%)				Milk Titratable acidity %	Dialysate* (%)			
		Org. P	Inorg. P	Ca	Mg		Org. P	Inorg. P	Ca	Mg
I. Milk	0.14	9	41	31	63	0.15	7	41	31	52
II. Milk + sodium lactate	0.14	8	40	32	62	0.15	9	42	32	50

Time of shaking ...	14 + 14 days				
	Milk Titratable acidity %	Dialysate* (%)			
		Org. P	Inorg. P	Ca	Mg
I. Milk	0.15	4	41	30	42
II. Milk + sodium lactate	0.16	5	42	32	44

* In all tables and comments the P, Ca and Mg constituents of the dialysate are calculated as % of the total P, Ca and Mg present respectively in the original milk.

(g) *Effect of saturation of milk with chloroform*

It was found necessary, during certain experiments where milk had to be kept for considerable periods, to maintain the milk saturated with CHCl_3 at a low temperature ($0-5^\circ$). This procedure was adopted after experiments had shown that under these conditions (1) the increase in acidity is reduced to a minimum, and (2) chloroform does not affect the results of dialysis.

SECTION II. THE REVERSIBILITY OF THE ACID EFFECT (ADDED LACTIC ACID) ON THE DIALYSABLE CONSTITUENTS OF MILK-POWDER SOLUTION AND OF RAW MILK

The main object of these experiments was to trace the changes in the amounts of the dialysable constituents under consideration with increasing acidification of the milk and with neutralization of that acidity, such neutralization being achieved in stages. At the same time the experiments were designed to give information on two further points:

(a) The effect of the time elapsing between acidification and subsequent neutralization on the dialysable salts.

(b) The effect of the time elapsing between neutralization and dialysis on the dialysable milk salts.

(a) *Milk-powder "solution"*

A preliminary experiment, in which milk-powder solution was acidified with lactic acid and immediately neutralized, having shown that the amounts of the dialysable inorganic P and Ca did not revert to those of the untreated solution, an experiment was conducted in which milk-powder solution (sample *b*) was acidified (to approx. 0.36% titratable acidity¹), and then kept for different periods before being neutralized to different acidities.

¹ It should be remarked that the acidified milk was obtained in all cases without any precipitation of casein. Indeed by slow development of acidity, during souring caused by bacteria at $0-5^\circ$ and in the presence of chloroform, titratable acidity of 0.63% has been attained without such precipitations. All the inorganic P and Ca were then dialysable.

The method of acidifying the milk-powder solution and of neutralizing it subsequently is evident from Table III, while Tables IV *a* and *b* and curves *C*, *D*, *E* and *F* on Figs. 1 and 2, show results obtained (the curves are from a series of experiments different from those recorded in Tables IV *a* and *b*).

Table III. *The addition of increasing amounts of alkali to acidified milk*

Acidified milk solution (cf. Method I, Table I)			Alkali subsequently added		
Milk powder (approx. 18%) solution ml.		Lactic acid (approx. 0.5%) solution ml.	N/2 NaOH solution ml.		Water ml.
500	+	400	0	+	100
500	+	400	11.2 (≡100 ml. 0.5% lactic acid)	+	88.8
500	+	400	22.3 (≡200 ml. 0.5% lactic acid)	+	77.7
500	+	400	33.8 (≡300 ml. 0.5% lactic acid)	+	66.2
500	+	400	44.7 (≡400 ml. 0.5% lactic acid)	+	55.3

Table IV *a*. *Effect of holding milk-powder solution in acidified condition for different periods*

Treatment of milk		Milk Titratable acidity %	Dialysate (%)		
Titratable acidity after acidification %	Period held at high acidity		Org. P	Inorg. P	Ca
Untreated samples		0.16	8	36	34
		0.16	12	36	33
		0.16	9	39	36
0.21	4 min.	0.16	11	41	39
0.21	24 hr.	0.16	11	41	38
0.21	48 hr.	0.16	11	42	40
0.36	4 min.	0.16	11	42	41
0.36	24 hr.	0.16	11	42	41

Table IV *b*. *Effect of holding partly neutralized acidified milk-powder solution for different periods*

Treatment of milk			Milk Titratable acidity %	Dialysate (%)		
Titratable acidity after acidification %	Period held at high acidity	Period held partly neutralized days		Org. P	Inorg. P	Ca
0.36	18 hr.	0	0.16	10	42	40
0.36	18 hr.	2	0.16	9	42	42
0.36	18 hr.	7	0.17	—	40	40
0.36	7 days	0	0.17	10	42	42
0.36	7 days	2	0.17	10	42	42
0.36	7 days	7	0.17	8	42	43

If the average of the % of dialysable constituents (as shown in Tables IV *a* and *b*) be calculated the following results:

Table IV *c*

	Dialysable constituents (%)		
	Org. P	Inorg. P	Ca
Original milk-powder "solution"	10	36	34
Acidified and neutralized milk-powder "solution"	10	41	40

(b) *Raw, separated milk*

Two samples of raw, separated milk (samples *d* and *e*) were acidified with lactic acid and then treated with NaOH. The volumes of acid and alkali were such that 1000 ml. of milk were eventually diluted to 1020 ml. These milks were dialysed. Analysis of the dialysates gave the results shown in Table V.

Table V

Titratable acidity of milks (%)		Analysis of dialysates (%)				
Original	After lactic acid and NaOH additions	Total P	Inorg. P	Org. P (by diff.)	Ca	Mg
Sample <i>d</i> :						
0.15*	0.15	54	42	12	34	62
0.35	0.15	53	43	10	37	63
Sample <i>e</i> :						
0.14*	0.14	51	39	12	34	66
0.36†	0.14	51	40	11	37	67

* Untreated.

† Maintained 20 hr. in acidified condition.

(c) *Discussion of the results*

(i) *Milk-powder "solutions"*. Examination of the results obtained with milk-powder solution shows (Tables IV *a*, *b* and *c*) that neutralization of the added acid does not restore the dialysable Ca and inorganic P to the equilibrium obtaining before the addition of acid, for, after such neutralization, the amounts of dialysable Ca and inorganic P are appreciably higher than those obtained initially.

Furthermore, this conclusion is not modified if the period during which the "solution" remains acid is extended to as long as 7 days or by keeping the subsequently partly neutralized liquid for 7 days before dialysis. Under the conditions of the experiment the dialysable Ca and inorganic P in the treated milk powder "solutions" (first acidified by lactic acid and subsequently treated with NaOH equivalent to the added acid) remained 6 and 5% respectively higher than in the untreated "solution". (With milk-powder solution, sample *a* (Table VII), corresponding figures were 7 and 4%.)

The change in dialysable Ca and inorganic P on acidification of the milk-powder solution, followed by neutralization shown as curves *D*, *E* and *F* on Figs. 1 and 2 (a separate series of experiments), confirms the conclusions drawn from the results shown in Tables IV *a*, *b* and *c*. Acidification and neutralization appear to effect no appreciable change in the amount of dialysable organic P.

(ii) *Raw, separated milk*. In the case of raw, separated milk (Table V) it will be seen that the original % of dialysable Ca is not regained after acidification and neutralization (it remains 3% high) whereas the corresponding concentrations of inorganic P and Mg (also determined in these tests) are regained.

The incomplete re-establishment of the salt equilibrium of milk by neutralization of added acid is not surprising in view of the work of Holt *et al.* [1925] who showed that a considerable period is necessary for the establishment of the phosphoric acid-calcium hydroxide equilibrium. They state that continuous shaking for 10 days results in the establishment of an equilibrium in that system which normally, in the absence of shaking, requires 2-3 months to become effective.

The experiments with milk, hitherto described in this paper, are concerned with the re-establishment of an equilibrium somewhat similar to that mentioned above, but in no case has a time greater than 9 days, the solutions remaining quiescent, been allowed for re-establishment of equilibrium.

In view of this, experiments were instituted in which continuous shaking for 14 days was employed.

SECTION III. THE EFFECT OF SHAKING (14 DAYS) ON THE SALT EQUILIBRIUM OF RAW, SEPARATED MILK

Some evidence had been obtained that when separated milk was agitated a change often took place in the amounts of the dialysable Ca and P. Before proceeding therefore with experiments on the restoration of salt equilibrium of acidified milk in which agitation was employed, this phenomenon was investigated. A sample of raw, separated milk (sample *f*) was divided into three portions; the first portion was dialysed immediately, the second shaken in a glass container on a mechanical shaker (at 0–5°, in presence of CHCl_3) for 14 days and then dialysed, the third similarly treated except that 28 days' shaking was given. Table VI gives results obtained typical of the majority of such experiments. It should be noted that shaking does not invariably cause such effects (Table II).

Table VI. *Effect of shaking on the dialysable Ca and P of raw, separated milk*

Treatment	Titratable acidity of milk before dialysis (%)	Analysis of dialysate (%)			
		Total P	Inorg. P	Org. P (by diff.)	Ca
Not shaken	0.15	57	43	14	40
14 days shaken	0.17	52	45	7	—
28 days shaken	0.18	53	48	5	35

It will be noted that the effect of shaking untreated, raw milk has been to increase the amount of inorganic P and to decrease the amounts of dialysable organic P and Ca, the last change being somewhat remarkable in view of the increase in titratable acidity. It appears that at least five possible reactions have to be considered:

- (i) The pure acidity effect (increase in titratable acidity from 0.15 to 0.18 %, causing increases in both dialysable Ca and inorganic P.
- (ii) The decomposition of non-dialysable organic P compounds by bacterial action¹ which may result in increased dialysable organic or inorganic P.
- (iii) The action of phosphatase whereby dialysable organic P compounds are partly changed into dialysable inorganic P.
- (iv) The utilization by the bacteria present of organic and/or inorganic P, thereby reducing the amount of either or both dialysable forms.
- (v) The change of equilibrium of the Ca, P and Mg induced by the agitation of the liquid.

¹ Van Slyke & Bosworth have shown [1916] and it has been confirmed that in extreme cases of souring the whole of the Ca and P becomes soluble. Berggren [1932] finds that the casein of milk loses P on dialysis of milk. She does not state it specifically but it would appear that this was caused by bacterial action. On the other hand in the course of work on the action of *B. subtilis* on milk, results have been obtained (not yet published) which show that the protein precipitated from milk in which *B. subtilis* has been cultured is higher in P than normal, suggesting preferential attack on the non-P groupings of the protein.

The first four possible reactions could only reduce the amount of dialysable Ca in an indirect way, namely by increasing the amount of inorganic P with subsequent precipitation of calcium phosphate. This possibility has been partly investigated, but as yet with no positive results. It is concluded therefore that the fifth reaction, namely a change in salt equilibrium, is probably responsible for the decrease in the amount of dialysable Ca.

SECTION IV. THE EFFECT OF 14 DAYS' SHAKING, AT 0-5°, ON THE REVERSIBILITY OF THE "ACID EFFECT" (LACTIC ACID ADDITION AND NATURAL SOURING) OF MILK

Separated milk-powder (9%) solution and raw separated milk were each acidified with lactic acid; other samples of raw separated milk were also acidified by "natural" souring. These milks were then neutralized with NaOH and shaken for 14 days with CHCl_3 . The additions of acid and of alkali were such that the milks were in all cases diluted only from 1000 to 1020 ml.

(i) *Milk-powder solution—acidified by added lactic acid.* The results (Table VII), obtained with a solution of milk-powder (sample *a*), show that during the 14 days' shaking period no material change occurs in the untreated solution.

Table VII. *Milk-powder solution*

	Initial					After 14 days' shaking				
	Milk solution Titratable acidity %	Dialysate (%)				Milk solution Titratable acidity %	Dialysate (%)			
		Org. P	Inorg. P	Ca	Mg		Org. P	Inorg. P	Ca	Mg
Untreated milk	0.16	7	38	32	62	0.16	9	38	33	62
Milk acidified to 0.36% titratable acidity and neutralized immediately	0.16	8	42	39	62	0.16	10	40	35	56

The acidified and neutralized solution, however, shows an initial difference in dialysable constituents of 7 and 4% of Ca and inorganic P respectively (cf. Table IVc), while after 14 days' shaking that difference is reduced to 2% in both cases. The dialysable Mg which remained unchanged in the first case, after the shaking treatment fell to 6% below the original figure.

(ii) *Raw, separated milk—acidified by added lactic acid.* In view of the results recorded in Section III, it was decided, in this instance, to ensure that equilibrium be established by shaking acid-treated milk before the addition of alkali. The milk (sample *f*) was therefore shaken for 14 days with the added acid and for a further 14 days after the addition of the alkali. The results, which are self-explanatory, are given in Table VIII.

(iii) *Raw separated milk—acidified by "natural" souring.* Raw, separated milk (sample *g*) was allowed to sour "naturally", and samples of the original milk and of the final, soured milk dialysed. During the progress of the souring, samples were taken and adjusted to a titratable acidity of 0.13% by the addition of NaOH. These partly neutralized milks were shaken for 14 days, dialysed and the dialysates analysed. Table IX, A records the results obtained. It will be observed that a preliminary 14 days' shaking was not given as it was thought unnecessary in a case where acidity increased very gradually (souring).

Table VIII

Period of shaking ...	0 days					14 days				
	Milk Titratable acidity %	Dialysate (%)				Milk Titratable acidity %	Dialysate (%)			
		Org. P	Inorg. P	Ca	Mg		Org. P	Inorg. P	Ca	Mg
(1) Milk	0.15	14	43	40	62	0.17	7	45	—	—
(2) Milk + lactic acid	0.20	14	49	42	73	0.22	11	49	43	—
(3) Milk + lactic acid	0.30	13	58	60	83	0.31	13	56	60	—
Period of shaking ...	14 days					14 + 14 days				
	Milk Titratable acidity to which adjusted %					Milk Titratable acidity %	Dialysate (%)			
		Org. P	Inorg. P	Ca	Mg		Org. P	Inorg. P	Ca	Mg
(1) Milk	—					0.18	5	48	35	76
(2) Milk + lactic acid	0.17					0.18	5	48	36	70
(3) Milk + lactic acid	a. 0.17					0.18	5	48	38	73
	b. 0.22					0.21	8	49	43	73

Table IX

	Initial					After 14 days' shaking						
	Milk Titratable acidity %	Dialysate (%)				Milk Titratable acidity (%)	Dialysate (%)					
		Org. P	Inorg. P	Ca	Mg		To which adjusted	After shaking	Org. P	Inorg. P	Ca	Mg
A. Original milk	0.15	15	41	42	83	0.13	0.13	10	41	35	67	
Souring milk	0.18	—	—	—	—	0.13	0.13	9	41	35	67	
„	0.21	—	—	—	—	0.13	0.13	10	41	35	63	
„	0.26	—	—	—	—	0.13	0.15	9	41	33	65	
„	0.30	13	65	82	100	0.13	0.15	8	41	34	72	
B. Original milk	0.16	12	42	35	63	0.07	0.09	0	42	21	57	
Soured milk	0.21	8	50	42	73	0.09	0.11	1	43	24	57	
						0.12	0.14	2	45	26	69	
						0.13	0.15	3	46	28	61	
						0.14	0.16	3	47	30	68	
						0.15	0.16	3	46	29	68	

The same comments also apply to the next experiment (Table IX, B) in which milk (sample *h*) was allowed to sour in a similar fashion but was then neutralized to different acidities before the 14 days' shaking. These results, obtained by analysing the dialysable constituents, are also recorded as curve *G* on Figs. 1 and 2.

Discussion of results

The experiments conducted with shaking, in order to hasten equilibrium of the milk salts under investigation, have introduced a number of points which increase the difficulty of drawing concrete conclusions. The two chief difficulties are (1) the gradual development of acidity during the extended shaking period, although carried out at 0–5° and in the presence of chloroform, and (2) the decrease in dialysable organic P, presumably caused by phosphatase activity. These two effects were noted particularly when using fresh, or naturally soured,

raw milk; the former effect is minimized and the latter eliminated in a pasteurized milk.

The gradual change in acidity will of course affect the dialysable inorganic constituents, thus complicating any reversibility test. For example, taking an increase of titratable acidity of 0.15–0.18 % (Table VI), on the basis of Figs. 1 and 2, curves *A* and *B*, increases of 2 % of dialysable inorganic P and of 4 % Ca should result; whereas the increase of inorganic P was 5 % and the decrease of Ca 5 %.

It is not known how or whether the phosphatase activity affects the dialysable inorganic constituents but, as previously mentioned, there is the possibility that it may cause an increase in dialysable inorganic phosphate which again may in turn affect the equilibrium of the dialysable Ca and Mg.

Table X. *Analyses of milk-powder solutions and raw separated milks used*

	(All figures as % except pH)							
	Milk-powder solutions		Raw, separated milks					
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	<i>h</i>
Total solids	9.06	9.01	9.46	9.45	9.46	9.17	8.92	9.42
Non-fatty solids	8.96	8.93	9.54	9.42	9.42	9.10	8.83	9.41
Titratable acidity	0.16	0.16	0.14	0.15	0.14	0.15	0.15	0.16
pH (using glass electrode)	6.62	6.50	6.82	6.77	6.87	6.55	6.80	6.71
Total P	1.05	1.07	1.02	1.01	0.99	1.07	1.07	1.00
Inorg. P	1.74	0.72	0.73	0.74	0.66	0.77	1.72	0.75
Ca	1.34	1.38	1.29	0.31	1.27	1.41	1.34	1.34
Mg	0.117	—	0.130	0.128	0.117	0.150	0.135	0.124

GENERAL CONCLUSIONS AND DISCUSSION

The results recorded in this paper have all been tabulated suitably and therefrom generalized conclusions drawn.

(a) *Shaking (14 days) milk-powder solution and raw, separated milk (at 0–5°, with CHCl₃)*

This treatment has no effect on the dialysable constituents of milk-powder solution, but the amounts, both of dialysable Ca and of Mg, in raw, separated milk may be reduced. Furthermore, in the case of raw, separated milk the dialysable organic P is also reduced, not presumably through shaking, but as a result of some other factor, possibly phosphatase activity.

(b) *Acidification (by lactic acid) and neutralization (NaOH) with and without shaking (at 0–5°, with CHCl₃)*

Acidification and neutralization of milk-powder solution result in higher concentrations of dialysable inorganic P and Ca than in the original milk. When the milk is agitated the amounts of these two constituents are reduced, but even so they remain slightly higher than in the original milk, while the dialysable Mg is reduced to an amount well below that of the untreated milk. No change in the amounts of dialysable organic P was observed during the above treatments.

In the case of fresh milk, the increased amounts of dialysable Ca, inorganic P and Mg caused by acidification are reduced by neutralization to quantities approximating to those of the original milk but, nevertheless, there is a tendency

for the amount of dialysable Ca still to remain slightly high: agitation brings this nearer that of the original. The amount of dialysable organic P is unaffected by addition of acid and by subsequent neutralization but it is reduced during the shaking period, as was found with untreated raw, separated milk.

(c) *The effect of bacterial ("natural") souring of raw, separated milk followed by neutralization and shaking (14 days) (at 0–5°, with CHCl_3)*

No clear conclusions can be drawn from the results obtained under this heading. This might be expected, as "natural" souring is ill-defined and the effects will no doubt be dependent upon the type of organisms present. It can be said however that the results obtained show that after neutralization of the acidity and 14 days' agitation the amounts of dialysable Ca, Mg and organic P were usually well below those of the original milk.

If it be taken that the evidence presented is conclusive in showing that the shaking of milk causes a decrease in the amounts of dialysable Ca and Mg, it follows that mechanical treatment is capable of effecting a change in the salt equilibrium of milk. It may therefore be inferred that the salt complex in milk is normally in an unstable equilibrium, true equilibrium being approached by extended shaking. Moreover, it appears that the processes through which milk passes in the production of milk-powder stabilize the equilibrium so that mechanical agitation no longer changes the amounts of dialysable constituents.

The incomplete reversibility, as regards dialysable inorganic P and Ca, by acidification and neutralization of milk agrees well with the above-mentioned hypothesis, as does also the conclusion that shaking aids in the reversion to the original concentration of dialysable constituents.

Whilst it must be admitted that the dialysable Mg results do not, in every case, follow those of the dialysable Ca, as might be expected, it must be remembered that Ca is present in much higher concentration and that there must be some "competition" between Ca and Mg ions for the cation $\text{PO}_4^{=}$. Equilibrium between Mg and Ca, controlled to some extent by the relative solubilities of Ca and Mg phosphates, may itself be somewhat upset by acidification and neutralization. Furthermore, phosphatase activity, as mentioned earlier, may have some interfering effect by affecting the phosphate equilibrium.

The reduction in the concentrations of dialysable Ca and Mg obtained when neutralized sour milk is shaken may be ascribed to the agitation which shifts the equilibrium beyond that existing in the original milk.

SUMMARY

1. Acidification with a relatively strong lactic acid solution has the same effect on the amount of the dialysable constituents as has the same amount of that acid added as a more dilute solution.

2. The effect of neutralization by NaOH of such acidity may be studied, as Na and lactate in such concentrations have no effect on the dialysable constituents of milk.

3. An approximately straight-line relationship exists between the concentration of dialysable Ca and inorganic P of milk-powder solution and titratable acidity in the acidification stage, but neutralization of the added acid does not result in the original amounts of these dialysable constituents being regained. Furthermore, the length of time (up to 9 days) the milk is held at the high acidity does not affect this non-reversibility. The amount of dialysable organic P is not affected by these treatments.

4. Neutralization of acidified, raw, separated milk results in an almost complete recovery of the original amounts of dialysable Ca, Mg and inorganic P, although the figure for Ca remains slightly above the normal.

5. Extended agitation (14 days' shaking) has been found to:

(a) Reduce the amounts of dialysable Mg and Ca and increase the amount of dialysable inorganic P in raw, separated milk.

(b) Have no effect on these constituents in milk-powder solution.

(c) Reduce these constituents in neutralized, acidified milk-powder solution and in similarly treated raw, separated milk.

6. By its aid (shaking) an approximate reversibility of the dialysable inorganic constituents of milk-powder solution after acidification and neutralization may be obtained. In the case of raw, separated milk, shaking is not entirely necessary and in any case such a prolonged treatment introduces difficulties concerned with the control of acidity and with apparent phosphatase action on the dialysable organic P.

7. A suggestion is made that the salt equilibrium of milk is normally unstable and that this equilibrium may be shifted by agitation of the milk. The treatment of milk in the preparation of milk powder appears to stabilize the salt equilibrium.

8. Comparison of the results of the dialysis of milks of differing acidity is not possible by adjusting the milks before dialysis to a predetermined standard acidity, since the reactions caused by acid (e.g. incipient souring) are not strictly reversible.

9. The following ranges of analytical figures (superseding previous results published by the authors) have been obtained to date on raw, pasteurized and dried, separated, "average" milks.

	Total %	Dialysable (as % of total present)
Inorganic P	0.63-0.77	33-43
Organic P	0.31-0.38	7-15
Ca	1.27-1.44	25-42
Mg	0.10-0.15	62-83

The amounts of the dialysable constituents have been determined by the "static" method under controlled conditions.

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