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Rennet Hysteresis and the Calcium Phosphate of Milk

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(Received 6 July 1945)

Heated milk exhibits a phenomenon known as rennet hysteresis, i.e. its time of coagulation shows a progressive increase with increase in the interval between heating and addition of rennet. Mattick & Hallett (1929) observed the effect in milk heated to temperatures ranging from 40 to 100°, and showed that a maximal and constant renneting time was attained in about 5 hr. Powell & Palmer (1935) later showed that the phenomenon was in all probability a property of the calcium caseinate-calcium phosphate complex of milk, demonstrable in artificial complexes of this type but not in calcium caseinate alone. They further observed that rennet hysteresis occurred both in solutions of the calcium caseinate-calcium phosphate complex to which gelatin had been added, and in gelatin-protected solutions of calcium phosphate to which calcium caseinate had been added. The nature of the protecting protein thus appeared to be a matter of indifference for the phenomenon, and the two forms of phosphate protection, by caseinate and by gelatin, seemed to differ little if at all. Earlier experiments by the writer (Pyne, 1934) had shown, on the other hand, that these two systems reacted differently with formaldehyde, whence it appeared that the nature of the protection was essentially different in the two cases; protection of calcium phosphate by caseinate in milk being, it was suggested, chemical rather than physical. In view of the apparently divergent behaviour of the system the experiments of Powell & Palmer were repeated by the author, who was unable to confirm them, the type of hysteresis being found to depend on the nature of the protecting protein. An explanation of the apparent origin of these differences is given later.

In view of these findings it was decided to examine further the whole phenomenon. The great sensitivity

of the rennet coagulation to changes in the concentration of calcium ions suggested that the shift in equilibrium of the caseinate-phosphate complex postulated by Powell & Palmer might take the form of a temporary change in the concentration of these ions in milk serum as a result of heating. Heat treatment, especially if severe, is known to produce permanent changes of this kind and it seemed possible that it might also give rise to short-time changes hitherto overlooked owing to the relatively long interval generally elapsing between heating and analysis. The comparative slowness with which phosphate equilibrium is attained in milk, as exemplified by the changes in soluble calcium content which occur when milk is shaken for prolonged periods (Lampitt, Bushill & Filmer, 1937), lends support to such a suggestion.

EXPERIMENTAL

Freshly separated skim milk was used and only one heat treatment was in general employed, 80–85° for 30 min. Calcium caseinate-phosphate complexes were prepared as by Powell & Palmer (1935), colloidal calcium phosphate protected by gelatin by the method of De Toni (1921). Renneting was carried out in a thermostat at temperatures ranging from 30 to 40°, and in general without addition of calcium salts, though such addition was necessary for the artificial caseinate-phosphate-gelatin complexes. Rennet, either as the commercial liquid extract or from tablets, was used in such amounts as to give convenient renneting times (2–10 min.). Coagulation times were determined for larger samples as by Powell & Palmer (1935), and for small samples by direct observation of curd formation.

For conductivity measurements the Kohlrausch bridge and telephone with valve amplification was employed, for freezing-point determinations, the Hortvet cryoscope.

Analytical methods for calcium and phosphate, where not otherwise stated, were those used by Pyne (1940).

RESULTS

Serum changes accompanying hysteresis

The relatively short life of the hysteresis phenomenon—about 5 hr.—made it necessary to employ rapid methods of investigation in order to detect any accompanying serum changes. Physical means, especially conductivity measurements, had obvious advantages here.

(a) *Conductivity and other physical methods.* Preliminary experiments with heated milk were negative, the conductivity of the rapidly cooled samples remaining apparently unchanged on further standing. When, however, chlorides, which account for the greater part of the conductivity, were largely removed by dialysis, either against successive changes of artificial chloride-free milk serum, or better still, against dilute M/1400-calcium phosphate solutions of pH 6.7, a marked rise in conductivity was found to accompany the changes of renneting time throughout the hysteresis period. Milk dialyzed in this way showed, incidentally, the characteristic rennet hysteresis of normal milks. Table 1 gives some typical results.

The concentration changes suggested by conductivity measurements were too slight for detection by freezing-point determinations on milk whether dialyzed or undialyzed. An attempt to employ the polarographic method for the same purpose was also unsuccessful. With barium caseinate-barium phosphate solutions (calcium cannot be satisfactorily estimated) it was found that the colloid constituents contributed so greatly to the diffusion current as to mask any small change in barium ion concentration which might accompany hysteresis. This matter is receiving further attention.

The changes in concentration of calcium and phosphate, suggested by conductivity measurements, were next investigated more quantitatively by chemical analysis.

(b) *Rapid dialysis.* The following conditions were adopted. NaCl (0.25 ml. of a 1% solution) containing a trace of saponin (to ensure wetting of membrane) was placed in a dialyzer consisting of a short length of 22 mm. glass tubing sealed at one end with a stretched cellophan membrane. The dialyzer was placed in about 50 ml. of the milk under investigation and the whole gently rocked for 30 min. This time of contact ensured a diffusion of ions (50–70%) adequate for comparative purposes.

Table 1. *Conductivities and renneting times at various intervals after heating of milk dialyzed against M/1400-calcium phosphate and heated for 30 min. at 85°*

	Specific conductivity (mhos $\times 10^{-6}$ at 15°) after (min.)							Renneting time at 35° (sec.) after (min.)	
	5	15	30	60	120	180	240	5	240
A	408	413	419	427	432	436	439	370	488
B	399	404	409	—	425	430	—	382	450
C	481	485	490	498	—	510	514	282	515

This definite rise in conductivity of dialyzed milk which occurs on standing after heating provided the first clear evidence that the hysteresis phenomenon was associated with a progressive increase in the amount of soluble material, the latter consisting undoubtedly of calcium and phosphate released into solution from the caseinate-phosphate complex. Incidentally the rate of change of conductivity with time, at first rapid, later slower, shows a remarkable parallelism to that of the renneting time over the hysteresis period (cf. Mattick & Hallett, 1929).

without allowing the dialysis time to extend too widely over the hysteresis period.

Inorganic phosphate and calcium were determined on 0.1 ml. portions of the dialysate, the former by the Kuttner & Lichtenstein technique (1930), the latter by a micro adaptation of the method of de Loureiro & Janz (1944).

The results (Table 2) show that there is a definite increase in the concentration of soluble calcium and phosphate throughout the hysteresis period.

(c) *Permutite action.* Davies (1937) has shown that contact of milk with barium permutite brings

Table 2. *Calcium and phosphate contents of dialysates from heated (85°, 30 min.) milk at various intervals after heating*

		Sample						
Ca (mg./100 ml.)		A	B	C	D	E	F	G
		(5 min. after heating)	(5 min. after heating)	(5 min. after heating)	(5 min. after heating)	(5 min. after heating)	(5 min. after heating)	(5 min. after heating)
	(240 min. after heating)	15.2	13.0	16.0	15.2	—	—	—
		19.6	17.2	19.6	17.2	—	—	—
Phosphaté (mg. P/100 ml.)	(5 min. after heating)	26.5	25.3	—	—	33.5	23.4	24.7
	(240 min. after heating)	28.9	27.4	—	—	35.9	25.9	26.1

about, without appreciable change in reaction, a rapid replacement by barium of the soluble calcium, followed by a slower replacement of the insoluble or colloidal calcium. The method seemed applicable to the detection of the changes in the concentration of serum calcium which might accompany rennet hysteresis.

The heated milk was rapidly cooled, immediately shaken for 10 min. with one-fourth its weight of barium permutite, drawn off, centrifuged free from suspended matter, and analyzed for calcium and phosphate. A second sample of the milk was similarly treated some 4 hr. after heating and cooling.

Table 3. *Calcium and phosphate contents and pH values of heated (85°, 30 min.) milk treated with barium permutite at various intervals after heating.*

	Sample			
	A	B	C	D
Ca (mg./100 ml.)				
(5 min. after heating)	106	132	89.2	94.4
(240 min. after heating)	94	121	86.0	88.4
Phosphate (mg. P/100 ml.)				
(5 min. after heating)	47.5	56.8	40.2	—
(240 min. after heating)	40.5	52.5	36.6	—
pH				
(5 min. after heating)	6.49	6.76	7.00	6.53
(240 min. after heating)	6.52	6.87	7.03	6.70

In all cases the residual calcium and phosphate of the permutite-treated milks are seen to have diminished with increase in the time since heating (Table 3), i.e. the amounts of these ions taken up by the permutite has increased during the period. Freshly heated milk is thus lower in rapidly replaceable or soluble calcium and also in soluble phosphate than milk that has stood for some hours after heating. The permutite results thus confirm, at least qualitatively, those obtained by dialysis. Quantitatively the agreement between them is naturally less marked, and the values found for calcium replaced by barium are in general low compared with those reported by Davies, who, however, remarks on the very different replacement activities of different samples of permutite.

Table 4. *Calcium and phosphate contents and pH values of rennet whey prepared at 30° from milks at various intervals after heating (85°, 30 min.)*

		Sample				
		A	B	C	D	E
Ca (mg./100 ml.)	(5 min. after heating)	37.8	36.4	—	—	—
	(240 min. after heating)	40.6	38.8	—	—	—
Phosphate (mg. P/100 ml.)	(5 min. after heating)	39.8	38.5	27.5	37.6	31.5
	(240 min. after heating)	41.0	39.8	28.6	39.3	33.1
pH	(5 min. after heating)	6.38	6.52	—	—	—
	(240 min. after heating)	6.51	6.61	—	—	—

The reduction in the concentration of soluble phosphate following contact with barium permutite, observed in these experiments, suggests that the reaction between this substance and milk is not limited merely to basic exchange.

(d) *Rennet whey.* Analysis of rennet wheys formed at different stages of the hysteresis also indicated that the phenomenon is accompanied by an increase in soluble calcium and phosphate. In preparing these wheys, renneting was carried out at 30°; with addition of 1% of commercial liquid extract. A curd sufficiently firm to yield a clear whey by centrifuging and filtration was obtained in 20–30 min.

Analyses of rennet curd (Grimmer & Paape, 1930) and of whey (Pyne, 1940) suggest that an adsorption of soluble calcium and phosphate occurs at some time during the renneting process. The analyses recorded (Table 4) are therefore presumably composite values resulting from the superimposition of this tendency on the contrary changes properly due to hysteresis. Nevertheless, the characteristic feature of the hysteresis phenomenon—the progressive release into solution of calcium and phosphate—is again evident.

Composition of the adsorbed and subsequently released phosphate

The amounts of calcium and phosphate released from the caseinate-phosphate complex during the hysteresis period are small. Nevertheless, it is possible from consideration of the data in Tables 2 and 4 to form a rough estimate of the type of phosphate involved in these changes. The data, where complete, suggest a Ca/P ratio of approximately 2 (Ca/P for $\text{Ca}_3\text{P}_2\text{O}_8 = 1.94$); the phosphate released therefore appears to correspond to that normally present in the calcium caseinate-calcium phosphate complex (Pyne, 1934). Indirect evidence that release of a relatively alkaline phosphate actually occurs during hysteresis is furnished by the pH values of the various wheys and permutite filtrates (Tables 2 and 4), since the course of the hysteresis is seen to be accompanied by a slight shift to a more alkaline reaction.

The relation of hysteresis to the various stages of rennet action

Rennet reaction is generally regarded as occurring in two stages: (a) enzymic scission of the caseinate (or alternatively digestion of some protecting substance) with formation of paracasein, (b) coagulation

A second sample of the same milk which had been allowed to stand for some hours at ordinary temperatures, after heating and cooling, was similarly treated. Table 5 gives the average relative coagulation times at 40° for a series of six milk samples submitted to this treatment. The initial coagulation time of the sample renneted immediately after heating and cooling is taken as 100 in all cases.

Table 5. *Average relative coagulation times at 40° of heated and rapidly cooled milk which had, at two very different intervals after heating (85°, 30 min.) been submitted to rennet action at 16° for various times*

		Min.									
Duration of rennet treatment at 16°		½	5	10	15	20	25	30	40	50	60
Time of subsequent coagulation at 40° of milks treated with rennet	(5 min. after heating)	100.0	70.8	58.6	49.7	48.7	48.2	49.2	49.2	50.0	51.8
	(240 min. after heating)	157.4	125.5	112.0	95.7	91.2	87.0	83.5	82.2	77.3	73.4

of paracasein by calcium ions. Some adsorption of calcium is apparently involved in the second stage, judging from the composition of rennet curd (Grimmer & Paape, 1930) and whey (Pyne, 1940). Rapid coagulation is in general associated with higher concentrations of ionic calcium and vice versa.

It has been seen that rennet hysteresis is, on the other hand, characterized by a progressive decrease in the ability of milk to be coagulated by rennet, accompanied by a corresponding increase in soluble calcium content. This somewhat unexpected result suggests that it is not the concentration of soluble calcium *per se* which plays the essential part in the second stage of the rennet reaction but rather the amount of calcium adsorbed (presumably as phosphate) from solution. Moreover, calcium adsorbed in these hysteresis experiments as a result of the action of heat, and thus prior to the addition of enzyme, appears to be as effective in promoting coagulation as is calcium, adsorbed after the first stage of the rennet reaction, on the ordinary view of the renneting process.

In order to ascertain more definitely to what extent either or both stages of the rennet reaction were influenced by this previously adsorbed calcium phosphate a series of renneting experiments was carried out. Heated milk, immediately after cooling, was treated with rennet (1.25 % of a 2.5 % solution prepared from Bengel tablets) at about 16°, a temperature low enough to allow the first part of the reaction, but not, to any appreciable degree, the second, since this has a much higher temperature coefficient (Berridge, 1942). After being held at 16° for various periods, small samples were transferred to copper vessels and rapidly heated by immersion and gentle rocking in a water-bath at 40° in order to bring about the second or so-called calcium ion precipitation stage. Time of coagulation was taken as that required for the first appearance of particles of curd.

These results suggest that rennet hysteresis has its origin in the second or calcium precipitation stage, for all the milk samples show a rapid and fairly uniform fall in renneting time to a moderately constant value in about the same time—some 15 min. in these experiments—corresponding undoubtedly to the completion of the first or enzymic stage. Thereafter, the course of the renneting times diverges somewhat for the two groups. The milk submitted to rennet action 4 hr. after heating shows a further but much more gradual fall in renneting time; milk which has been renneted immediately after heating maintains, on the contrary, a renneting time which is almost unchanged but shows a slight tendency to rise again. This behaviour on the part of freshly heated milk is undoubtedly due to the supervention of the hysteresis phenomenon. The longer periods of contact with rennet at 16°, by permitting a greater degree of re-solution of adsorbed calcium phosphate, can thus give rise to a slower coagulation when the sample is later warmed to a temperature at which the second stage of the reaction can occur. It is of interest to note that this loss of calcium phosphate into solution, leading to a retardation of the coagulation, can proceed simultaneously with and apparently independently of the first or enzymic stage of the rennet reaction.

That the hysteresis phenomenon is associated with the second stage of the rennet reaction is further attested by the following facts: (a) milk heated to temperatures as low as 40–43° shows some degree of hysteresis, without, however, suffering any permanent reduction in renneting ability as compared with the corresponding raw milk (Mattick & Hallett, 1929). Hysteresis can thus occur under circumstances where no injury to the enzymic reaction appears to have taken place; (b) again, differences in renneting time are observed also in milk subjected to different heat treatments even where these are so mild (holding at 0–2° and

at 30° respectively) as to exclude the possibility of injury to the enzymic reaction proper (see Table 6). The samples investigated here were first of all submitted to flash pasteurization at 75° in order to obviate possible bacterial change during the subsequent holding at the lower temperatures; (c) the extreme sensitiveness of the renneting time of artificial calcium caseinate-calcium phosphate complexes to the previous heat treatment of these complexes, even where this is relatively mild (Holter, 1932), is apparently an instance of the same kind, accentuated probably by the dilute nature of the solutions investigated.

Table 6. *Coagulation times with rennet at 40° of milk previously held at different relatively low temperatures*

	Coagulation times (sec.)		
	A	B	C
Held 24 hr. at 0–2°	106	162	452
Held 24 hr. at 0–2°, then 5 hr. at 30°	88	122	306

Hysteresis in calcium caseinate-calcium phosphate-gelatin systems

As mentioned earlier, the present investigation owes its inception to divergent observations on the behaviour of these heated systems to rennet. The results of Powell & Palmer (1935) with mixtures of calcium caseinate and gelatin-protected calcium phosphate indicate the existence of a normal hysteresis similar to that of milk; those of the writer, on the other hand, indicate a reversed hysteresis (Table 7).

Table 7. *Average coagulation times with rennet at various intervals after heating (85°, 30 min.) of different types of calcium caseinate-phosphate-gelatin systems*

System	No. of exp.	Average coagulation time at 35° (sec.) after (min.)	
		5	240
Calcium caseinate-calcium phosphate complex + gelatin	9	104	133
Calcium phosphate protected by gelatin + calcium caseinate	11	184	157

The mixtures investigated contained in general 2.5% calcium caseinate, 2.5% gelatin, and were about $M/300$ – $M/150$ in $Ca_3P_2O_8$. Owing to their low content of soluble calcium it was necessary, following Powell & Palmer, to add a small quantity of a soluble calcium salt, 2–5% of 0.14M- $CaCl_2$, just before renneting in order to obtain satisfactory coagulation. Sufficient rennet extract was then added to cause coagulation in about 2–5 min. at 35°.

The divergent observations on the hysteresis of these systems appear to have their origin in differences in the methods of preparing the gelatin-protected calcium phosphate. The writer, following De Toni (1921), prepared the colloid by slowly running a solution of trisodium phosphate into a boiling gelatin solution of calcium chloride. De Toni emphasizes the need for boiling, without which combination between calcium and phosphate proceeds too slowly in the presence of gelatin to form appreciable amounts of the inorganic colloid. Prepared in this way the colloid forms an almost opaque, porcelain-like solution. Powell & Palmer's preparation, on the other hand, appears to have been made in the cold from lime and phosphoric acid solutions. It is said to have been translucent—a description which implies that its formation was at least incomplete. Its behaviour on heating is in accordance with this supposition. A marked shift in reaction towards acidity was found to occur, suggesting that the formation of tricalcium phosphate, accompanied by that of the monocalcium salt, was at this stage taking place from the elements of the dicalcium salt. If this view is correct, it would appear probable that when this gelatin-calcium phosphate solution was added to one of calcium caseinate and the mixture heated for the study of its hysteresis, some formation of tricalcium phosphate also took place, but this time in the presence of calcium caseinate. Consequently the system obtained in this way is unlikely to have differed essentially from that prepared by adding gelatin to the calcium caseinate-calcium phosphate complex. That both should have shown the same type of hysteresis is therefore not surprising.

From the present experiments it is clear that the hysteresis phenomenon is not identical for caseinate-gelatin-phosphate systems in which the phosphate is protected by caseinate and by gelatin respectively. The equilibrium between dissolved calcium phosphate and the complex appears to differ, as regards the direction and magnitude of its displacement by heat treatment in the two cases. The nature of the protection exercised by the two proteins can therefore hardly be the same.

DISCUSSION

It is clear from the experiments here reported that rennet hysteresis merely reflects a general phenomenon of milk—a heat-induced displacement of the equilibrium between calcium phosphate in solution and the colloidal calcium caseinate-calcium phosphate complex. Rise of temperature produces a temporary transfer of some of the soluble calcium and phosphate to the colloid complex, and this transfer is slowly and (if the heating has been more than moderate) only partly reversed on subsequent

cooling; chilling produces naturally the opposite effect (see Table 6). Other things being equal, the greater the degree of adsorption of calcium phosphate by the complex, the more rapidly will the system coagulate when acted upon by rennet. The enzymic attack proper is not, however, concerned in these changes, which appear instead to influence the renneting time mainly, if not exclusively, by their effect on the second or so-called calcium ion precipitation stage. Severe heat treatment, of course, can independently affect the sensitivity of the caseinate molecule to rennet enzyme attack and retard the coagulation as a whole. This, however, is apparently an additional effect of heating and does not in general mask the hysteresis phenomenon unless it is so severe as to inhibit coagulation altogether.

It is difficult to reconcile these results with the view that the enzymic part of rennet action consists of an alteration of a constituent of the original casein which acts, before this alteration, as a protective colloid for the main cation-sensitive group of constituents (Linderstrøm-Lang, 1928; Holter, 1932). Adsorption of calcium phosphate from solution by the caseinate complex as a result of heat treatment seems to affect the second stage (while leaving the first stage of the reaction unaffected) in much the same way as if the adsorption had occurred during this stage and subsequent to the enzyme reaction proper. This behaviour appears to conform with the older Hammarsten view of rennet action.

As to the differences observed in the type of hysteresis exhibited by caseinate-phosphate-gelatin systems according to whether caseinate or gelatin is the protecting colloid, the facts are best explained by assuming that the caseinate-phosphate complex

is essentially a calcium caseinate-phosphate, i.e. a compound in which the union of phosphate and caseinate has taken place through calcium atoms common to both. The gelatin-protected phosphate would, on the other hand, appear to resemble a gold sol, and to consist presumably of a calcium phosphate nucleus coated with gelatin. Obviously the composition of the postulated caseinate-phosphate will not be fixed, but can vary according to the number of phosphate groups present. Moreover, the heterogeneity of casein is not relevant in either case; there is no suggestion that all the casein fractions are equally capable of forming these complexes.

SUMMARY

1. The rennet hysteresis of heated milk arises from the adsorption of calcium phosphate by the calcium caseinate-calcium phosphate complex during heating, followed by its gradual release again at lower temperatures.

2. Calcium phosphate thus adsorbed affects the renneting time primarily through its acceleration of the second or so-called calcium ion precipitation stage of the coagulation. This result seems to accord best with the Hammarsten theory of rennet action.

3. Rennet hysteresis of calcium caseinate-calcium phosphate-gelatin systems differs according to whether the phosphate is protected by caseinate or by gelatin. This behaviour is interpreted as favouring the existence of a chemical union between the two constituents in the calcium caseinate-calcium phosphate complex of milk.

I wish to thank Prof. J. J. McHenry for advice on the conductometric technique and Mr D. B. O'Loughlin for assistance in some of the experimental work.

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The Distribution of Myrobalanitannin

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(Received 15 February 1945)

Myrobalanitannin (I: luteoic acid 5-biglucoside, $R = C_{12}H_{21}O_{10}$) present in myrobalans, the fruit of *Terminalia chebula* Retz., was first obtained by Nierenstein (1910) as a well-crystallizing substance

which gave, on hydrolysis either with dilute sulphuric acid or with emulsin, 1 mol. ellagic acid (II) and 2 mol. glucose. The hydrolysis with emulsin excludes the possibility that myrobalanitannin is