Alkaline Phosphatase of Milk

1. ASSOCIATION OF THE ENZYME WITH A PARTICULATE LIPOPROTEIN COMPLEX

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(*Received* 4 *June* 1953)

In order to obtain a highly purified alkaline phosphatase for detailed studies of this enzyme, especially in regard to possible transferase activity, milk was selected as a source of enzyme which might have some advantages over animal tissues. It is known that the enzyme of animal tissues is intimately associated with insoluble cellular particles (Kabat, 1941; Hers, Berthet, Berthet & de Duve, 1951). However, there was no evidence to suggest such an association of the enzyme with insoluble particles in milk. It therefore seemed possible that the autolytic (Albers & Albers, 1935) or tryptic (Schmidt & Thannhauser, 1943) procedures found necessary for release of the enzyme from animal tissues might be avoided by use of milk as the source of enzyme.

Although a phosphatase was recognized in milk by Demuth (1925), and later by Wilson & Hart (1932) and Graham & Kay (1933), the enzyme attracted little interest until Kay & Graham (1933) showed that the survival of this enzymic activity in pasteurized milk could be used as a sensitive method of detecting unsatisfactory heat treatment. Because of the importance of this 'phosphatase test' in the dairying industry, the relative heat-stability of the milk alkaline phosphatase has received considerable attention, as shown in reviews by Kay, Aschaffenburg & Mullen (1949), Sanders (1949) and Sjöström (1949). Comparatively few studies have been concerned with other properties of the enzyme.

Kay & Graham (1933) found that a portion of the phosphatase in milk was associated with the fat-globule membrane, from which it was released into the buttermilk by churning separated cream. A preparation of the fat-globule membrane from cream obtained by Hansson, Solberg & Sjöström (1946) was found to have considerable phosphatase activity. However, it seemed probable that much of the phosphatase activity remained in the separated milk after removal of the fat globules, since Zittle & Della Monica (1950) achieved some purification of the phosphatase using whey obtained from separated milk. These latter workers employed tryptic digestion in their purification procedures.

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With such limited information available, it was clearly necessary to examine the relationship of the alkaline phosphatase to the other milk components. The distribution of the enzyme among the major milk fractions was therefore investigated. The results of these studies are reported in this paper.

It has been found that the milk alkaline phosphatase is firmly bound to insoluble particles of a lipoprotein nature. A method for almost quantitative separation of the enzyme from these particles, by the controlled use of *n*-butanol, has been developed. By this means, the milk phosphatase has been obtained in true solution and this has permitted purification as described in the following paper (Morton, 1953*a*). A brief account of the use of *n*-butanol for the purification of milk alkaline phosphatase and other enzymes has already been published (Morton, 1950).

MATERIALS AND METHODS

Enzyme unit. The unit of activity is defined as the amount of enzyme which liberates from sodium β-glycerophosphate, 1 μg. of inorganic phosphate P/min. at 38°. The specific activity is expressed either as units/mg. of protein N, as determined under optimal conditions, or, to facilitate comparison with published preparations, as Q_p values, i.e. the equivalent μl. of P liberated by 1 mg. of protein in 1 hr. at 38° (Engelhardt & Lyubimova, 1939). The factor 6·25 has been used throughout this work to convert protein N into equivalent dry weight.

Alkaline phosphatase activity. For the standard test, 1 ml. of the appropriately diluted enzyme preparation was added to 9 ml. of the buffered substrate at 38°, the final concentrations of the reactants being as follows: sodium β-glycerophosphate, 0·02 M; sodium veronal buffer, pH 9·8 at 38°, 0·04 M; $MgCl_2$, 0·01 or 0·001 M (see below). For estimation of activity of milk or milk products, or of crude enzyme preparations, the concentration of $MgCl_2$ was 0·001 M, higher concentrations being inhibitory. For all purified enzyme preparations, the concentration was 0·01 M.

The reaction was stopped after the appropriate interval by the addition of 5 ml. of 25% (w/v) trichloroacetic acid (TCA). Any precipitate was removed by filtration through no. 42 Whatman paper. Inorganic phosphate was then determined on a suitable sample of the filtrate and washings, and on a similar sample from a control tube stopped at zero time.

Because of the high phosphate blank, the amount of untreated milk, or of milk products, was adjusted to give approximately 10% hydrolysis of the substrate in 15 min. at 38°. For all more purified enzyme preparations, the amount of enzyme was adjusted to give hydrolysis of less than 5% of the substrate in 5 min. at 38°. The initial velocity thus determined was found to be proportional to the amount of enzyme.

Butter was tested by holding a weighed quantity at 38° until separation of the butterfat as an oil. The material was centrifuged, the cloudy serum removed with a syringe connected to a fine capillary, the fat washed with distilled water, and a sample from a known volume of mixed serum and washings used for determination of activity.

With the more highly purified enzyme preparations obtained in the later stages of the work, no protein precipitate was obtained with either TCA or with the molybdate-sulphuric acid reagent of Fiske & Subbarow (1925). Therefore, the volume of the final reactants was reduced to 5 ml. and the reaction stopped directly with the acid molybdate reagent, adjusted as to acid strength to allow for the alkali content of the buffer (see Delory & King, 1943). Inorganic phosphate was then estimated directly.

Inorganic orthophosphate. This was determined by the method of Fiske & Subbarow (1925).

Organic phosphate. This was determined by ashing a suitable small sample (about 0·2 ml.) with 0·5 ml. of 10N-H_2SO_4, and adding one drop of conc. HNO_3 to the charred material while hot, to complete oxidation. After being cooled and diluted with 2 ml. of distilled water, the tubes were heated on a boiling-water bath for 10 min. to hydrolyse any pyrophosphate. Then 1 ml. of 2·5% (w/v) ammonium molybdate was added, the contents were mixed and held for 5 min. and the colour was finally developed by addition of reducing reagent and adjustment to a final volume of 10 ml.

Protein nitrogen. The micro-Kjeldahl method was used. Dialysis in cellophan sacs against distilled water was used where necessary to remove non-protein N. The maximum absorption in the range 270–280 mμ. was also taken as a measure of protein concentration in later studies. The values were converted into protein nitrogen by frequent calibration with micro-Kjeldahl determinations.

Dry weight. The material was dried in a vacuum over conc. H_2SO_4 for 48 hr.

Lipid. This was initially estimated as the loss in dry weight following repeated extraction of the TCA precipitate, by centrifuging with 30% (v/v) ether in ethanol, at room temperature. Later, *n*-butanol was substituted for ether: ethanol, following observations to be described, and preliminary acid precipitation was omitted.

Butterfat of milk and dairy products was estimated essentially by the Gerber method (see Ling, 1945). Owing to lack of standardized equipment for this method the estimations were kindly carried out by the laboratory staff at the Milk Marketing Board Creamery, Norwich.

pH. All pH values were determined with the glass electrode, and are given for a temperature of 20°, unless otherwise indicated.

Solvents. Unless otherwise indicated, solvents used were of laboratory reagent grade.

RESULTS

The alkaline phosphatase activity of normal whole milk

There is a considerable variation in the activity of normal milk, as shown from the results in Table 1.

The mean value for two large bulks of milk show fair agreement with the mean of milk supplied from a small mixed herd, and may be considered as fairly representative. The increased activity with advanced lactation confirms the earlier findings of Folley & Kay (1936–7).

Distribution of activity among milk components

In order to establish the quantitative distribution of the phosphatase among the major components, milk was separated into several fractions as follows:

A sample (1 l.) of milk, obtained immediately after milking, was heated to 38° and centrifuged in 250 ml. cups for 45 min. at 2000 *g*. The cream, skim milk and sediment were carefully collected as different fractions by cooling the centrifuge cups and contents to 5° for 2 hr., and then mechanically removing the solidified cream layer. The separated milk was carefully collected by suction through a fine-glass tube and the sedimented material dispersed in

Table 1. *The alkaline phosphatase activity of normal whole milk*

(Sources of milk: (1) Pooled night and morning milk, supplied between September 1949 and April 1950 by a retail producer milking a mixed herd. (2) Milk from a small experimental herd. This was obtained through the courtesy of Prof. Beveridge and Dr W. McLymont, of the Department of Animal Pathology, University of Cambridge. (3) Milk from two groups of cows taken on the one day, kindly supplied by the retail producer. (4) Samples kindly supplied by the Milk Marketing Board Creamery, Norwich, each taken from 2000 gal. of pooled milk.)

Source	Mean* activity (units/ml.)	Mean* specific activity (Units/mg. N)	Q_p
(1) Commercial herd milk	11·9±0·5 (18)	2·5±0·18 (5)	17·3
(2) Experimental herd milk	28·3±4·3 (6)	3·9±0·13 (6)	26·6
(3) Commercial herd milk			
(*a*) cows in advanced lactation	14·0 (1)	.	.
(*b*) cows in early lactation	8·0 (1)	.	.
(4) Pooled milk	14·0 (2)	2·9 (2)	20·5

* Figures in brackets denote number of samples.

distilled water. Of the separated milk fraction, 300 ml. were then centrifuged in 50 ml. cups in a Servall-SS1 high-speed centrifuge at 14000 *g* for 30 min.

The small, surface-fat layer, the yellow translucent serum A, and the solid deposit of sedimented casein and other material (precipitate A) were again separately collected. The casein deposit was dispersed in distilled water, giving a stable white suspension. The serum A was re-centrifuged under the same conditions for 2·5 hr., and the precipitate (precipitate B) again separated from the yellow supernatant serum B and dispersed in distilled water.

The cream obtained from the first centrifuging was cooled and held at 5° for 14 hr. and then shaken in a glass jar on a reciprocating shaking machine until small butter granules appeared. The cloudy 'buttermilk' was carefully decanted, the butter granules pressed free of liquid with a spatula, the butter washed by 'working' it with distilled water, and the washings added to the buttermilk.

Phosphatase activities were determined at all stages. The results shown in Table 2 are calculated for an initial 1 l. of whole milk and are representative of those obtained with three different milk samples.

About 30 % of the alkaline phosphatase is removed with the fat globules in the cream fraction separated at low centrifugal force. Neither adjustment of the pH of the whole milk over the range 4·6–9·5 nor addition of a cationic detergent (cetyltetramethylammonium bromide, 0·02 % at pH 6·6) made any substantial difference to the distribution of the activity between the cream and separated-milk fractions. The activity of the initial whole milk was very much depressed following treatment at the lower pH values at which some precipitation of casein occurred. Repeated washing of the cream (at pH 6·8) by centrifuging (2000 *g*, 30 min.) with an equal volume of distilled water at 18° removed about 70 % of the activity in the cloudy aqueous layers. As shown in Table 2, about 65 % of the activity of the cream fraction is recovered in the buttermilk when the phase-inversion occurs on churning. There appears to be some destruction of enzyme during the churning process, presumably through surface denaturation.

Preliminary fractionation of separated milk

It is clear from Table 2 that over 50 % of the phosphatase activity of whole milk remains in the fat-free component, and hence it seemed desirable to examine possible methods of separating the enzyme from the major protein constituents, casein and β-lactoglobulin, as an essential preliminary to purification. As a general procedure for these experiments, whole milk was heated to 38°, centrifuged (1500 *g*, 45 min.), and the cream removed and churned. The buttermilk so obtained was added to the separated milk, and the mixed milk recentrifuged. The product was a fat-free milk of enhanced phosphatase activity, containing about 70 % of the original enzyme of the whole milk.

Sedimentation of casein. The results shown in Table 2 indicated that separation of the coarser casein particles (precipitate A), representing about 30 % of the casein of the milk, removed less than 6 % of the alkaline phosphatase.

In attempting to apply these findings on a larger scale, separated milk was centrifuged at 25° in two continuous-flow centrifuges, one having a multi-disk bowl, speed 6000 rev./min., the other a hollow bowl, speed 23000 rev./min. With each centrifuge a flow rate of approximately 200 ml./hr. gave a yellow opalescent serum, the combined sera having a specific activity of 2·5 units/mg. nitrogen. The sedimented casein was collected as a faintly opalescent, gelatin-like ribbon from the inside of the centrifuge bowls. It readily dispersed in distilled water to give a completely stable suspension.

Although some 10 l. of milk were fractionated by this method, effecting some purification of the enzyme, the method was slow and tedious, and

Table 2. *Distribution of alkaline phosphatase activity among milk components*

Treatment	Fractions obtained	Activity of fractions: Vol. (ml.)	Units/ml.	Total units	Protein N (mg./ml.)	Specific activity (units/mg. N)	Recovery (% whole milk)	Purification
Whole milk, 2000 *g*, 45 min.	Whole milk	1000	13·4	13400	4·9	2·7	100	1
	Separated milk	935	8·1	7574	4·8	1·7	56·5	0·6
	Cream	68	60·0	4080	2·1	28·6	30·4	10·6
	Sediment	8	6·7	54	.	.	0·4	.
Separated milk, 14000 *g*, 30 min.	Serum A	922	7·3	6731	2·9	2·2	50·2	0·8
	Ppt. A	160	4·6	736	13·6	0·3	5·5	0·1
	Fat	10	23·0	230	.	.	1·7	.
Serum A, 14000 *g*, 2 hr. 30 min.	Serum B	870	4·6	4002	2·7	1·7	29·9	0·6
	Ppt. B	30	83·0	2490	11·6	7·2	18·6	2·7
Cream, shaken	Buttermilk	28	91·0	2548	4·4	20·7	19·0	7·7
	Butter*	15	27·4	411	.	.	3·1	.

* Figures expressed in terms of emulsion obtained at 38°.

ultimately considered impracticable for large-scale purification work.

Effect of pH. The pH was adjusted from the normal (pH 6·7) with 0·2 N sodium hydroxide up to pH 10·9, and with 0·2 N acetic, lactic and hydrochloric acids to pH 4·4, at two temperatures (0 and 18°). The results were essentially the same with the three acids and at both temperatures. After centrifuging (approx. 5000 *g*, 15 min. using the high-speed attachment, International no. 1 centrifuge), the supernatant was adjusted to pH 7·5, and any precipitate was rubbed into distilled water and adjusted to pH 8·0 to favour dispersion of the casein particles. There was little change in activity over the pH range 5·5–9·5, but rapid loss of activity below pH 5·0 and above pH 10·0 was observed. At pH above 9·0, the milk became progressively more viscous and yellow owing to swelling and dissolving of casein particles. With the decline in pH from 5·5 to 4·6, there was increasing precipitation of casein (until complete at the isoelectric point, pH 4·6), and progressive loss of enzyme activity. No purification was obtained by casein precipitation in this way.

Fractionation with salts. In general, sufficient solid was added to separated milk, maintained at pH 6·5–7·0 by appropriate additions of N sodium hydroxide or N hydrochloric acid, until visible precipitation of the casein complex occurred. After standing for 30 min. to 1 hr., at controlled temperature, the milk was either filtered through fluted paper (Whatman no. 1) or centrifuged (1500 *g*, 30 min.). The precipitates were dispersed in distilled water and dialysed free of salt before testing. The filtrates were treated similarly. Slow filtration through the precipitated protein gave clear filtrates, which were invariably devoid of activity, although slight activity was sometimes found in the opalescent supernatants obtained by centrifuging. Most of the enzyme, however, was associated with the casein precipitate. In no case was there any purification of the enzyme.

Precipitation of casein with the following salts used at neutral pH and at the percentage saturation indicated (in brackets) gave less than 10 % total recovery of the enzyme: NaCl (satd.); $(NH_4)_2SO_4$ (60 %); $MgCl_2$, $12H_2O$ (satd.); $CaCl_2$, $6H_2O$ (satd.); anhyd. $MgSO_4$ (satd.) and anhyd. Na_2SO_4 (satd.). However, when the casein was precipitated by saturation with K_2HPO_4 and NaH_2PO_4, added in the proportions required to keep the pH at 6·6, about 40 % of the total activity was recovered. The casein obtained by this procedure was much more flocculent and more readily dispersed in water than that obtained with any other salt. It was also observed that the addition of 5 g. Na_2HPO_4/100 ml. milk, prior to precipitation of the casein with NaCl or $CaCl_2$, $6H_2O$, considerably improved the recovery of enzyme but sodium citrate was not similarly effective. The addition of phosphate did not alter the low recovery of enzyme obtained when ammonium sulphate was used to precipitate the casein.

Fractionation using rennin and pepsin. Separated milk at 37° was adjusted to pH 6·0 with N hydrochloric acid after addition of 2 ml. of M calcium chloride per 100 ml. milk. Sufficient rennin or pepsin was added to cause a firm clot in 10 min. The coagulum was cut with a sharp knife into small cubes, the yellow, slightly turbid whey decanted from the curd at regular intervals over 1 hr., and then centrifuged. The whey was adjusted to pH 7·5, and the paracasein precipitate or 'curd' was dispersed by grinding in a mortar with distilled water and adjusting to pH 8·0, before testing. The results are shown in Table 3.

Addition of 10 % (v/v) calcium phosphate gel (32 mg. dry weight/ml.) to rennin whey (pH 5·8), previously dialysed against running tap water (4 hr.) and distilled water (4 hr.), caused adsorption of most of the enzyme. However, while repeated elution of the precipitate with 10 % ammonium sulphate solution at pH 8·0 recovered about 20 % of the activity, the extract was opalescent and there was no purification. Centrifuging the dialysed whey (at pH 5·5) at 14000 *g* for 1 hr. gave a small upper layer of fat and clear supernatant of low activity, while the precipitate contained most of the enzyme.

Following digestion at pH 8·8, by addition of 0·01 % crude trypsin and incubation for 18 hr. at 25°, a sample of the same whey was adjusted again

Table 3. *Alkaline phosphatase activity of fractions obtained with rennin and pepsin*

Treatment conditions					
pH	Precipitation with	Fraction	Specific activity (units/mg. N)	Recovery* (% whole milk)	Purification
6·5	Rennin	Milk	1·32	100	1
		Curd	0·3	15	0·3
		Whey	2·6	41	2·0
6·0	Pepsin	Milk	3·90	100	1
		Curd	0·2	8	0·1
		Whey	7·8	62	2·0

* Low total recoveries are due to occlusion of alkaline phosphatase by the paracasein precipitate (see text).

to pH 5·8 and centrifuged as before. Approximately 65 % of the total activity now remained in the supernatant, indicating considerable modification of the enzyme association by this treatment. This result suggested a similarity in behaviour between the alkaline phosphatase in milk, and that reported for the enzyme in animal tissues (Kabat, 1941).

Fractionation with acetone. The methods and the apparatus described by Askonas (1951) for fractionation of soluble enzymes of muscle were used. It was found that the optimum pH for fractionation of dialysed separated milk was in the range pH 6·3–6·6. Magnesium acetate (final concentration 0·015 M) was used to adjust the ionic strength and gave better results than did sodium acetate. In the absence of sufficient magnesium (or calcium) ions, difficulty was experienced in centrifuging down the casein precipitate.

The form of the enzyme recovery curve obtained with acetone fractionation of separated milk and based on a number of separate experiments is shown in Fig. 1. Small changes in the conditions of treatment did not markedly affect the form of the curve (curve 1), but the use of sodium phosphate (0·008 M), at pH 6·6, to adjust the ionic strength markedly delayed precipitation of the enzyme. This is indicated by the distinctly different form of the enzyme recovery curve (curve 2) as shown in Fig. 1. The effect is similar to that reported by Askonas (1951) for muscle proteins, although more marked in this case. This may be partly due to the interaction of inorganic phosphate with the casein complex.

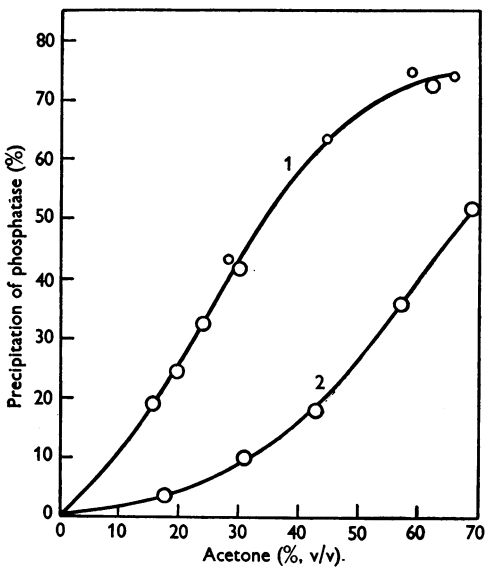

Fig. 1. Effect of acetone concentration on recovery of alkaline phosphatase during fractionation of dialysed separated milk. Curve 1, initial concentration of magnesium acetate, 0·015 M; pH 6·4. Curve 2, initial concentration of sodium phosphate, 0·008 M; pH 6·6.

The casein fraction of the milk proteins was precipitated at acetone concentrations between 35 and 50 % (v/v), and the greater part of the enzyme accompanied this fraction. As shown in Table 4, however, there was some small purification of the enzyme in the higher acetone fraction, but the yield was low. All fractions with enzymic activity were either distinctly cloudy, or opalescent, as had been found in fractionation studies using salts.

The high enzyme recoveries obtained during acetone fractionation of separated milk contrast markedly with those obtained using either acids or salts. The acetone-precipitated casein readily dispersed in distilled water, and gave completely stable suspensions, resembling closely the behaviour of native, undenatured casein obtained by sedimentation. This was in marked contrast to the products obtained with acid and salts, excepting with sodium phosphate.

In a single experiment, separated milk was fractionated with acetone at room temperature (20°). There was very low recovery of the phosphatase (total recovery about 20 %), and the casein was resistant to dispersion.

These observations have indicated a close relationship between the extent of denaturation of casein and the phosphatase activity of the casein fraction. The findings suggest that casein prepared by acid precipitation is already largely denatured, and hence the product so extensively studied by protein chemists may be considerably different from the native protein.

It was found that ethyl ether (A.R. grade), previously cooled to − 15° and added to the dialysed milk at 0° to give a final concentration of 9·5 % (v/v) prior to acetone fractionation, considerably improved the total recovery of the enzyme since much less acetone was required for precipitation. The use of ether, added before addition of acetone, has

Table 4. *Alkaline phosphatase activity of fractions obtained with acetone*

Treatment conditions					
pH	Precipitation with acetone	Fraction	Specific activity (units/mg. N)	Recovery (% whole milk)	Purification
6·5		Milk	2·02	100	1
	0–46 %, v/v	Ppt.	1·15	49	0·6
	46–65 %, v/v	Ppt.	5·64	27	2·8

been found to improve the fractionation of the enzyme during later purification stages and has been adopted as a useful modification of the acetone-fractionation procedure for general use.

However, the addition of ether, up to 20 % (v/v), together with acetone, did not alter the evident association of the phosphatase with particulate components of the milk.

Preparation of an enzymically active lipoprotein complex

On the basis of the results obtained during the above preliminary investigations, and combining several procedures as shown in Table 5, a 43-fold purification of the phosphatase from 10 l. of separated milk was obtained. The dialysed product of stage 6 (termed preparation A) was an almost colourless, faintly opalescent solution. When frozen and dried in vacuum over sulphuric acid, it gave a white powder, which was readily taken up in dilute buffer (0·02 M veronal-hydrochloric acid), pH 7·5, giving an apparent solution.

When adjusted to pH 4·6 only faint turbidity appeared, and preparation A was therefore considered essentially free of casein. However, when dialysed against distilled water (12 hr.) and centrifuged at 14000 *g* for 45 min., 82 % of the activity was found in the white precipitate. While considerably purified, the enzyme was not in true solution.

Analysis indicated that preparation A contained about 10 % lipid, which appeared to be largely phospholipid (lipid P, 3·2 %). Trypsin digestion of rennin whey had indicated that some enzyme remained in true solution following this treatment. These two observations suggested that phosphatase in milk was associated with a lipoprotein complex, which had been considerably purified as preparation A. A number of lipid solvents were therefore examined as means of disrupting this complex.

Removal of lipid with butanol. Samples (5 ml.) of the opalescent dispersion of preparation A, containing 1·5 mg. protein nitrogen/ml., and at pH 7·5 in veronal-hydrochloric acid buffer (0·01 M), were treated by stirring and gently shaking (30 sec.) with excess of organic solvent or bile salt at room temperature (18°). After centrifuging (approx. 5000 *g*, 30 min.) and carefully removing the aqueous layer with a fine capillary attached to an all-glass syringe, the aqueous layer was suitably diluted and tested for phosphatase activity immediately. Table 6 records the activity and the qualitative appearance of the aqueous material after removal of excess of solvent and dilution with an equal volume of distilled water. In contrast to the opalescent or turbid extracts obtained with all other organic solvents or bile salts, both *n*- and *iso*butanol yielded water-clear, colourless aqueous extracts with good recovery of the initial activity. The remainder of preparation A was therefore treated similarly with *n*-butanol. The clear aqueous layer was removed, filtered through a thin layer of Hyflo on a Büchner funnel, dialysed against distilled water until free from butanol (18 hr.), and dried over sulphuric acid in a vacuum. The resultant material (termed preparation B) was completely soluble in distilled water, and no precipitate appeared after centrifuging for 2 hr. at 14000 *g*. No further lipid could be extracted from the trichloroacetic acid precipitate with 30 % (v/v) ether in ethanol, indicating that the single extraction with *n*-butanol removed virtually all lipid material.

Examination of whole milk and products, prepared as previously described, showed that in all cases *n*-butanol disrupted the lipoprotein complex as effectively as in the case of preparation A, releasing the bound alkaline phosphatase. In order to precipitate the casein complex in the same treatment, the following procedure was developed. The material (at pH 7·5) was treated with excess of (20 %, v/v) *n*-butanol and stirred vigorously or shaken (30 sec.). After heating to 38°, the pH was adjusted to 5·2 with N acetic acid, and the emulsion held for a further 5 min. at 38° before centrifuging. The water-clear aqueous layer was separated and

Table 5. *Partial purification of phosphatase from separated milk*

(Stages of preparation: (1) Initial whole milk, pH 6·7. (2) Milk separated, dialysed, and adjusted pH 6·3. (3) Precipitate obtained at 0–46 % (v/v) acetone at −2°. (4) Precipitate dialysed, adjusted to 0·01 M calcium acetate and to pH 5·7. Heated, 40°, 15 min., in litre batches. (5) Precipitate with ether (8 %, v/v) and further acetone (0–18 %, v/v). (6) Dialysed and repeated stage 4. Centrifuged 3500 *g*, 30 min., pH 7·2. Dried in vacuum. 'Preparation A'. (7) Preparation A in 20 ml. water, at pH 7·5, shaken (20 secs.) with 10 ml. *n*-butanol and held 38°, 5 min. at pH 5·2. Aqueous layer dialysed and dried in a vacuum. 'Preparation B'.)

Stage of preparation	Vol. (ml.)	Activity (units/ml.)	Total (units × 10^3)	Protein N (mg./ml.)	Specific activity (units/mg. N)	Yield (%)	Purification
(1)	10 000	20·1	201·0	5·11	3·9	100	1·0
(2)	8 900	12·1	108·0	3·52	3·4	54	0·9
(3)	3 940	13·2	52·0	6·20	2·1	26	0·5
(4)	4 190	9·9	41·5	0·81	12·3	21	3·2
(5)	400	41·3	16·5	0·83	49·6	8	12·7
(6)	390	29·6	11·6	0·22	134·5	6	34·5
(7)	15	446·0	6·7	0·48	930·0	3	238·0

Table 6. *Effect of lipid solvents on alkaline phosphatase activity*

	Material treated*			
	Preparation A		Cream	
Solvent or bile salt	Enzyme recovery (%)	Relative† turbidity	Enzyme recovery (%)	Relative† turbidity
(Untreated)	100	+ + +	100	+ + + +
Ether:ethanol (1:3)	45	+ +	N.D.	N.D.
n-Butanol	82	–	76	–
*iso*Butanol	68	–	66	–
n-Amyl alcohol	N.D.	N.D.	75	+ +
Ethyl acetate	69	+ +	N.D.	N.D.
Carbon tetrachloride	82	+ + +	69	+ + + +
Chloroform	68	+ + +	53	+ + +
Toluene	88	+ +	72	+ + +
Deoxycholate (1%)	72	+ +	N.D.	N.D.
Taurocholate (1%)	94	+ +	N.D.	N.D.
Petroleum ether	N.D.	N.D.	85	+ + +

* Preparation A treated at pH 6·8, cream at pH 5·5.

† Scale of relative turbidities of aqueous phase after treatment and dilution with equal volume distilled water is as follows: opaque, + + + +; opalescent, + +; clear, –; not determined, N.D.

brought to pH 7·5. In all cases it contained between 50 and 70% of the initial phosphatase in true solution. The results obtained with organic solvents in comparative trials with cream at pH 5·5 are shown in Table 6. Similar results were obtained with both milk and buttermilk. Improved yields obtained with dialysed milk indicated that low ionic strength was desirable during treatment of enzymes with *n*-butanol.

The combined procedures giving preparation B, and summarized in Table 5, yielded a very active enzyme, completely water-soluble and purified 238 times when compared with the enzyme in normal milk. However, such is the activity of milk phosphatase that preparation B must be considered less than 5% pure. These preliminary experiments, however, provided the basis of the method for obtaining the highly purified enzyme.

DISCUSSION

The low specific activity of normal cow's milk (2·5 units/mg. N) indicates that it compares very unfavourably as a source of alkaline phosphatase with animal tissues. Untreated dispersions of bovine kidney and intestinal mucosa, for example, have specific activities of the order of 20–25 and 70–100 units/mg. nitrogen respectively (Morton, 1953*b*) while Abul-Fadl, King, Roche & Thoai (1949) have reported values of 100–135 and 650–800 units/mg. nitrogen respectively for autolysates of these tissues.

In contrast to most animal tissues, however, the major proteins of milk have been fairly well defined, and the enzymes present seem limited in number (see review by McMeekin & Polis, 1949). Hence it appeared quite probable that alkaline phosphatase would be readily separable from other constituents by application of standard techniques. The failure of such methods can undoubtedly be attributed to the binding of milk phosphatase in a particulate complex.

Hitherto, milk has been considered to contain fine particles belonging to three categories, namely: (1) coarse, readily sedimentable foreign matter, such as udder cells, and leucocytes; (2) fat globules surrounded by a protein membrane; (3) colloidal casein particles (the casein-calcium phosphate complex). The fractionation of milk by differential centrifuging (Table 2) clearly showed that little or no alkaline phosphatase was associated with the coarse material of category 1. However, up to 30% of the enzyme was associated with the fat globules, most of which could be removed by centrifuging and collection of the cream layer.

Kay & Graham (1933) first observed the higher phosphatase activity of cream compared with milk, and more recently Kay *et al.* (1949) have directed attention to it in relation to tests for adequate pasteurization. However, there has been no previous quantitative estimate of the amount of activity associated with the milk fat. Following careful removal of the cream, the separated milk is found to contain the remaining 70% of the phosphatase activity, but it is probable that the smaller fat globules in this phase are more efficient in adsorbing enzyme than are the larger globules obtained in the cream layer. However, between 50 and 60% of the total phosphatase activity of whole milk is undoubtedly associated with the fat-free phase, although, as later work has shown, the enzyme is not present in true solution.

The results confirm that the enzyme associated with the fat phase is attached to the fat-globule membrane (Kay & Graham, 1933; Sjöström, 1940), probably by an adsorption mechanism, since repeated washing of the fat elutes the enzyme into the turbid aqueous extract. This evidence is now supported by electron micrograph studies (Morton, 1953*c*). It is possible that all the phosphatase enters the milk attached to the fat globules and largely dissociates from the membrane in the milk cistern and during subsequent handling, especially during centrifuging. There has been no investigation to decide between this and the alternative hypothesis that some of the particulate enzyme is adsorbed after secretion of the fat globule.

The enzyme is most conveniently recovered from the fat phase by churning to butter, release of the enzyme accompanying the phase inversion. In this process some of the smaller fat globules do not coalesce, but escape into the buttermilk. Between 10 and 15% of the activity of the buttermilk is associated with these small globules. It has been confirmed that the washed butterfat produced by churning has no activity (Kay & Graham, 1933).

Because of the increased phosphatase activity and lower protein content, cream (and its aqueous washings) and buttermilk all have higher specific activity than milk (Table 2). However, fractionation studies have indicated that buttermilk behaves very much the same as separated milk, so that the essential association of the enzyme with particulate matter, as discussed below, is not altered by adsorption on the fat globules in milk.

The predominant particles of the separated milk are those of category 3 above, namely colloidal particles of the casein-calcium phosphate complex. Centrifuging (14 000 *g*, 30 min.) deposits the coarser particles from separated milk as a translucent, faintly blue fraction (Table 2, precipitate A), without significant removal of the phosphatase. The small precipitate (Table 2, precipitate B), obtained by longer centrifuging (14 000 *g*, 2 hr. 30 min.) is similar in appearance but contains some phosphatase activity, so that the specific activity of this fraction is 2·7 times that of the original milk. It is clear, therefore, that in normal milk the enzyme is attached to particles capable of sedimentation with a definite fraction of the casein particles.

The results obtained with chemical fractionation studies are consistent with the attachment of the phosphatase to a distinct particulate component and indicate further that the complex is not dissociated by normal fractionation procedures.

As would be expected for a particulate protein fraction, precipitation of the enzyme occurs in the pH range 4·6–5·2, and at comparatively low salt and low acetone concentrations. In all such fractionation studies, the water-clear supernatants were uniformly devoid of activity.

The marked loss in activity at pH 4·6, the isoelectric point of casein, and in all cases with salt fractionation, warrants further consideration. As shown in the following paper (Morton, 1953*a*), the partially purified phosphatase in true solution shows only small loss of activity over the range pH 4·6–7·5, although marked irreversible denaturation occurs close to pH 4·4. Furthermore, the enzyme in true solution was frequently fractionated with ammonium sulphate with quite normal recovery (70–80%). Thus there occurs the very unusual phenomenon of an enzyme having apparently greater stability when highly purified than when examined in the crude source material.

The explanation appears to be connected with the particulate nature of the enzyme in milk. Differential centrifuging (Table 2) indicates that particles carrying phosphatase activity must be of about the same size and in intimate association with the exceedingly more numerous casein particles. This has now been confirmed by studies with the electron microscope (Morton, 1953*c*). Under conditions favouring co-precipitation and change in the casein complex, there is some occlusion of the particulate enzyme so that it is no longer accessible to the substrate during the activity test. Hence treatments which change the physical state of the precipitated casein alter the relative accessibility of enzyme and substrate and so change the estimate of activity. Thus the readily dispersable casein precipitate obtained with mixed phosphates gave higher enzyme recovery than the markedly granular precipitate obtained with ammonium sulphate. The relative rate of acidification has also been found to alter the physical state of casein precipitated near the isoelectric point and similarly also the phosphatase activity. This is no doubt the explanation of the variable effect of acidification reported by Sjöström (1949) but ascribed by him as due to other causes. Sjöström (1949) considered his earlier evidence to support the reversible dissociation of phosphatase into co-enzyme and apo-enzyme, but found difficulty in explaining results of later studies.

While occlusion of the particulate phosphatase is the major factor, there is some evidence that the particles carrying the enzyme are themselves modified to some extent by low pH and by salts, and that this may affect their apparent enzymic activity. While the enzymically active particles are largely co-precipitated with casein during acetone fractionation, the casein remains undenatured and readily dispersable to form stable suspensions in distilled water. Such dispersions show their full enzymic potential in the activity test and, when treated with butanol, the bound enzyme is released into true solution. However, butanol treatment of

acid- or salt-precipitated casein is not so effective in releasing the bound enzyme. This further suggests that the casein thus precipitated may have undergone partial denaturation.

The specific precipitation of casein as paracasein (Table 3) effects some purification, leaving much of the particulate-bound enzyme largely in the whey fraction. This procedure was not adopted because of the desire to avoid proteolytic enzymes of any type. It has been used as a basic procedure for milk phosphatase purification by Zittle & Della Monica (1950), who obtained a 60-fold purification of the enzyme as compared with the activity in separated milk. These workers appreciated that the enzyme was associated with lipid material, which remained attached to the enzyme, even following vigorous treatment with chloroform. Zittle & Della Monica (1951) reported difficulties in elution of milk phosphatase adsorbed on 'Celite' from an opalescent preparation obtained following tryptic digestion of whey, and compared their observations with those reported for certain particle-bound enzymes and certain virus preparations. These workers have applied the butanol method (Morton, 1950) for the purification of milk phosphatase from separated milk and have confirmed the unique action of this solvent (Zittle & Della Monica, 1952).

Although Kekwick, Mackay & Record (1946) found ether valuable for fractionation of human blood plasma and preparation of fibrinogen, ether alone did not precipitate any protein from milk under the experimental conditions employed. However, the addition of ether (10 %, v/v) prior to fractionation of milk with acetone has been found to give both higher recoveries of enzyme and some improvement in fractionation. It is noteworthy that Martland & Robison (1929) employed a mixture of ether and ethanol at room temperature for precipitation and fractionation of bone phosphatase, but the present investigation appears to be the first study of the use of ether and acetone together for low-temperature fractionation of enzymes.

The enzymically active material isolated as preparation A was shown to be a lipoprotein complex. Treatment of the aqueous opalescent suspension of preparation A with *n*- or *iso*butanol was found to extract the lipid material (mostly phospholipid) and to release simultaneously the alkaline phosphatase into true solution (Table 6). Butanol was similarly effective in releasing the enzyme into true solution directly from milk, cream and other milk products (Table 6).

The immediate change in the properties of the alkaline phosphatase following butanol treatment confirms the hypothesis that the phospholipid is responsible for the binding of the enzyme in the particulate complex. This contention also gains support from the finding by Sjöström (1940) of a positive correlation between the phospholipid contents and alkaline phosphatase activities of various milk and milk fractions. More recently it has been verified by the isolation and examination of the enzymically active lipoprotein particles of cow's milk (Morton, 1953*c*).

Although the enzymic activity was found to be fairly stable to various organic solvents (Table 6) usually considered to be excellent lipid solvents, with the exception of butanol these were quite ineffective in disrupting the lipoprotein complex of preparation A sufficiently to release the bound enzyme into true solution. It is notewothy also that treatment of preparation A with ether at $-50°$, a procedure found to disrupt the lipoproteins of blood plasma (McFarlane, 1942), did not release the alkaline phosphatase into solution. Moreover, the enzyme was not obtained in solution when an acetone powder of preparation A was extracted with 0·85 % sodium chloride. These findings indicate a unique action of butanol which has been fully confirmed by later experience (Morton, 1950; Zittle & Della Monica, 1952).

SUMMARY

1. The alkaline phosphatase activity of normal cow's milk is very low as compared with organs such as kidney and intestine. The Q_p value is about 20.

2. About 30–40 % of the enzyme is adsorbed to the butterfat globules and may be concentrated by separation of cream. The enzyme is released into the aqueous phase (buttermilk) on churning cream to butter, or by elution with distilled water. The butterfat itself is devoid of activity.

3. The phosphatase in fat-free separated milk and buttermilk is not in true solution in the serum, but is associated with an insoluble particulate lipoprotein complex. This particulate material may be sedimented together with a portion of the casein particles by high-speed centrifuging.

4. Precipitation of casein from separated milk with acids, salts or organic solvents results in coprecipitation of the lipoprotein particles together with the associated phosphatase. The more specific precipitation of casein with rennin (or pepsin) permits of some separation of the casein and lipoprotein particles.

5. Precipitation of casein with acid and certain salts produces partial denaturation, resulting in occlusion of the phosphatase in the protein precipitate and apparent loss of enzymic activity. Inorganic phosphate partially protects casein from denaturation during salt precipitation.

6. The lipoprotein complex which binds milk alkaline phosphatase may be disrupted by treatment of the aqueous material (milk or its products) with

n- or *iso*butanol. Removal of the lipid is accompanied by simultaneous release of the enzyme into true solution. No other organic solvent tested was effective in releasing the bound phosphatase, indicating a unique action of butanol.

I wish to thank Dr M. Dixon, F.R.S., for his encouraging interest and advice during this work. This work was carried out while holding a Travelling Scholarship awarded by the Gowrie Scholarship Trust of Australia, whose assistance is gratefully acknowledged.

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Alkaline Phosphatase of Milk

2. PURIFICATION OF THE ENZYME

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(*Received* 4 *June* 1953)

It has been shown in the previous paper (Morton, 1953*a*) that alkaline phosphatase in milk is intimately associated with insoluble lipoprotein particles. In this respect the enzyme resembles the alkaline phosphatases of animal tissues (Kabat, 1941; Hers, Berthet, Berthet & Duve, 1951), but unlike the tissue enzymes (see Roche & Thoai, 1950) milk phosphatase is only partly released from the particulate material when this is treated with trypsin or lipase (Zittle & Della Monica, 1952).

A far more effective method of obtaining the phosphatase in true solution, and one which avoids undesirable exposure of the enzyme to proteolysis, is the treatment of the whole milk or milk product with butanol (Morton, 1950, 1953*a*). Using this procedure to obtain the enzyme in true solution, and by further treatments, the alkaline phosphatase has been highly purified. The final product is considered to be essentially homogeneous protein. The details of the method are described in this paper.

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METHODS

The analytical methods have been described in the previous paper (Morton, 1953*a*).

Preparation of activated charcoal. Animal charcoal (British Drug Houses) (180 g.), was treated successively with 5 l. of 0·1 N-HCl, 2 l. 5% KCl, 200 ml. 0·1 M-$Ba(OH)_2$, and finally with 2 l. of 5% KCl. After each treatment the charcoal was sucked dry over no. 42 Whatman paper on a large Büchner funnel. After the last treatment it was dried at 100° in an oven, and stored in an air-tight jar. I am indebted to Dr P. D. Mitchell for suggesting this procedure for obtaining an active charcoal, free of inorganic phosphate.