XVIII. THE REACTION AND CALCIUM CONTENT OF MILK AS FACTORS IN THE COAGULATION PROCESS.

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The two main factors which govern the coagulant action of rennin on caseinogen are the reaction and the calcium content of the medium. Within recent years the influence of variations in the reaction upon this process have been studied especially by van Dam [1908], Allemann [1912] and Michaelis and Mendelssohn [1913], all of whom have employed the electrometric method for the estimation of the actual acidity or hydrogen ion concentration, $[H']^1$. Van Dam, who adopted a rather ingenious method of determining the coagulation time in specimens of milk which were only slightly diluted, came to the conclusion that the rate of coagulation was directly proportional to the $[H']$ for the comparatively limited range which he investigated. After studying the action of $CaCl_2$ upon the coagulation period, he came to the conclusion that in so far as this salt raised the $[H']$ of the mixture, it also hastened the formation of soluble casein from caseinogen. The specific action of the calcium was dependent not upon the calcium salts in solution in the milk, but upon the amount of calcium in colloidal combination. Allemann estimated the $[H']$ of milk after the addition of equivalent amounts of hydrochloric and acetic acids and found that the $[H']$ was the same for equivalent concentrations of the two acids although the degree of dissociation in the two cases was so different. This is evidently due to the fact that the milk acts as a reaction regulator from its alkaline phosphate content and the regulating power is lost when all the secondary is transformed into primary phosphate, acid precipitation of the caseinogen occurring at this point. The author also pointed out that prior to the precipitation of caseinogen by acid—stated quantitatively, with lower $[H']$ than $1.25 \cdot 10^{-5} N$

¹ $[H']$ =Hydrogen ion concentration.

$p_{H'}$ =The negative exponent of the same when the normality is expressed as a power of 10.

(from $0.64 \cdot 10^{-5}$ to $1.25 \cdot 10^{-5}$)—a zone of opalescence is met with and it is within this zone that the coagulant action of rennin takes place.

Michaelis and Mendelssohn investigated the subject in rather a different way from the others. They determined the optimum zones of $[H']$ for the coagulant action of rennin and the precipitation of caseinogen by acid. They found the optimum zone for rennin action in the presence of soluble calcium salts to lie between $0.4 \cdot 10^{-6}$ and $1 \cdot 10^{-6}$ N. It was not possible to narrow this zone down further, but in their opinion $1 \cdot 10^{-7}$ and $3 \cdot 10^{-6}$ lay outside the region of optimal activity. The optimum zone for acid precipitation of caseinogen in pure solution or in milk was found to lie about $2.5 \cdot 10^{-5}$ N, while in $m/40$ $CaCl_2$ solution optimum precipitation was obtained with higher $[H']$, namely about $3 \cdot 10^{-4}$ N. The authors therefore state that there is a region between 10^{-6} and 10^{-5} in which neither optimal rennin action nor optimal acid precipitation takes place. They therefore do not regard the zone of rennin action as simply an extension of the acid precipitation zone. Making use of acetate regulator mixtures to eliminate the rise in $[H']$ produced by the addition of $CaCl_2$, they came to the conclusion that the Ca^{++} action in rennin coagulation is not simply an expression of the $[H']$ rise. In studying the parts played by variations in $[H']$ and in the soluble calcium content on the coagulation process, it is essential to devise experimental conditions which render it possible to effect a change in the one without at the same time altering the other. It is necessary therefore to avoid the $[H']$ variations produced by the addition of calcium chloride to milk, and hence the use of regulator mixtures is required. In the investigation however of the rennin coagulation process, it is also necessary to determine whether during the course of enzyme action there is any change in $[H']$ such as occurs, for example, in the case of peptic digestion [Sørensen, 1908, 1912; Christiansen, 1912], in which at the outset the combining power of the digestion products for bases is increased and subsequently decreased. In the case of such an investigation, as also in the study of the effects produced on $[H']$ by substances which accelerate or retard coagulation, it is necessary to avoid the use of reaction regulators.

At the outset therefore the $[H']$ of fresh milk was determined before and during the course of digestion and without the addition of a reaction regulator mixture.

The method employed for the determination of the $[H']$ was the electrometric one as described by Sørensen and others. I have given certain details as to my own method of procedure in another paper [1914]. It is quite

unnecessary to give a list of all the determinations of the $[H']$ of fresh milk, but as the values depend partly upon the method employed for saturating the milk specimen in the electrode with hydrogen, it is necessary to refer briefly to the effect of bubbling the gas through the milk. When one employs the "stationary" hydrogen electrode (as used by Hasselbalch, Michaelis and others) the values are slightly higher than when the gas is bubbled through the milk, even when the precaution is taken of passing the gas through a series of wash bottles containing milk before it enters the electrode. The fall in $[H']$ is evidently due to the removal of CO_2 , but this can be greatly diminished by passing the gas through a number of milk tubes before it enters the electrode.

THE $[H']$ OF FRESH UNDILUTED MILK.

From a very large number of specimens examined, the range of $[H']$ in fresh milk was found to be from $0.158 \cdot 10^{-6} N$ (p_H . 6.80) to $0.310 \cdot 10^{-6} N$ (p_H . 6.50) as determined by the stationary hydrogen electrode method. When hydrogen is bubbled through the milk in the electrode, with the precautions taken which have just been mentioned, the $[H']$ range is from $0.140 \cdot 10^{-6}$ to $0.158 \cdot 10^{-6} N$. The great majority of the fresh milk specimens have a $[H']$ from $0.18 \cdot 10^{-6}$ (p_H . 6.75) to $0.25 \cdot 10^{-6}$ (p_H . 6.60) as determined by the stationary electrode method.

THE $[H']$ DURING THE COAGULATION PROCESS.

This is most easily estimated either in milk which has been diluted or in milk which has been first rendered non-coagulable by the addition of alkaline oxalate. The coagulation process can be readily followed in the oxalated milk on the addition of $CaCl_2$. The addition of potassium oxalate to milk causes a fall in $[H']$ owing to the hydrolytic dissociation of the salt.

Thus 1 cc. $K_2C_2O_4 \cdot 4H_2O$ (3 %) added to 10 cc. milk—i.e. about $m/80$ oxalate concentration—gives rise to a fall in $[H']$ from $0.19 \cdot 10^{-6} N$ to $0.06 \cdot 10^{-6}$ (p_H . 6.70 to 7.22). That is to say the addition of a sufficient quantity of potassium oxalate to render milk non-coagulable lowers the $[H']$ to such an extent that the change in reaction alone, apart altogether from the calcium precipitation, would place the milk outside the range of Michaelis' coagulation zone. One must therefore bear in mind that the depression of coagulability produced by the addition of oxalate must be in part due to the lowered $[H']$.

If rennin be added to an "oxalate" milk and the mixture kept in a water-bath at 38° the $[H']$ is not found to change during the period of digestion.

An example may be given of such a milk specimen which remained unclotted throughout the experiment.

TABLE I.

		$[H'] \times 10^6 \text{ N.}$	p_H
Fresh milk		0.477	6.70
Same containing $m/80 \text{ K}_2\text{C}_2\text{O}_4$, after 10 min. digestion		$[H'] \times 10^7$	p_H
"	"	0.707	7.15
"	"	0.724	7.14
"	"	0.724	7.14
"	"	0.741	7.13

When CaCl_2 is added to an "oxalate" milk the $[H']$ rises, but the milk remains uncoagulable until the $[H']$ reaches a higher level than p_H . 6.86. The following examples of different specimens of milk are given in order to show the effects produced by oxalate and CaCl_2 on the $[H']$ of milk subjected throughout to the action of rennin at 38° .

The E.M.F. of the fluid is given at different periods and the calculated p_H in order that any change in the $[H']$ throughout the course of the experiment may be observed.

TABLE II.

- (1) Milk 10 cc., $\text{K}_2\text{C}_2\text{O}_4$ (3 %) 1 cc., Rennin (0.25 %) 1 cc. at 38° .

Period of digestion in minutes	E.M.F.	p_H	Remarks
5	0.7552	7.23	fluid
10	0.7546	7.22	"
15	0.7544	7.22	"
	added 1 cc. CaCl_2 (1 %)		
	0.6962	6.21	curdling
6	0.6948	6.18	clotted
10	0.6947	6.18	"
20	0.6949	6.18	"

- (2) Another specimen of milk containing the same amount of oxalate and rennin at 38° .

Period of digestion in minutes	E.M.F.	p_H	Remarks
1	0.7508	7.15	fluid
5	0.7500	7.14	"
10	0.7496	7.13	"
15	0.7494	7.13	"
	added 0.75 cc. CaCl_2 (1 %)		
1	0.7103	6.46	fluid
2	0.7110	6.47	"
5	0.7127	6.49	curdling
10	0.7135	6.50	clotted
15	0.7135	6.50	"
30	0.7135	6.50	"

(3) Another specimen of oxalate milk and rennin at 38°.

Period of digestion in minutes	E.M.F.	p_H	Remarks
1	0.7420	7.00	fluid
5	0.7400	6.97	"
10	0.7391	6.97	"
added 0.3 cc. CaCl_2			
1	0.7343	6.87	fluid
3	0.7340	6.86	"
6	0.7335	6.86	"
10	0.7338	6.86	"
again added 0.3 cc. CaCl_2			
10	0.7166	6.56	clotted
20	0.7141	6.52	"
25	0.7140	6.52	"

Other specimens gave similar results. It is evident in the first place that the oxalate lowers the $[H^+]$ while the CaCl_2 raises it and that coagulation occurs when the $[H^+]$ reaches the level of that possessed by a normal coagulable milk.

During the period, however, when the ferment is acting on milk either containing an excess of oxalate or of CaCl_2 there is no definite change of reaction.

It is certain that in specimen (3) after the addition of 0.3 cc. CaCl_2 , when the $[H^+]$ has been raised approximately to the lowest level of the optimum coagulation zone, viz. p_H 6.86–6.87, ferment action must have been proceeding during the ten minutes in which the potential estimations were made and yet there was no appreciable alteration in the E.M.F.

Therefore during the early period of the rennin action prior to the separation of a clot there is no indication of accompanying $[H^+]$ alterations such as are evident in pepsin action. Again, in the actual passage from the fluid to the clotted state there is also no definite indication of an alteration in the $[H^+]$ such as might occur from the fixation of calcium by the soluble casein giving rise to an increase in the $[H^+]$ of the fluid.

EFFECT OF VARIATIONS IN THE CALCIUM CONTENT AND THE REACTION OF MILK ON COAGULABILITY.

As alkaline oxalate in addition to its effect upon the soluble calcium content of the milk also lowers the $[H^+]$, it was necessary either to counteract this effect upon the $[H^+]$ as, for example, by the addition of oxalic acid, or to adopt some other method of diminishing the soluble calcium content. It has been for a long time well known that milk which has been kept at a

temperature over 80°C . is gradually rendered less and less coagulable by rennin. Otto de Vries and Boekhout [1901] stated that the calcium content of such poorly coagulating milk is not lower than that of normal milk, although they granted that the addition of CaCl_2 rendered the milk coagulable. This was not due, however, in their opinion to an increased soluble calcium content of the milk, and they therefore concluded that the rise in acidity was the governing factor. Van Dam estimated the total calcium content of milk of normal coagulability and that of milk which either did not coagulate at all or only after a longer period than normal, and he found that the former contained about 0.154–0.174 % CaO , while the latter contained 0.135–0.150 % CaO . Klein and Kirsten [1901] state that heated milk on addition of CaCl_2 clots more readily with rennin than unheated milk.

Milk rendered uncoagulable by heating.

The milk was kept at a temperature slightly below boiling point for one hour, by placing it in the inner vessel of a double saucepan, the outer one containing boiling water. Such milk, which shows greatly reduced coagulability, was made use of in the experiments which follow. After the milk had been heated for one hour it was cooled, made up to the original volume and filtered after again being warmed. The first point to determine was the $[\text{H}']$ of the heated milk as compared with that of the same milk prior to heating. So far as I know, the $[\text{H}']$ of heated milk has not hitherto been determined.

The following table gives the $[\text{H}']$ of different specimens of milk before and after heating.

TABLE III.

$[\text{H}']$ of Milk before and after heating.

	Before			After		
	E.M.F.	$[\text{H}'] \times 10^7 \text{ N}$	p_{H}	E.M.F.	$[\text{H}'] \times 10^7 \text{ N}$	p_{H}
(1)	0.7174	2.63	6.58	0.7126	3.25	6.49
(2)	0.7254	1.90	6.72	0.7162	2.81	6.55
(3)	0.7274	1.77	6.75	0.7174	2.63	6.58
(4)	0.7273	1.77	6.75	0.7190	2.51	6.60
(5)	0.7304	1.58	6.80	0.7190	2.51	6.60
(6)	0.7310	1.55	6.81	0.7250	1.95	6.71

In every case the heated milk shows a higher $[\text{H}']$ than the same milk before heating.

The $[\text{H}']$ of heated milk specimens remains also much more constant than that of fresh milk. Heating therefore produces exactly the opposite effect on the $[\text{H}']$ from that produced by the addition of potassium oxalate.

In order to understand the cause of this rise in acidity attention requires to be directed to the calcium content of the milk. The percentage of CaO in different specimens of milk before and after heating is shown in the following table.

TABLE IV.

Percentage of CaO in Milk before and after heating.

	Before	After
	CaO %	CaO %
(1)	0.1738	0.1345
(2)	0.1738	0.1524
(3)	0.1630	0.1416
(4)	0.1577	0.1437
(5)	0.1542	0.1447

These results show an average of 0.1645 % CaO in unheated and 0.1434 % CaO in heated milk. The rise in $[H^+]$ which occurs on heating milk is evidently due therefore to the separation of calcium phosphate in more basic form than it previously existed in the milk, the transformation probably being of the following nature:

The removal of carbonic acid from the milk by heating lowers the solubility of the calcium phosphates.

According to Conradi [1901] the diminished coagulability of heated milk with rennin goes hand in hand with an increase in coagulability on heating with calcium chloride. This is undoubtedly the case, for if one takes two 5 cc. specimens of fresh and heated milk, one finds that after adding 0.5 to 0.6 cc. $CaCl_2$ (1 %) to each, the heated specimen coagulates on boiling, while the fresh one does not coagulate until 0.8–0.9 cc. is added.

Action of rennin on heated milk.

As above stated, the coagulant action of the ferment on heated milk is very much diminished and that this decrease in activity is not due to the alteration in reaction is evident from the fact that the increase in $[H^+]$ produced by heating milk ought of itself to increase the coagulability. That the diminished coagulability is due to the lowered calcium content of the milk is evident from the fact that on raising the percentage of calcium in heated milk to the same level as in the milk before heating, the milk coagulates as readily as the fresh milk. As already stated, however, the addition of calcium chloride to the milk raises the $[H^+]$ unless the milk be diluted with some reaction regulator such as the acetate mixture employed by Michaelis.

When heated milk is diluted with sodium acetate ($m/4$) the $[H']$ is lowered owing to the hydrolytic dissociation of the alkaline acetate. On adding $CaCl_2$ to this mixture the $[H']$ at first rises owing to the lower dissociation constant of the calcium acetate; with further additions of $CaCl_2$, however, the $[H']$ only alters slightly. Thus, for example, the $p_{H'}$ values of certain acetate and acetate- $CaCl_2$ milk mixtures are as follows:

	$p_{H'}$
(1) Heated milk (not diluted)	6.56
(2) Heated in $m/4$ sodium acetate (milk 1 in 4 dilution) ..	6.97
(3) Same as (2) but containing $m/200$ $CaCl_2$	6.81
(4) Same as (2) but containing $m/40$ $CaCl_2$	6.73

In the subsequent experiments N sodium acetate was added to the milk.

Addition of $CaCl_2$ to heated milk.

The following series illustrates the action of $CaCl_2$ on the coagulability of heated milk diluted as follows,

Milk	10 cc.
Sodium acetate N ..	2 cc.
Water to	40 cc.

To this mixture various amounts of $CaCl_2$ were added and the $[H']$ and coagulation time noted.

TABLE V.

1 % $CaCl_2$	E.M.F.	$[H'] \times 10^6$ N	$p_{H'}$	Coagulation time in minutes
2 cc.	0.7307	0.15	6.81	6
4	0.7257	0.19	6.72	5
6	0.7207	0.23	6.63	3
8	0.7198	0.24	6.62	2.5
10	0.7195	0.24	6.61	2

Although it is evident that the addition of the $CaCl_2$ solution has produced a rise in $[H']$, this is inappreciable after the initial increase. The shortening in the coagulation period to one-third of the original has taken place with an 80 % increase in the $[H']$. In many cases the rise in $[H']$ produced by the addition of $CaCl_2$ is even less marked, as in the specimens previously referred to where a $m/200$ $CaCl_2$ in milk-sodium acetate had a $p_{H'}$ of 6.81, while a $m/40$ $CaCl_2$ showed a rise in $[H']$ only to $p_{H'}$ 6.73. Yet in this case the shortening in the coagulation time was approximately the same as in the last mentioned series.

The factor concerned in the shortening of the coagulation period in this series has evidently not been the slight rise in $[H']$ because such a rise

otherwise produced than by addition of CaCl_2 would scarcely have shortened the coagulation period.

If we now determine the rise in $[\text{H}']$ which is required in order to render heated milk coagulable without the further addition of CaCl_2 we obtain results which make it evident that the calcium chloride does not produce its effect simply from the rise in $[\text{H}']$ which it produces.

The coagulation zone of heated milk diluted with sodium acetate and acetic acid.

Heated milk may be again rendered coagulable by rennin not only by an increase in the soluble calcium content without an appreciable rise in the $[\text{H}']$, but also by a rise in the $[\text{H}']$ without an increase in the soluble calcium content except in so far as the increasing acidity may produce a slight effect.

When heated milk is diluted with sodium acetate, the $[\text{H}']$ falls. This fall is prevented by the addition of acetic acid. The following table gives the $[\text{H}']$ and observations on the coagulation of milk-acetate-acetic acid mixtures of different $[\text{H}']$.

TABLE VI.

Milk 10 cc., Sodium acetate (N) 10 cc., Rennin (0.25 %) 2 cc.,

Water up to 40 cc.

	E.M.F.	$[\text{H}'] \times 10^7 \text{ N}$	p_{H}	
Without acid	0.7416	1	7.00	no clot
0.6 cc. acetic acid (N)	0.6920	7.24	6.14	"
1.2 " "	0.6717	16.69	5.78	clotted
1.5 " "	0.6661	20.42	5.69	"
2 " "	0.6577	28.84	5.54	"

From a comparison of Tables V and VI it is evident that coagulation takes place at a much lower $[\text{H}']$ when CaCl_2 is added to the heated milk than when acetic acid is added, namely in the case of the former with $[\text{H}']$ from 0.15 to $0.24 \times 10^{-6} \text{ N}$, in the latter on the other hand only when the $[\text{H}']$ is higher than 0.724×10^{-6} . There are therefore two variables to be considered in the case of the rennin action on caseinogen, namely the $[\text{H}']$ of the fluid and its calcium content.

Before discussing the relationship of these variables to one another, it is advisable to refer briefly to the acid precipitation zone of caseinogen in the absence of ferment, in order to see whether the ferment zone is not after all simply an extension of the acid precipitation zone.

If heated milk be diluted with sodium acetate-acetic acid mixture as in the preceding series and the $[\text{H}']$ determined at the point when caseinogen

is precipitated from the milk, one obtains results which show that the precipitation zone lies on the acid side of the coagulation zone. The milk-acetate-acetic acid mixtures were kept at 38°.

Milk	10 cc.
Sodium acetate (N)	10 cc.
Acetic acid N from	1 cc. upwards
Water to	40 cc. at 38°

Until the $[H']$ reached $0.95 \cdot 10^{-5}$ there was no sign of a precipitate. The figures below give the acidities at which precipitation takes place.

E.M.F.	$[H'] \times 10^5 N$	p_H	Remarks
0.6377	0.630	5.20	No ppt. in 1 hour
0.6275	0.955	5.02	ppt. in 6 min.
0.6236	1.122	4.95	ppt. in 4 min.

On repeating this experiment but with 2 cc. $CaCl_2$ (1 %) added the acid precipitation of caseinogen took place at practically the same $[H']$ as in the absence of the $CaCl_2$. A precipitate was obtained in 6 min. with a $[H']$ of $1.04 \cdot 10^{-5}$ (p_H 4.98).

Michaelis and his fellow workers give, as the most favourable $[H']$ for the precipitation of caseinogen in the absence of $CaCl_2$, $2.5 \cdot 10^{-5} N$ (p_H 4.60), which is a more acid reaction than the one I have just given. They employed, however, caseinogen solutions, and the conditions as regards temperature of mixture and sodium acetate concentration were different in the two cases.

Michaelis and Mendelssohn found that in the presence of $CaCl_2$ (in greater concentrations than those used by me) the acid precipitation zone of caseinogen is shifted still more to the acid side.

Although there is no doubt that the acid precipitation zone of heated milk lies some distance on the acid side of the coagulation zone, it is certain that with still further lowering of the calcium content of the milk, the zone of rennin action would approach more nearly that of acid precipitation.

The $[H']$ of milk therefore requires to be highest for the precipitation of caseinogen by acid from solutions of its salts.

The separation of caseinogen from its solutions by the action of rennin takes place at lower $[H']$, the exact point at which coagulation takes place being dependent upon the soluble calcium content. It is highest when the calcium content is low and gradually falls as the latter is raised.

What then are the exact relations of these two variables to one another?

The value of the one variable $[H']$, which corresponds to a definite value of the other variable (calcium) for a fixed coagulation time, has been determined in a series of milk-acetate-acetic acid mixtures. The coagulation time

which was taken was 5 min., and the calcium content and $[H']$ of the various milk-acetate mixtures determined which showed coagulation by rennin within that time.

Beginning with a milk-acetate mixture made up as follows:

Heated milk	10 cc.
Sodium acetate (N)	10 cc.
Rennin (0.25 %)	2 cc.
Water to	40 cc.

the $[H']$ and CaO percentage of the diluted mixture were determined and found to be p_H 7.00 and 0.035 % CaO respectively. This mixture did not clot.

To a series of milk-acetate mixtures of the above composition acetic acid (normal) was added (starting with 0.1 cc.) until one was obtained which clotted in five minutes.

It was found that a milk-acetate-acetic mixture of $[H']$ $0.112 \cdot 10^{-5}$ clotted in ten minutes; one of $0.157 \cdot 10^{-5}$ in seven minutes; and one of $0.177 \cdot 10^{-5}$ in five minutes.

Thus a milk specimen of 0.035 % CaO required to have the $[H']$ raised to $0.177 \cdot 10^{-5}$ to obtain coagulation in five minutes.

The calcium content of the diluted milk was then gradually increased by the addition of small quantities of $CaCl_2$ (1 %). After each addition of $CaCl_2$ a series of milk-acetate-acetic acid mixtures of the same calcium content but of different $[H']$ was prepared, and the $[H']$ of the one which showed coagulation in five minutes was accurately determined.

The following table gives the results:

	CaO %	$[H'] \times 10^6 N$	p_H
(1)	0.035	1.778	5.75
(2)	0.036	1.660	5.78
(3)	0.038	0.871	6.06
(4)	0.042	0.309	6.51
(5)	0.050	0.230	6.64
(6)	0.060	0.186	6.73
(7)	0.073	0.154	6.81
(8)	0.085	0.135	6.87
(9)	0.122	0.126	6.90
(10)	0.135	0.107	6.97

From this table it is evident that when the calcium content is brought below 0.06 % and especially when it has fallen to 0.042 % the $[H']$ requires to be proportionately much more increased in order to enable coagulation to take place within the five-minute period. Thus in the range from 0.060 to 0.035 % CaO, the $[H']$ requires to be increased tenfold. This range in

calcium content represents the difference between fresh milk and heated milk diluted to the same extent with the acetate mixture.

The specimens of heated milk diluted with the acetate mixtures which possess hydrogen ion concentrations corresponding to those of fresh coagulable milk have a CaO percentage similar to those of fresh coagulable milk diluted to the same extent, namely specimens (4), (5) and (6). In the specimens above (6) NaOH (N/10) was added in order to obtain the lower $[H^+]$.

THE RÔLE OF THE CALCIUM SALTS IN THE FORMATION OF SOLUBLE CASEIN.

In the preceding experiments attention has been merely directed to the separation of the casein clot, and practically no reference was directly made to the earlier part in the coagulation process, namely the formation of soluble casein. The part which the calcium content of the milk plays in this earlier process is difficult to dissociate from that played by the accompanying reaction changes.

The question to decide is whether the transformation of caseinogen to soluble casein takes place at the same rate in the case of a milk specimen of lowered calcium content as in one possessing a normal amount. Various methods were employed in order to decide this question. A few examples illustrating these methods may be given.

Two diluted milk specimens of the following composition were taken:

(1) Milk (heated)	10 cc.	(2) Same but without $CaCl_2$
$CaCl_2$ (1 %)	2 cc.	
Rennin (0.25 %)	2 cc.	
Water to	40 cc.	

These specimens were kept at 38° . Clotting took place in specimen (1) in two minutes. Immediately on the appearance of the clot in this specimen, 2 cc. $CaCl_2$ (1 %) were added to the other specimen, which up to this time had shown no clot. Coagulation occurred two minutes later. On this being repeated with a smaller amount of rennin solution, coagulation occurred in specimen (1) in 3.5 minutes, and in specimen (2) 3.5 minutes after the addition of $CaCl_2$. That is to say in neither of these cases did the preliminary digestion in a diluted milk specimen without the addition of $CaCl_2$ shorten the subsequent period of coagulation after the addition of $CaCl_2$.

One must bear in mind, however, that the addition of $CaCl_2$ not only increases the calcium content of the milk but it also, especially in milk simply diluted with water, raises the $[H^+]$, and thus specimen (1) was

placed under more favourable circumstances, as regards reaction, for the activity of the rennin. The addition of water alone lowers the $[H']$ and so specimen (2) was placed under more unsatisfactory conditions for the enzyme action.

It is necessary therefore to compare two diluted milk (heated) specimens of approximately the same $[H']$, the one containing $CaCl_2$, the other without this addition as was done in the following experiment:

(1) Milk (heated)	10 cc.	(2) Same but without $CaCl_2$
Sodium acetate (N)	10 cc.	
Acetic acid (N/10)	0.5 cc.	
$CaCl_2$ (1 %)	1 cc.	
Rennin (0.25 %)	2 cc.	
Water to	40 cc.	

Both specimens had a $[H']$ of approximately $2 \cdot 10^{-7}$ N. Specimen (1) clotted in 5.75 min. Immediately on appearance of coagulation in this specimen 1 cc. $CaCl_2$ (1 %) was added to the other, which at this time showed no sign of coagulation. Coagulation occurred in this second specimen 2.75 min. later. That is to say the preliminary digestion with rennin in a milk-acetate mixture to which no $CaCl_2$ was added has shortened the period of coagulation after the addition of $CaCl_2$ by rather more than one half. Evidently therefore, although as was to be expected the transformation of caseinogen into soluble casein takes place in such a mixture when no $CaCl_2$ has been added, the rate of transformation is slower than in the presence of a small quantity of that salt, even when the $[H']$ in both cases is the same.

That the addition of $CaCl_2$ does not act simply as a precipitant of soluble casein is shown also from the following experiment.

20 cc. heated milk were taken, diluted 1 in 4 with water and kept at 38° . The mixture was divided into three portions. One of these was poured into a flask containing 2 cc. rennin and 1 cc. $CaCl_2$ (1 %), the other two portions into flasks containing 2 cc. rennin alone, and all were kept at 38° . The first specimen clotted in four minutes. One of the remaining specimens was then brought to boiling point, in order to destroy the rennin, and then cooled to 38° , while the third specimen was kept at 38° . When 1 cc. $CaCl_2$ (1 %) was added to each of these specimens after four minutes, the one in which the rennin had been destroyed did not clot, while the third specimen clotted in rather more than one minute.

In both of these specimens during the four minutes' action of rennin the caseinogen must have been acted upon by the ferment to such an extent that on the addition of 1 cc. $CaCl_2$ coagulation would take place after the

lapse of little over a minute, but this occurred only in the case where the ferment was allowed to continue its action during that extra period.

It is evident therefore that the action of the soluble calcium salt is not simply to complete a process the earlier stages of which do not require its presence.

From the beginning of the ferment action until its close the rate of transformation of caseinogen into the final product of digestion is dependent upon the calcium content and reaction of the medium.

CONCLUSIONS.

1. During the course of rennin action there is no change in the $[H']$ of milk, either in the earlier stage or in the actual separation of the clot.
2. The addition of an alkaline oxalate to milk lowers, while that of $CaCl_2$ raises, the $[H']$.
3. Fresh milk which has been subjected to a temperature slightly below boiling point for one hour shows a rise in $[H']$, and a fall in the calcium content. Such milk is only very slowly acted upon by rennin.
4. The coagulability of heated milk may be raised either by the addition of $CaCl_2$ or by raising the $[H']$. The former does not act simply by raising the $[H']$ nor the latter from its effect upon the soluble calcium content.
5. The acid precipitation zone of caseinogen lies on the acid side of the rennin zone of action, but the latter gradually approaches the former as the calcium content of the mixture is lowered, so that in all probability the latter is an extension of the former towards the neutral point.
6. Calcium chloride, apart from its effect upon $[H']$, increases the activity of the rennin ferment from the beginning of the digestion process.

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