

THE ACID COAGULATION OF MILK.

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It is well known that when milk is allowed to sour, the rise in acidity is not directly proportional to the increase in the number of acid-forming bacteria. There is a distinct phase in the process during which numerical increase of these organisms takes place, accompanied only by slight acid production, after which the acidity rises very rapidly to a maximum.

Two reasons have been assigned for this delay :—

(I) The necessity for a certain lapse of time, during which the organisms produce an enzyme, the function of which is the eventual formation of lactic acid from the lactose present, and,

(II) That the lactic-acid-producing bacteria do not find in milk their normal habitat, and only acquire the power to produce acid after a certain lapse of time.

The experiments here recorded are the outcome of some preliminary trials to throw some light, if possible, on the causes of the delay in acid formation, and though that end has not been attained, the results are in themselves interesting, and elucidate some of the chemical changes which take place progressively as milk sours.

It may, however, be briefly remarked that as no chemical basis for the delay has been found, it is highly probable that the power to produce acid is only developed by such organisms as *Bacillus acidi lactis*, after a certain time of growth, just as may be observed in the case of certain organisms of the "coli" group, which, when inoculated into glucose-broth, often show no signs of gas production during the first 24 hours (in spite of vigorous growth), yet in the ensuing 24 hours produce gas very rapidly till the maximum evolution is reached.

It would be a perfectly reasonable supposition, and in the face of the experiments of some other observers, a very likely supposition that the lactic acid produced is at first neutralised, completely or partially, by some constituent of the milk, and so removed from the sphere of action. This, however, is not the case, as the following experiments prove:—

Definite quantities of milk, immediately after milking, were mixed with varying amounts of a solution of ordinary lactic acid of known strength, and the increase in acidity of the milk determined in each case after the addition. By the word "acidity" throughout this investigation, is to be understood that which is measured by the addition of decinormal soda to a neutral point determined by phenolphthalein. As milk itself immediately after milking shows a considerable acidity to this indicator, the increase of acidity above this number is taken to be due entirely to lactic acid, either added or produced. Determinations of acidity are best carried out in the following manner:—10 c.c. of milk are placed in each of two long Nessler glasses of 50 c.c. capacity, 10 c.c. of water added to each, and to one, 2 or 3 drops of the indicator. The contents are mixed by rotary shaking and being held side by side in one hand, N/10 NaOH is run in till the pink colour is just permanent, the blank by the side enabling this point to be determined with great exactitude.

For the experiment mentioned above a solution of N/10 lactic acid was employed, and the following results obtained:—

Experiment I.

10 c.c. milk required	1.45 c.c. $\frac{N}{10}$ NaOH
1 c.c. lactic acid solution added to this required				0.9 ,, ,, (in addition)
10 c.c. milk and 1 c.c. lactic acid solution required				2.5 ,, ,,

Experiment II.

10 c.c. milk required	1.45 c.c. $\frac{N}{10}$ NaOH
3 c.c. lactic acid solution added to this required				2.9 ,, ,, (in addition)
10 c.c. milk and 3 c.c. lactic acid solution required				4.5 ,, ,,

Experiment III.

10 c.c. lactic acid solution required	2.2 c.c. $\frac{N}{10}$ NaOH
10 c.c. milk and 10 c.c. water required				1.75 ,, ,,
10 c.c. milk and 10 c.c. lactic acid solution (titrated at once)	4.05 ,, ,,
10 c.c. milk and 10 c.c. lactic acid solution (after 35 minutes)	4.05 ,, ,,

These experiments show conclusively that, within the limits of error of such titrations, lactic acid is not neutralised (as far as phenolphthalein is concerned) either when added to milk already neutralised, or mixed with milk and the combined acidity determined, neither has time any effect on the titration.

Though from such experiments it might be concluded that lactic acid does not combine with any constituent of the milk, it actually does so, but in such a loose combination, that it can be still readily determined by caustic soda in the presence of phenolphthalein. Hammarsten¹ concluded that casein did not combine with lactic acid, because this acid could be completely removed from casein by triturating with water, and our own experiments detailed later, show also that the combination of casein and lactic acid, as it occurs in milk, is very susceptible to the presence of water, and is readily decomposed by it. The researches of Slyke and Hart², and later of Laxa³, have undoubtedly proved the existence of such lactates, but the experiments of these observers are open to the objection that they cannot in themselves be taken as conclusive evidence of the existence or formation of such substances in milk itself, and for this reason, viz. that the casein employed by them had been separated from milk by more or less drastic methods of solution and precipitation, repeated again and again, and it is impossible to assert that the substance obtained eventually by such methods is identical in all its properties with the original proteid as it exists in milk. It can be scarcely doubted that the severe methods which must at present be employed in the separation and purification of proteids give rise to the formation of small quantities of other substances, which have more than once been mistaken for bodies existent in the original source.

In addition to the compound, or compounds, of casein and lactic acid, the proof of the existence of which is of comparatively recent date, there are the compounds of casein and lime salts, which have long been known. The actual constitution of these compounds is very imperfectly understood, though definite substances containing 1 mol. CaO and 2 mol. CaO have been described by Söldner⁴, and Lehmann⁵ also gives the percentage of CaO in casein. Eugling⁶ has

¹ Hammarsten, *Jahresb. der Thierchemie*, Vol. VII. p. 160.

² Slyke and Hart, *Amer. Chem. Journ.*, Vol. XXXIII. p. 461.

³ Laxa, *Milchwirtsch. Zentralblatt*, Vol. I. p. 538.

⁴ Söldner, *Landwirtsch. Versuchs-Stat.*, Vol. XXXV. p. 351.

⁵ Lehmann, *Pflüger's Archiv*, Vol. LVI. p. 558.

⁶ Eugling, *Landw. Versuchs-Stat.*, Vol. XXXI. p. 392.

asserted that the calcium is combined as calcium triphosphate, and the presence of this substance, together with CaO, has also been mentioned. Our own experiments entirely confirm the results of Eugling and others that the calcium is combined as calcium triphosphate $\text{Ca}_3(\text{PO}_4)_2$ and not in any other form, though the nature of the compound cannot be in any way understood. If, on the other hand, CaO itself were present in the casein molecule, it might reasonably be expected that added lactic acid would neutralise this, and so be removed from the sphere of action; but as our experiments above show, this is not the case.

Our first experiment was to determine the amount of calcium compounds in combination with the casein, during the progressive stages of "souring." In attempting this, an immediate difficulty presented itself, in that a method of separating the casein without the addition of acid or any agent that might bring about chemical change, *had* to be employed.

Two ways alone seemed practicable:

- (I) Lehmann's method of filtration through porous earthenware;
- (II) Precipitation with alcohol.

Of these the former was to be preferred. Practical difficulties, however, presented themselves. Every attempt to obtain the casein by filtration through porous earthenware proved abortive or useless regarding the end in view, but a slight modification of Lehmann's original method using porous plates, succeeded admirably. The method finally used is as follows:—

An ordinary porous soup plate such as is used for drying crystals, is turned upside down, the edge being raised off the bench to allow free access of the air to the underneath surface; 20 c.c. of milk can, with care, be placed on the base of the plate without running over the edge. A saucer is placed over the milk to prevent evaporation (a trace of formalin being added to the milk beforehand to prevent further decomposition). The plate gradually absorbs everything but the fat and casein; when dry 10 c.c. of a very dilute formalin solution are run on and spread over the surface of the casein, and after replacing the cover, allowed to absorb as before. This is once repeated, by which time all soluble substances have been removed from the casein and can be seen as a ring far down the side of the plate. The layer of casein and fat is removed as completely as possible with a knife, and placed in a vacuum desiccator for 48 hours to dry. It is then extracted with dry ether in a Soxhlet extractor, and again dried in the desiccator.

The casein is thus obtained in perfectly white friable strips, though not, however, completely free from traces of fat. The average amount of this fat was estimated in several samples by the Schmidt method (solution in HCl), and allowed for in the weight of casein used in each experiment. This is not of course strictly correct, but the error is too small to appreciably affect the results.

In the casein so obtained estimations of calcium and phosphoric acid were made in the following manner:—

A weighed quantity of the casein is carefully incinerated in a platinum dish. (A curious property of the casein may be noted; as the casein becomes free from calcium salts, it swells up more and more on incineration, and when practically free from them, is difficult to keep in the dish.) The incineration is continued till an almost white ash is obtained, a result most easily brought about by moistening the char with a few drops of dilute HCl, evaporating to dryness, and then completing the incineration. The calcium salts are dissolved out with hot dilute HCl and filtered (to remove any fragments of porcelain that might be present) into a small beaker. In the solution so obtained the calcium and phosphoric acid are estimated as follows:—

The contents of the beaker are heated to boiling on a sand-bath, and 2 c.c. of a saturated solution of ammonium oxalate added. Allow to cool somewhat and add dilute ammonia till just alkaline to litmus (the calcium oxalate now precipitates). Make distinctly acid with dilute acetic acid and again heat to boiling, and allow to stand overnight. Filter through a small filter, and after incineration weigh as CaO. The filtrate is concentrated to about 10 c.c. on the water-bath and the phosphoric acid estimated by precipitation with magnesia mixture as magnesium pyrophosphate. After the first experiment, the calcium was estimated volumetrically, as on account of the small quantities dealt with, the error in the gravimetric method is rather large. In this case the oxalate is filtered off through a small toughened filter (it is not necessary to clear the beaker of oxalate), the filter is pierced, and the precipitate washed into a small flask with a fine-nosed washed bottle. About 10—15 c.c. of 1:10 H₂SO₄ are heated to boiling, and the beaker washed out with a few drops of this on to the filter, and the filter well washed with the remainder. The contents of the flask are heated to about 50° C. and titrated with permanganate solution (1 c.c. = 0.0025 grams calcium) set against pure sodium oxalate.

Experiment I.

Milk obtained under very careful conditions and inoculated at once with a large quantity of *B. acidi lactici*, this organism being chosen as it does not complicate the experiment by the production of gas.

2000 c.c. of the milk (a large amount is necessary in order that the daily withdrawals may affect the remainder as little as possible), were obtained in a large bottle, arranged with a side siphon tube so fitted that this could be washed out each day after withdrawal of milk, with sterile water.

To this milk were added 5 c.c. of a coagulated milk culture of the organism distributed in about 20 c.c. sterile water. The whole was thoroughly mixed, and kept at 65°—67° F. At intervals of 24 hours about 50 c.c. were siphoned off into a sterile flask, the contents of the bottle being well mixed just previously. Of the amount so withdrawn 20 c.c. were placed on the porous plate, 10 c.c. were used for an acidity determination, and a dilution was made for a bacterial count.

The following table shows the results obtained:—

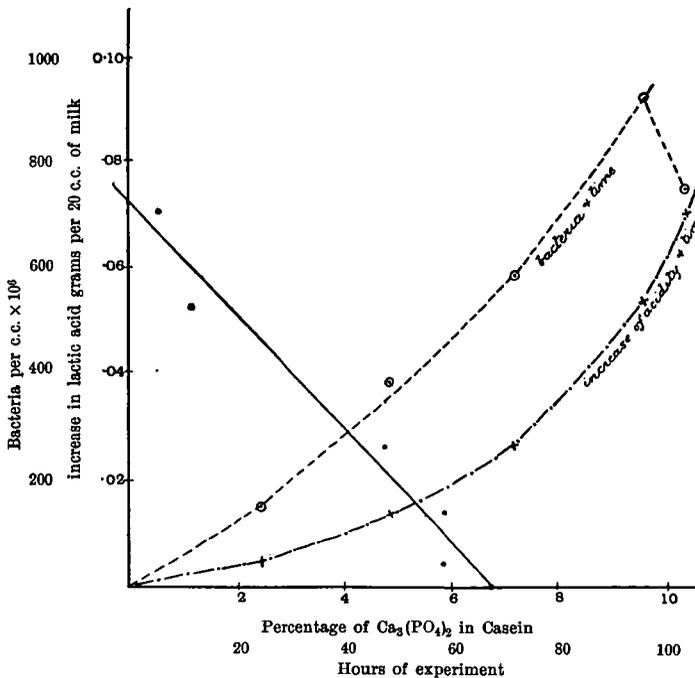
Time	Lactic acid in grams per 20 c.c.	No. of bacteria	Weight of casein (20 c.c.)	Calcium		Mg ₂ P ₂ O ₇			Ca ₃ (PO ₄) ₂ per cent. in casein
				CaO found	Calc. as Ca ₃ (PO ₄) ₂	Found	Calc. for Ca ₃ (PO ₄) ₂	Excess	
Start	—	26 × 10 ⁶	·5528	·0203	·0374	·0287	·0269	·0018	6·77
24 hrs.	·0046	150 × 10 ⁶	·5091	·0161	·0297	·0256	·0213	·0043	5·83
48 „	·0142	380 × 10 ⁶	·4878	·0155	·0286	·0242	·0205	·0037	5·86
72 „	·0257	596 × 10 ⁶	·4943	·0127	·0234	·0195	·0168	·0027	4·73
96 „	·0545	925 × 10 ⁶	·4915	·0026	·0048	·0146	·0034	·0112	0·97
116 „	·0714	758 × 10 ⁶	·4204	·0018	·0033	·0042	·0023	·0019	0·79

It will be seen at once that the percentage of Ca₃(PO₄)₂ steadily decreases as the acidity increases, and if the results be plotted as a curve (see curve A) with percentages of Ca₃(PO₄)₂ as abscissae, and lactic acid in 20 c.c. as ordinates, an approximation to a straight line curve is obtained. The results are rather irregular, owing to the rather large error of experiment, but in the later experiments (as will be seen) the approximation to the straight line is much closer. The point at which the percentage of Ca₃(PO₄)₂ would become zero, is the moment of precipitation of the casein. Perfect freedom from Ca₃(PO₄)₂ is in practice not obtainable.

From the fact that a straight line curve is obtained, we further deduce that the amount of Ca₃(PO₄)₂ present in the casein is directly

proportional to the amount of lactic acid present until the milk coagulates.

As the CaO as estimated decreases, so also does the phosphoric acid, and on calculating the amount of magnesium pyrophosphate which should be found if the CaO estimated were entirely derived from $\text{Ca}_3(\text{PO}_4)_2$ and subtracting this from the magnesium pyrophosphate as estimated in each case, a slight excess of pyrophosphate is always present, which is naturally to be expected, seeing that the proteid itself furnishes a small amount of phosphorus which will be estimated together with that in combination with calcium.



Curve A.

As no other combination of the phosphoric acid and calcium oxide than that of calcium triphosphate will agree with the estimated quantities of these substances, and further as they decrease in the same relative proportion, we infer that the whole of the calcium in casein as it exists in milk, is combined in the form of calcium triphosphate and is gradually eliminated with increasing acidity in this form, and this form only, or if not actually present in this form, in

such a state that on separation and incineration of the proteid, this substance results.

As experiments in which bacteria are the source of the acid are subject to complications of an unknown character, a few trials were made in order to determine whether the same results could be obtained by the addition of lactic acid itself. These having shown that the action was, as far as could be seen, identical, the first experiment was repeated as follows:—

Experiment II.

2000 c.c. of milk obtained as before, were mixed with 50 c.c. methylated ether, well shaken and cooled. Trial had already shown that such milk would keep at least 7 days at 65°—66° F. without any increase in acidity or the appearance of bacterial activity.

It was also determined, as a preliminary, that the action of the lactic acid is practically instantaneous, and that no further effect is produced by lapse of time.

A mixture of milk and lactic acid solution was made, one part being allowed to stand 10 minutes and the other 70 minutes before placing on the porous plate.

	Casein	Percentage of CaO
After 10 minutes	·4617	2·64
„ 70 „	·4721	2·65

The effect therefore of the acid is practically instantaneous.

An approximately N solution of lactic acid was then added in varying amounts to 100 c.c. quantities of the etherised milk, and as before the casein separated, and the calcium and phosphoric acid estimated.

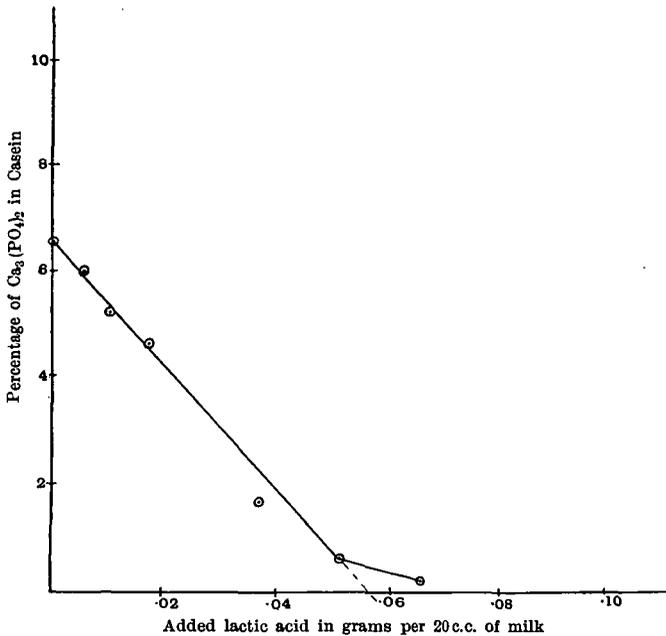
The results were as follows:—

Lactic acid in grams per 20 c.c.	Casein obtained in grams (20 c.c.)	Calcium		Mg ₂ P ₂ O ₇			Percentage of Ca ₃ (PO ₄) ₂ in casein
		CaO obtained	Calc. as Ca ₃ (PO ₄) ₂	Found	Calc. on Ca ₃ (PO ₄) ₂	Excess	
—	·534	·019	·035	·0275	·0253	·0022	6·60
·0056	·499	·017	·031	·0253	·0221	·0032	6·05
·0102	·554	·015	·029	·0241	·0208	·0033	5·20
·0170	·543	·013	·025	·0217	·0178	·0039	4·60
·0372	·525	·005	·009	·0103	·0065	·0038	1·70
·0514	·434	·001	·003	·006	·0020	·0040	0·60
·0646	·436	·0004	·001	trace	—	—	0·20

These results are plotted as curve B.

In this experiment the approximation to a straight line curve is very close.

Other experiments on the same lines gave identical results.



Curve B.

The experiments of Slyke and Hart might seem to throw a certain amount of doubt on these results, in view of the fact that they record the formation of a soluble casein lactate. If this substance be found in milk it is to be inferred that in such experiments as above recorded, this soluble lactate will pass into the porous plate, and consequently the casein obtained by this method will consist, not of the total casein of the milk, but of unchanged casein and insoluble lactate. As, however, the experiments to be recorded later throw considerable doubt on the formation of such a soluble lactate in milk under the conditions of these experiments, we are at liberty to conclude that the whole of the casein of the milk is retained by the porous plate, and that the direct proportionality between the percentage of $\text{Ca}_3(\text{PO}_4)_2$ in the casein, and the amount of lactic acid present in the milk holds good.

It is to be remarked that a much higher percentage of calcium salts in the casein has been obtained, than has hitherto been recorded by other observers; for instance, Söldner gives 2.36% CaO in the casein

of cow's milk, and Slyke and Hart confirm this. Lehmann, using the same method as in our experiments, gives 1.45—1.75%.

Using milk immediately it had been obtained from the cow, we have found the following results:—

- (I) 6.17% $\text{Ca}_3(\text{PO}_4)_2$ = 3.35% CaO.
 (II) 6.34% $\text{Ca}_3(\text{PO}_4)_2$ = 3.44% CaO.
 (III) 6.77% $\text{Ca}_3(\text{PO}_4)_2$ = 3.66% CaO.
 (IV) 6.60% $\text{Ca}_3(\text{PO}_4)_2$ = 3.58% CaO.

It does not seem at all likely that the compound of casein and calcium triphosphate is of a definite character, from the fact that the calcium salt is so easily eliminated by the acid, a process which seems to resemble a simple solution rather than a chemical reaction, and also, that the elimination of the triphosphate must take place equally throughout the mass of the casein, for otherwise it might be expected that the precipitation of the proteid would be similarly progressive instead of sudden, bearing in mind the fact that the acid coagulation does not take place until the elimination of the calcium triphosphate is practically complete.

We cannot confirm the statement of Slyke and Hart that "when acid is added to milk it unites with the calcium combined with the casein," or "that on the further addition of acid the casein combines with it to form a casein salt." The lactic acid only dissolves out calcium triphosphate from the casein, and as will be seen later the combination with lactic acid takes place concurrently with this.

As when milk is boiled a precipitation of calcium salts, especially phosphate, is supposed to occur; it seemed interesting to investigate the effect of boiling on the amount of calcium triphosphate combined with the casein.

Quantities of the same milk were used, one part being placed on the porous plate direct, and the other heated for half an hour in live steam, then rotated for a considerable time to deposit any precipitated calcium salts and the milk then placed on the porous plate. From the casein obtained in this second case $\frac{1}{3}$ th was deducted to allow for the albumin precipitated by the heat, and of course retained by the plate.

The following are the results:—

	Casein	Percentage of CaO
Unheated milk	.819	2.72
Heated milk	.786 (corrected for albumin)	3.34

There appears therefore to be a rise in the percentage of calcium salts in the casein of heated milk.

In order to eliminate any effect due to rotation and time, the experiment was repeated. The effect (if any) of rotation on the milk was first investigated:

	Casein	Percentage of CaO
Milk standing 3 hours	·8896	2·75
Same milk rotated 3 hours	·8719	2·73

Rotation therefore has no effect on the calcium salts of the casein.

A second experiment with heated milk gave the following results:—

	Casein	Percentage of CaO
Unheated milk	·777	3·01
Heated milk	·819	3·24

It seems certain therefore that there is no elimination of calcium salts from the casein when milk is heated to the boiling point of water.

The distribution of lactic acid during "souring."

In order to determine the distribution of lactic acid when either progressively produced in, or added to milk, it is necessary to again separate the casein from the other constituents of the milk after addition of varying quantities of lactic acid, and to determine what proportion of the acid is in combination with the casein, and what proportion remains in the residue after separation of the casein.

The method employed in the former part of this investigation was clearly inapplicable. Resort was therefore had to precipitation of the casein by alcohol. Attempts to filter the coagulum produced, and to wash it with dilute alcohol, completely failed, and it became necessary to separate the precipitated casein by rotation of the liquid, removing the clear menstruum, shaking the coagulum with dilute alcohol, rotating again, and so on till no more acid was removed. The following method was finally adopted:—

40 c.c. of absolute alcohol are placed in a 100 c.c. rotation tube and 20 c.c. of the milk run in from a pipette, the tube closed, well shaken, and rotated for about 10 minutes, the clear liquid is blown off with a small wash-bottle arrangement, the coagulum mixed with 20 c.c. of 66% alcohol, shaken, rotated, and the liquid blown off as before. This washing is once repeated. The acidity of the washings after the addition of 50 c.c. water, is then found by titration with N/10 NaOH to phenolphthalein, and the casein is washed out with water into a flask and similarly titrated.

As a number of obvious objections can be raised to this method, it was necessary to closely investigate these, before using the method for the end in view.

(I) Is albumin precipitated by the alcohol?

A very careful enquiry into this showed that little, if any, albumin is precipitated with the casein, so that it may be taken that casein alone is obtained in the alcohol precipitate, the albumin appearing in the washings.

(II) As the casein lactate is undoubtedly susceptible to the presence of water, may not lactic acid be removed from the casein in quantities depending on the number of washings?

To determine this, two parallel experiments were made as above, and in one case two washings were employed and in the other three washings, the acidities of the casein and of the washings being determined as c.c. of N/10 NaOH.

	Casein	Washings
Two washings	4.0 c.c.	4.0 c.c.
Three „	4.0 „	3.9 „

It is therefore evident that the acid can be removed in a definite manner by two washings, and is not dependent on the number of washings employed.

(III) Is the proportion of acid combined with the casein and remaining in the washings dependent on the strength of alcohol employed to precipitate and wash the casein?

To determine this three quantities of 20 c.c. of the same milk were precipitated by adding to 40 c.c. absolute alcohol, but after the first rotation and separation of the clear liquid, the washings of the casein were made with, (A) absolute alcohol, (B) 66% alcohol, and (C) 50% alcohol. The caseins and washings were then titrated in the ordinary way, the alcohol strength in each of the washings being adjusted to equal volumes and the same percentage (50%) of alcohol (see *infra* IV).

The results were as follows:—

Strength of alcohol	Casein	Washings
Absolute alcohol	5 c.c.	3.8 c.c.
66% „	4.7 „	4.0 „
50% „	4.45 „	4.5 „

It is evident that as the amount of water used in the washing increases, more lactic acid is split off from the casein.

(IV) Has the presence of alcohol any effect on the titration? Unfortunately alcohol has a very serious effect, as will be seen from the following experiment: milk was mixed with varying quantities of alcohol and the acidity estimated in each case:—

10 c.c. milk and 20 c.c. water	1.9 c.c. $\frac{N}{10}$ NaOH
10 " " { 10 " " }	2.25 " "
10 " " { 10 " alcohol }	2.9 " "
10 " " { 20 " " }	2.9 " "

(V) In view of the fact that Slyke and Hart state that casein mono-lactate is soluble in 50% alcohol, it would seem that alcohol is not a suitable agent for separating the casein. The authors have not been able in any way to detect the presence of this mono-lactate, or of any lactate soluble in the strength of alcohol used; and its presence seems highly problematical for these reasons:—

(1) When the washings are neutralised, no precipitation takes place, as would be the case if a lactate of casein were present. The only proteid present in the washings in appreciable quantity is the albumin of the milk.

(2) The experiment given under heading (IV) would have given very different results. These results simply show a varying distribution of acid in a perfectly regular gradation. There is every reason therefore to suppose that the whole of the casein is precipitated by the alcohol treatment, and that the method of procedure described above is perfectly adequate for the purpose, certain careful conditions being always observed. These conditions are obvious from the above discussion. (I) Exactly the same procedure, especially as regards volume of water and alcohol present, must be maintained throughout the experiment, and (II) as strong alcohol as possible must be used for washing.

To attain these conditions the milk was titrated alone for total acidity; the caseins were washed out with water after the third rotation, and the volumes of water and alcohol in the washings were always adjusted to be the same, both in total volume and in relative proportion of alcohol to water, this being made 1:1. Absolute alcohol was used in one experiment for washings, but 66% alcohol being more convenient in practice was used in all others.

The chief effect of the presence of the alcohol in the titrations is seen from the fact that the total acidity is not always equal to the sum of the acidities of the washings and the casein.

EXPERIMENT I.

Absolute alcohol used for washings.

Milk, mixed with ether immediately after milking, and used at once: varying quantities of an approximate N solution of lactic acid added with careful-mixing and the casein separated as described above. Results are given in c.c. of N/10 NaOH.

Total acidity			Increase in total acidity		
Milk	Casein	Washings	Milk	Casein	Washings
2.90	1.45	1.60	—	—	—
3.68	2.16	1.74	0.78	0.71	0.14
4.81	2.78	2.12	1.91	1.33	0.52
5.59	3.61	2.29	2.69	2.16	0.69
8.92	4.95	3.97	6.02	3.50	2.37
10.97	5.21	5.61	8.07	3.76	4.01
13.07	5.86	7.41	10.17	4.41	5.81

EXPERIMENT II.

66 % alcohol used for washings.

All conditions of experiment as in the above.

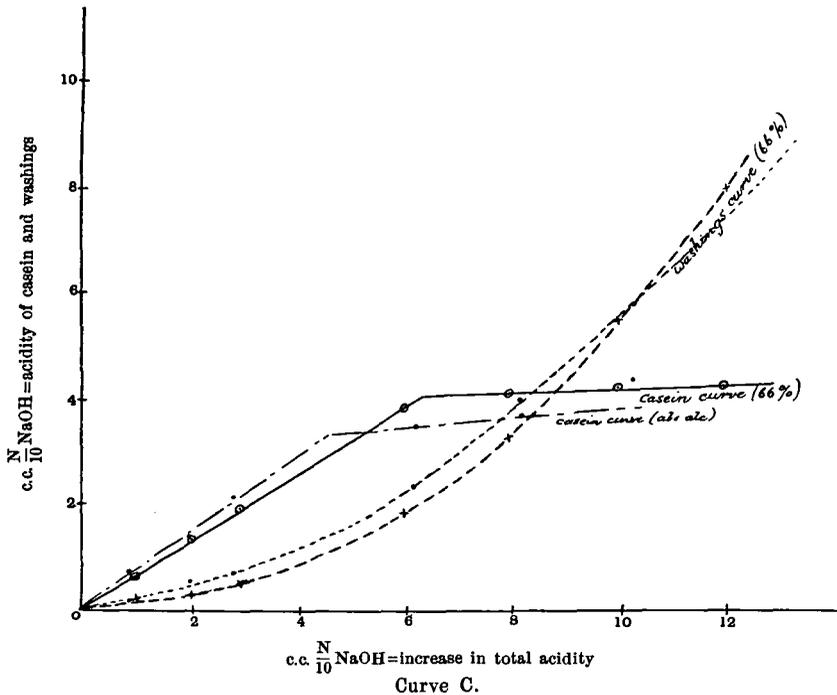
Total acidity			Increase in total acidity		
Milk	Casein	Washings	Milk	Casein	Washings
4.71	1.70	2.30	—	—	—
5.67	2.31	2.52	0.96	0.61	0.22
6.66	3.03	2.57	1.95	1.33	0.27
7.51	3.65	2.84	2.80	1.95	0.54
10.61	5.56	4.12	5.90	3.86	1.82
12.58	5.87	5.67	7.87	4.17	3.37
14.54	5.98	7.82	9.83	4.28	5.52
16.53	6.04	10.44	11.82	4.34	8.14

EXPERIMENT III.

66 % alcohol used for washings.

Total acidity			Increase in total acidity		
Milk	Casein	Washings	Milk	Casein	Washings
4.39	1.39	2.79	—	—	—
5.23	2.25	2.88	0.84	0.86	0.09
5.94	2.82	3.27	1.55	1.43	0.48
6.98	3.44	3.59	2.59	2.05	0.80
10.06	5.44	4.62	5.67	4.05	1.83
12.23	4.77 (?)	6.95 (?)	7.84	3.38 (?)	4.16 (?)
14.24	5.70	8.43	9.85	4.31	5.64

The values of Experiments I and II were plotted with c.c.'s of N/10 NaOH equal to rise in total acidity as abscissae, the corresponding number of c.c.'s equal to the rise in acidity of the washings and casein as ordinates. In both cases the curves obtained are closely similar, and show that up to a certain point the rise in acidity of the casein is directly proportional to the rise in total acidity: a point is then reached when an abrupt change takes place, and the casein appears to take up but little more acid. This maximum acidity, as it may be termed, is closely connected with both the precipitation of the casein and the disappearance of the calcium triphosphate from the casein, both taking place almost immediately after this point is reached.



The increase of the acidity in the washings follows a well marked curve, but no inference can be made from this, as the acidity is undoubtedly influenced by the alcohol present during the titration.

As the amount of lactic acid combined with the casein is directly proportional to the amount of lactic acid present, and the amount of calcium triphosphate combined with the casein is also directly

proportional to the amount of lactic acid present, it follows that at any moment the lactic acid combined with the casein is directly proportional to the calcium triphosphate present in the casein. Whether the lactic acid takes the place of the displaced phosphate it is impossible to say.

In conclusion, it may be inferred from these experiments that the compounds of calcium salts and of lactic acid with casein as they occur in milk, do not possess the definite compositions of those that have been formed with casein after its separation from milk, as described by Söldner for lime salts, and Slyke and Hart for lactates of casein, but that the proportions of calcium triphosphate and lactic acid in combination with the casein are at any moment before the milk coagulates the result of a sort of equilibrium between the casein and the total lactic acid present, and that at the moment of precipitation of the casein, the calcium triphosphate has been practically completely eliminated, and the combination with lactic acid has reached a maximum.