

ACID PHOSPHATASE IN CREAM AND IN SKIMMILK

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SUMMARY

Acid phosphatase activity in cream is about twice that in skimmilk. The phosphatase in both is relatively heat-stable; 70% of the activity remained after heating at 65° C. for 15 min. The enzymes from raw cream and raw skimmilk were each purified threefold. The cream enzyme was concentrated in the insoluble residue obtained by centrifuging the buttermilk fraction of washed cream at 50,000 × G for 1 hr. When skimmilk was centrifuged under the same conditions, most of the enzyme activity remained in the supernatant solution, which was further purified by ammonium sulfate treatment to yield a soluble form of acid phosphatase. Ultracentrifuge fractionation of buttermilk failed to concentrate the cream enzyme activity in a single fraction. Acid phosphatase, alkaline phosphatase, and xanthine oxidase activities were broadly distributed in these fractions. pH activity curves, K_M values, and the effect of inhibitors are reported for the purified acid phosphatase of cream and milk. These studies showed no significant difference between the two enzyme preparations.

The occurrence of acid phosphatase in whole cow's milk has been reported by Mullen (9), who investigated some of the properties of the enzyme but did not attempt its purification. The distribution of acid phosphatase in cream and in skimmilk had not been studied. Such data would be of interest for comparison with xanthine oxidase and alkaline phosphatase distribution figures, because related studies (7, 8, 13) indicate that many of the enzymes of milk occur in the fat globule membrane of cream. The present report describes acid phosphatase in cream and skimmilk, and compares some properties of the enzymes from the two sources.

METHODS

Assay of alkaline phosphatase. Enzyme activity was determined by a modification of the method used by Zittle and DellaMonica (11). The test material was incubated 5 min. at 38° C. with 0.00083 M disodium phenyl phosphate, 0.0025 M MgCl₂, and 0.067 M ethanolamine-HCl buffer, pH 9.8. The enzyme preparation made up 1 ml. of this 6-ml. incubation mixture. The liberated phenol was estimated colorimetrically. Activities are reported as micromoles phenol released when 1 ml. enzyme preparation is incubated with 5 ml. substrate for 1 hr. at 38° C. The unit of activity is defined as the amount of enzyme that will hydrolyze 1 μ M of substrate under the same conditions.

Assay of acid phosphatase. Two methods were used. In the first part of the experimental work the enzyme was determined by incubating the sample for 1 hr. at 38° C. with 0.00417 M disodium phenyl phosphate and 0.0417 M succinic acid-borax buffer, pH 4.0 (5). With cream and all fractions of cream and skim-

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milk, there was a linear relationship between amount of enzyme and phenol released. With skimmilk, there was some departure from linearity with the larger amounts of sample. Conversion of phenol to activity units was the same as for alkaline phosphatase. Subsequently, the acid phosphatase was estimated by Lowry's method (2), with disodium *p*-nitrophenyl phosphate as the substrate. This method was more rapid than the previous one, and at pH 5.2, the optimum pH with this substrate, precipitates of casein when present were finer and more easily suspended. One-tenth-milliliter enzyme was added to 1 ml. buffer-substrate containing 0.005 *M* disodium *p*-nitrophenyl phosphate and 0.1 *M* acetate buffer, pH 5.2. After 30 min. at 38° C., the reaction was stopped by the addition of 3 ml. 0.25 *N* NaOH, bringing the pH to 12.0. Blanks were included in every assay performed. Free nitrophenol was determined, using a Beckman Spectrophotometer, Model DU,² with a 1-cm. light path at 410 m μ . Activities are expressed as micromoles nitrophenol released when 0.1 ml. enzyme is incubated with 1 ml. substrate for 30 min. at 38° C. The unity of activity is defined above.

Assay of xanthine oxidase. The method of Zittle *et al.* was used (12).

Assay of protein. Protein was estimated by the method of Lowry *et al.* (6).

Raw skimmilk and raw cream from pooled milk was supplied by a local dairy.

EXPERIMENTAL PROCEDURE AND RESULTS

1. *Distribution.* Acid phosphatase of skimmilk and cream is of relatively low activity. When assayed with disodium phenylphosphate substrate, the activity in cream was 0.68 unit per milliliter; in skimmilk the activity was 0.37 unit per milliliter. Thus, in whole milk with a skimmilk:cream ratio of 7:1, 21% of the activity is located in the cream and 79% in the skimmilk. These values varied considerably with different samples and in some batches approached a 1:1 proportion.

The variable distribution of the acid phosphatase between skimmilk and cream appears from the work of others. In one instance (1) cream was reported to contain the highest activity, in the other instance (3a) the skimmilk.

2. *Heat treatment.* Mullen (9) reported that the acid phosphatase of whole cow's milk retained 80-90% of its activity under pasteurization conditions (62.8° C. for 30 min.), whereas the alkaline phosphatase was completely destroyed. This interesting property has been confirmed. Similar results have been reported by others (1, 3a). When skimmilk is heated at 65° C. for 15 min., 67% of the acid phosphatase activity remains. Cream retains 72% of its acid phosphatase activity; whereas, the purified buttermilk preparation retained only 36% of the activity. The alkaline phosphatase in all of these materials was completely destroyed.

3. *Preparation of acid phosphatase from cream.* Preparation of acid phosphatase from cream involved washing by dispersing 40% raw cream in two parts water and centrifuging for 15 min. at 2,000 G at 4° C. After repeating

²It is not implied that the U. S. Department of Agriculture recommends the above company or its product to the possible exclusion of others in the same business.

the centrifuging step, the washed cream was churned with one part water to give buttermilk. The buttermilk was separated from the milk fat by decantation. Small particles of milk fat were removed by centrifuging for 15 min. at 2,000 G. The buttermilk was then centrifuged at 50,000 G for 1 hr., a procedure which sedimented approximately 90% of the acid phosphatase. Each step of the procedure was studied in detail, to determine the optimum conditions for the purification of the acid phosphatase.

As alkaline phosphatase is present in cream and has been extensively studied (7, 8, 13), its activity was also determined. Some interesting differences between the two enzymes were revealed in the fractionation studies using the ultracentrifuge. When these differences became apparent, a few xanthine oxidase activities were determined to see whether this enzyme would be distributed like acid phosphatase or alkaline phosphatase.

(a) *Washing of cream.* Table 1 shows that 50% of the acid phosphatase and 50% of the alkaline phosphatase is removed by washing. Zittle *et al.* (13) found that 75% of the alkaline phosphatase was removed by washing the cream four times. To avoid such large losses, the cream was washed only twice in these procedures.

TABLE 1
Acid phosphatase and alkaline phosphatase in cream and washed cream

	Acid phosphatase (units/1 ml.)	Alkaline phosphatase (units/1 ml.)
Cream	.684	55.4
1st washed cream	.518	40.4
1st wash H ₂ O	.240	11.4
2nd washed cream	.351	28.8
2nd wash H ₂ O	.130	8.2

Disodium phenyl phosphate substrate was used. Results are calculated to the volume of the original cream.

An attempt was made to increase the yield of acid and alkaline phosphatase in buttermilk by washing with isotonic and hypertonic solutions that might be expected to retain the microsomal structure of the fat globule membrane (4). Accordingly, 0.25 M sucrose, 0.5 M sucrose, 0.15 M NaCl, and 0.45 M NaCl solutions were used. Enzyme losses, however, were identical to the loss when water alone was used to wash the cream.

(b) *Churning of cream.* After churning the washed cream, 103% of the original acid phosphatase and 65% of the alkaline phosphatase were present in the buttermilk (Table 2). The butter was washed once with water; additional activity was also obtained by melting the butter at 38° C. and siphoning off the serum. The buttermilk, wash, and serum account for 131% of the initial acid phosphatase and 74% of the alkaline phosphatase. The high yields of acid phosphatase exceeding those measured initially suggest the presence of an acid phosphatase inhibitor in cream.

(c) *Fractionation of buttermilk in the ultracentrifuge.* The acid phosphatase activity in the buttermilk was studied in the ultracentrifuge and its

TABLE 2
Acid phosphatase and alkaline phosphatase in washed cream and buttermilk fractions

	Total volume (ml.)	Acid phosphatase			Alkaline phosphatase		
		Units per ml.	Total units	Per cent of total	Units per ml.	Total units	Per cent of total
Washed cream ^a	750	.266	200	100	25.4	19,050	100
Buttermilk	430	.480	206	103	28.8	12,400	65
Butter wash	107	.099	10.7	5	5.76	617	3
Butter serum	57	.820	46.7	23	21.2	1,207	6

^a Washed cream was diluted with one part water. Disodium phenyl phosphate substrates were used for assays.

activity compared with that of alkaline phosphatase and xanthine oxidase. The Spinco Model L Ultracentrifuge ² (Rotors No. 40 and No. 21) was used for this work. The starting material was buttermilk prepared from cream washed in 0.25 M sucrose. This was first centrifuged at 2,000 G for 15 min. to remove unchurned cream and butter and then centrifuged according to the procedure shown in Table 3. The results are given in Table 4. After each centrifugation, the supernatant was decanted and the pellet dissolved in 0.25M sucrose. The supernatant was then recentrifuged at a higher centrifugal force. The unit, gravity (G) × min. (3), is used as a measure of centrifugation. Neither acid phosphatase nor alkaline phosphatase was obtained in a single fraction (Figure 1). Their broad distribution suggests that these enzymes are associated with

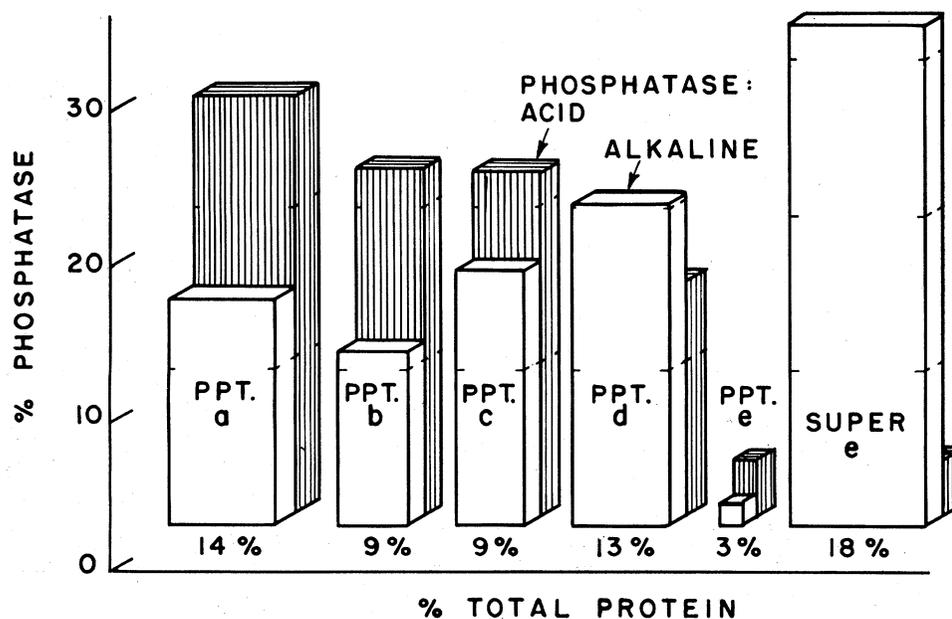


FIG. 1. Distribution of acid and alkaline phosphatase activity in buttermilk ultracentrifuge fractions (Table 3 gives procedure).

TABLE 3
Fractionation procedure for washed cream buttermilk

Fraction	Head No.	Centrifugal force at maximum speed ^a ($G \times 10^3$)	Duration of centrifugation at maximum speed (min.)	Total (G-min. $\times 10^3$)
a	21	11.4	8	91.2
b	21	11.4	16	182.4
c	21	26.3	10	263.0
d	40	105.4	15	1,581.0
e	40	105.4	30	3,162.0

^a Head 40 reaches top speed in 5 min., whereas Head No. 21 takes 20 min.

large micelles having a wide spectrum of size and density. In similar studies, Morton (8) centrifuged washed cream buttermilk and compared the two fractions obtained by centrifuging at $630 \times 10^3 G \times \text{min.}$ and $2,100 \times 10^3 G \times \text{min.}$ with the final supernatant. The first two fractions had properties that indicated that they were akin to microsomes. The supernatant contained only 8% of the total alkaline phosphatase and was assumed to contain no microsomes.

Table 4 presents the protein, acid phosphatase, alkaline phosphatase, and xanthine oxidase contents of the supernatants and precipitates obtained by the procedure outlined in Table 3. Great differences are evident in the sedimentation patterns of alkaline phosphatase on one hand and xanthine oxidase and acid phosphatase on the other. This is most apparent in supernatant *d* obtained after four successive centrifugations. This supernatant contained 39% of the

TABLE 4
Acid phosphatase, alkaline phosphatase, xanthine oxidase, and protein after centrifugal fractionation of buttermilk

Fraction	Acid phosphatase ^a (units/ml)	Alkaline phosphatase ^a (units/ml)	Xanthine oxidase (units/ml)	Protein (mg/ml)
Buttermilk	.273	12.24	40.9	1.39
supernatant	.174	10.54	29.5	.86
a				
ppt.	.073	1.82		.19
supernatant	.105	8.74		.62
b				
ppt.	.058	1.47		.123
supernatant	.042	7.34	4.0	.56
c				
ppt.	.062	2.10		.125
supernatant	.013	4.79	0.56	.29
d				
ppt.	.042	2.58		.18
supernatant	.011	4.06		.26
e				
ppt.	.010	0.19		.028

^a Determined with disodium phenylphosphate substrate. Results are calculated to the volume of the original buttermilk.

original alkaline phosphatase as compared to 4.7% of the acid phosphatase and 1.4% of the xanthine oxidase. The enzymes deposited in the pellet at $1,581 \times 10^3 \text{ G} \times \text{min.}$ (Fraction *d*) could be associated with cellular particles such as microsomes. The alkaline phosphatase remaining in solution may be a second enzyme or the same enzyme in a soluble form. By centrifuging washed cream buttermilk at $1,140 \times 10^3 \text{ G} \times \text{min.}$, Sasaki (10) presented evidence that the alkaline phosphatase in the supernatant differed from the alkaline phosphatase in the precipitate.

The above experiments pointed out conditions for the separation of acid phosphatase from washed cream buttermilk by which the specific activity of the buttermilk was increased from .018 to .062, a purification of better than three-fold. The procedure could be carried out in water as well as 0.25 *M* sucrose, indicating that the insoluble acid phosphatase exists in a stable form that is not solubilized in hypotonic medium. Lipase, trypsin, Triton 100, and freeze-thaw treatment all failed to solubilize the enzyme. *N*-butyl alcohol, which solubilizes alkaline phosphatase, inactivated the acid phosphatase. Further purification of the cream enzyme was, therefore, unsuccessful.

4. *Preparation of acid phosphatase from skimmilk.* Results of centrifugation studies (Table 5) showed that large amounts of acid phosphatase remain in the supernatant of skimmilk centrifuged at 37,500 G for 60 min. ($2,250 \times 10^3 \text{ G} \times \text{min.}$). This is in contrast to buttermilk in which the enzyme is almost completely sedimented. The obvious discrepancies in percentages reported in Table 5 are due to the difficulty in accurately assaying raw skimmilk. After

TABLE 5
Acid phosphatase^a distribution in differential centrifugation of buttermilk and skimmilk

	Buttermilk		Skimmilk	
	Units/ml	Per cent	Units/ml	Per cent
Original material	.400	100	.370	100
Centrifuge $150 \times 10^3 \text{ G} \times \text{min.}$				
Supernatant	.421	105.2	.394	106.5
Sediment	.057	14.3	.242	65.5
Supernatant recentrifuged				
$2,250 \times 10^3 \text{ G} \times \text{min.}$				
Supernatant	.061	15.2	.338	91.5
Sediment	.366	91.4	.382	103.5

^a Determined with disodium phenyl phosphate substrate.

dialyzing against 0.15 *M* NaCl for 24 hr. and 0.25 *M* sucrose for 15 hr., the enzyme activity was almost doubled. This is probably due to the presence of inhibitory substances in the raw skimmilk.

Figure 2 outlines the procedure for separating acid phosphatase from skimmilk. Although activity was present in both the supernatant and sediment after the initial centrifuging at 50,000 G for 1 hr., only the supernatant was retained for further purification. The yield of acid phosphatase in acid whey was better

than in rennet whey. A similar observation has been made by others (1). The soluble acid phosphatase recovered in the final step after dialyzing against distilled water had a specific activity of 0.36 as compared to 0.0121 for raw skimmilk. This represents a threefold purification. Greater purification can probably be achieved through salt fractionation and column chromatography techniques. Such work is under way and results will be reported in a subsequent paper.

SOME PROPERTIES OF PARTIALLY PURIFIED ACID PHOSPHATASES

The above experiments show the existence of two types of acid phosphatase—a particulate enzyme in cream and a soluble enzyme in skimmilk. The properties of the two enzyme preparations were compared.

1. *pH activity curve.* The optimum pH for the enzymes from both sources was determined using 0.1 M tris-maleate buffer (1a) and disodium *p*-nitrophenyl phosphate substrate. Figure 3 shows a pH optimum in the vicinity of pH 5.0 for the cream enzyme. A broad pH optimum in the same range was obtained with the skimmilk preparation. With 0.1 M succinate-borate buffer and 0.1 M acetate buffer, similar results with a more pronounced peak were obtained. The pH optimum is 4.0 when disodium phenyl phosphate substrate and succinate-borate buffer are used. In the presence of 0.01 M sodium fluoride, acid phos-

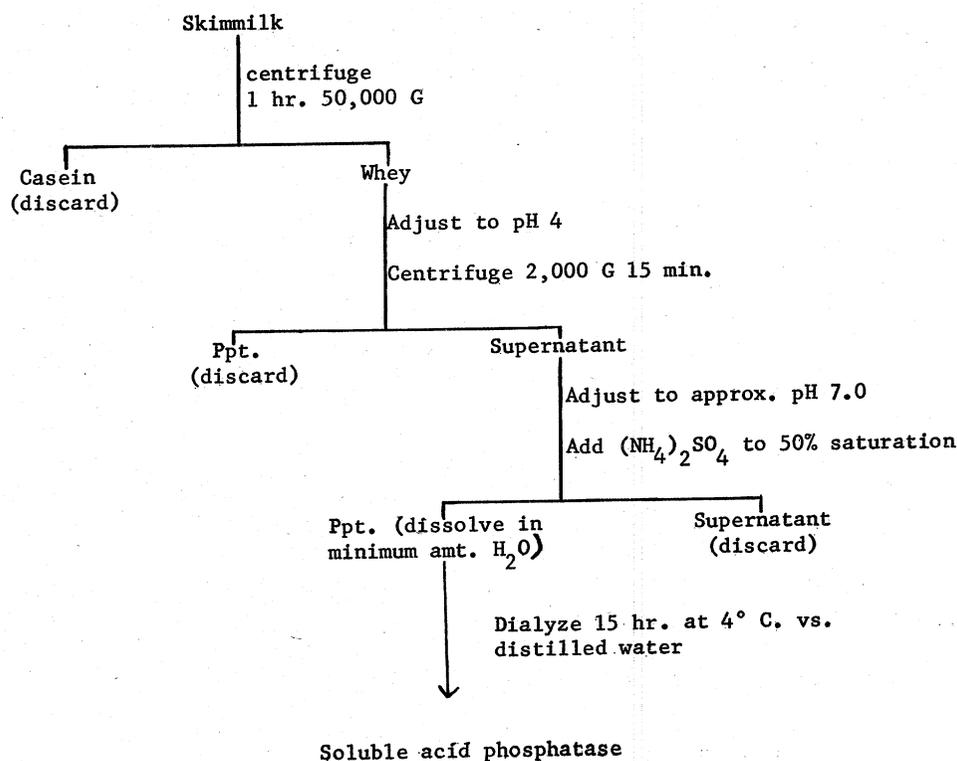


FIG. 2. Preparation of acid phosphatase from skimmilk.

phatase is inhibited but alkaline phosphatase (pH 8.2 and higher) is not affected.

2. *Michaelis constant.* Constants were determined for the two enzymes, using a broad range of concentrations of disodium *p*-nitrophenyl phosphate substrate in 0.1 *M* tris-maleate buffer, pH 5.0. The Michaelis constant for the purified cream acid phosphatase was 0.0010 as compared to 0.00051 for the purified skimmilk acid phosphatase. Further purification of the enzyme is desirable before it can be concluded that the two enzymes differ in this respect. The value for the cream phosphatase was 0.0020 when phenyl phosphate was the substrate. Mullen (9) reported a Michaelis constant of 0.0032 for the acid phosphatase in whole milk with phenyl phosphate substrate.

3. *Effect of inhibitors.* In these studies the enzyme, inhibitor, and 0.2 *M* acetate buffer, pH 5.0, were mixed and allowed to react at 38° C. for 20 min. before adding disodium *p*-nitrophenyl phosphate substrate and timing the assay. Each enzyme was tested with at least three concentrations of inhibitor. Results were plotted as reciprocals of per cent activity versus the concentration of the

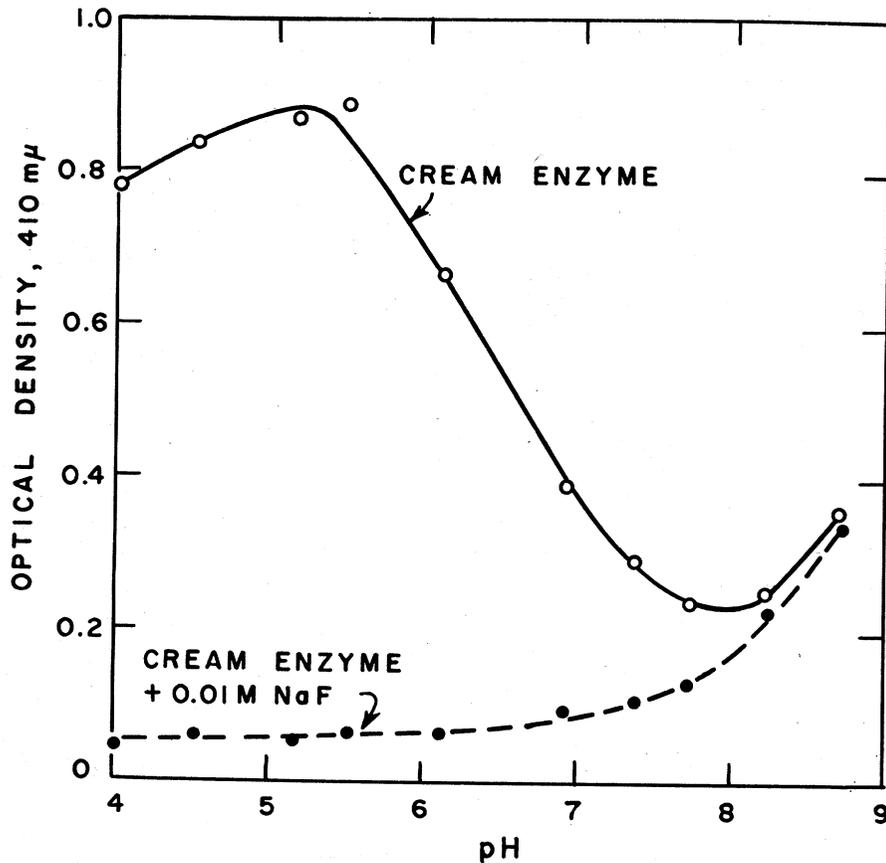


FIG. 3. pH activity curves for acid phosphatase prepared from raw cream. (Assayed with nitrophenyl phosphate substrate, with 0.1 *M* tris-maleate buffer.)

TABLE 6
Effect of inhibitors on acid phosphatases^a prepared from raw skimmilk and raw cream

Inhibitor	50% inhibition molar concentration	
	Skimmilk enzyme	Cream enzyme
Na ₂ H ₂ PO ₄	.005	.002
Na ₂ AsO ₄	.005	.006
CuSO ₄	>.001	.0007
Hg (C ₂ H ₃ O ₂) ₂	.0005	.0004
NaF	<.0001	<.0001

^a Determined with disodium *p*-nitrophenyl phosphate substrate.

inhibitor. Table 6 lists some substances exerting inhibitory action and gives the molarity causing 50% inhibition. The cream enzyme was more affected by NaH₂PO₄ and CuSO₄ than was the skimmilk preparation. Among other reagents studied, KCN was slightly activating to the cream enzyme but did not affect the skimmilk enzyme. There were no marked effects from MgCl₂, CaCl₂, FeCl₃, l-tartrate, or Versene (disodium ethylene diaminetetraacetate). The inhibitory effects of NaF and CuSO₄ had been observed previously (1).

DISCUSSION

The determination of acid phosphatase in milk and cream was hindered by several difficulties which must be considered. The acid phosphatase content is low and relatively large aliquots were necessary for the assays. Because of this, the presence of alkaline phosphatase, which is about 50 times greater in amount, is a source of concern. The shape of the pH activity curve and the influence of sodium fluoride, which inhibits acid phosphatase but not alkaline phosphatase, indicates that alkaline phosphatase does not exert any activity at pH 4 to 5.

The large amount of protein precipitated in the assay mixture is troublesome. This is particularly true in assaying skimmilk with phenyl phosphate substrate at pH 4.0. With nitrophenyl phosphate at pH 5.0, the precipitate is finer and more easily kept in a homogeneous suspension during the assay. The estimation of the acid phosphatase in milk has been investigated in detail (4a).

The results show also that direct assays of milk and cream are limited by the presence of an inhibitor, presumably phosphate, in view of the strong inhibition of the purified phosphatases by phosphate. To avoid this inhibition, samples of milk and cream for assay may be dialyzed. The inhibitor is not present in the purified samples. Phosphate is a stronger inhibitor of acid phosphatase than of alkaline phosphatase (11).

The pH optimum reported by Mullen (9) has been confirmed for this phosphatase, and the Michaelis constant is of the same magnitude as that found by Mullen. The marked resistance of this enzyme to heat has also been confirmed. It has been found further that this resistance lessens as the phosphatase is separated from other proteins.

The ultracentrifuge experiments with the cream preparation show that the acid phosphatase is easily sedimented and probably is associated with the fat globule membrane. The distribution of acid phosphatase, alkaline phosphatase,

and xanthine oxidase indicate that the fat globule membrane fraction is not homogeneous but has a broad spectrum of size and density. The ultracentrifuge experiments also indicate that the acid phosphatase in skimmilk does not sediment readily and is presumably dissociated from the lipoprotein complex. It does not, however, appear to be in equilibrium with the complex in the cream, since the cream complex resisted efforts to dissociate it. The dissociated form of the acid phosphatase in skimmilk seems to be a likely form for purification and attempts along this line appear to be promising.

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