

THE FAT-GLOBULE MEMBRANE OF MILK: ALKALINE
PHOSPHATASE AND XANTHINE OXIDASE
IN SKIMMILK AND CREAM

C. A. ZITTLE, E. S. DELLAMONICA, J. H. CUSTER, AND R. K. RUDD
Eastern Regional Research Laboratory,¹ Philadelphia, Pa.

Recent studies of Morton (11, 13) show that the fat-globule membrane of milk has many properties in common with the enzyme-containing, lipoprotein complexes of animal tissues termed microsomes. The complex nature of the fat-globule membrane suggests that isolation methods involving pasteurization (3), the use of low pH (6), and lipide-solvents (3, 6) should be avoided to isolate this material in its native state. Further, by avoiding pasteurization, enzyme assays (xanthine oxidase and alkaline phosphatase in the present studies) can be used to follow the fractionation. In the present report the lipoprotein fraction from cream is compared with a similar fraction from skimmilk in behavior in the ultracentrifuge and in filtrability, chemical composition, and enzyme content.

METHODS

Alkaline phosphatase. Phenol phosphate is used as substrate and the assay is performed at pH 9.6 in ethanolamine buffer at 37° C. (18). Enzyme activities are expressed in terms of optical absorbance in an 18 mm. diameter calibrated test tube at 660 m μ . An absorbance of 0.105 (10.5 units) is equivalent to the release of 1.0×10^{-7} moles of phenol.

Xanthine oxidase. The method is based on the ability of xanthine oxidase to catalyze the reduction of the colorless triphenyltetrazolium chloride to a red substance when xanthine or other suitable substrate is present. Details of the method are described elsewhere (17). The formation of 3×10^{-7} moles of reduced triphenyltetrazolium under the conditions of the test is defined as 10 units of oxidase.

Determination of lipides by extraction with organic solvents. Five hundred to 800 mg. of freeze-dried solids were extracted twice with 40 ml. acetone and finally with 40 ml. ethyl ether. When it was desired to dissociate the lipoprotein (10), the acetone extraction was followed by 40 ml. *n*-butanol and finally with ethyl ether.

Determination of nitrogen and protein. The nitrogen content was determined by the Nessler method. Comparative protein estimations were made routinely with the biuret reagent.

Determination of phosphorus. Samples were digested with sulfuric acid and hydrogen peroxide. Phosphorus was estimated with the ammonium molybdate reagent after reduction with ferrous sulfate.

¹ A laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

Ultracentrifuge. The Spinco preparative ultracentrifuge was used with No. 40 rotor and 13.5-ml. plastic tubes. In the experiments described, the ultracentrifuge was run for 1/2 hour at 40,000 r.p.m. ($105,400 \times G$). All solutions were centrifuged first at 3,000 r.p.m. (approx. $2,500 \times G$) for 10 minutes to remove gross particles.

EXPERIMENTAL PROCEDURE

Preparation of lipoprotein membrane from cream. The procedure of Jenness and Palmer (9) was followed closely for preparation of the "membrane" by using unpasteurized cream obtained commercially, which contained 25% butterfat. The enzyme distribution in a typical experiment through successive washings and final churning of the cream is shown in Table 1. The enzyme in the

TABLE 1
Xanthine oxidase and phosphatase in cream, washed cream, and the buttermilk

	Xanthine oxidase (units/1 ml.)	Alkaline phosphatase (units/1 ml.)
Cream*	51	123
1st washed cream	42	76
1st wash water	14	40
2nd washed cream	22	48
2nd wash water	14	20
3rd washed cream	16	35
3rd wash water	8.6	15
4th washed cream	9.1	30
4th wash water	7.5	5
Buttermilk	7.0	18

* All assays are calculated to the volume of the cream (14.0 l., obtained by dilution of 3.5 l. of 25% cream with water). The volumes of wash water were actually 11 l., volumes of the 4th cream 2.25 l., and the buttermilk obtained from this 1.12 l.

washed cream and the wash usually adds up to the enzyme in the previous cream, indicating that there is no serious destruction of the enzymes in this procedure. In this experiment, 15% of the phosphatase and 14% of the xanthine oxidase were recovered in the buttermilk. The losses of phosphatase into the water during the washing are considerably greater than those reported by Rimpila and Palmer (16). This might be due to the chilling to which the starting cream had been subjected, since this increases the dissociation of the lipoprotein from the cream (9). Aging of whole milk at low temperature has also been reported (2) to increase the portion of xanthine oxidase in skim milk.

The proteins of the washed cream buttermilk were concentrated by precipitating with 3.2 M ammonium sulfate. The damp salt precipitates were stored at 7° C. The retention of activity in the salt cakes has been variable, but frequently the loss in activity was as little as 10% in 30 days. The preparations have also been dialyzed, which process in itself causes some loss in xanthine oxidase activity, and then dried from the frozen state. Freeze-drying caused, on the average, a 15% loss of the alkaline phosphatase but a 46% loss of xanthine oxidase. The

freeze-dried product contained approximately 5 units of xanthine oxidase and 15 units of alkaline phosphatase per 1 mg. of solids. A rapid additional loss in xanthine oxidase followed on storage of the dried material at 7° C., and activity was almost completely lost by the end of 30 days. Alkaline phosphatase, on the contrary, was quite stable.

The freeze-dried buttermilk preparations contained 60 to 70% of material that was extractable with acetone and ether. Others (16) have found with similar preparations that like amounts (56 to 78%) could be extracted with alcohol and ether. Much of this extracted fraction is probably butter oil. The phosphorus content of the fraction obtained by acetone and ether extraction was 0.25%, indicating that only 6% (16) of the extracted fraction is phospholipide. The residue, after acetone-ether extraction, contained 11.0% nitrogen and 0.53% phosphorus, values in agreement with previous reports (6) for the membrane component. Additional material (18%, containing 1.7% phosphorus) was extracted from the acetone-ether residue with *n*-butanol, a reagent which appears to be especially effective in dissociating lipoprotein (10). The residue, after this treatment, contained 11.8% nitrogen and 0.24% phosphorus. Even when these extractions were performed at 5° C., a considerable loss in enzyme activity occurred, particularly of the xanthine oxidase. The alkaline phosphatase activity of this product was about 40 units per 1 mg. solids. The membrane fraction (microsomes) obtained by ultracentrifugation has been reported (13) to contain 1% nucleic acid phosphorus, equivalent to approximately 10% nucleic acid. By using the same analytical methods, less than 0.05% nucleic acid phosphorus was found in the present studies. Even if all the phosphorus (0.24%) in the solvent-extracted residue belonged to nucleic acid, this would be equivalent to only 2.4% nucleic acid. Further studies by Morton (14) have shown that microsomes from the milk of individual cows show considerable variation in nucleic acid content but that microsomes from bulk milk contain about 0.25% nucleic acid.

Preparation of lipoprotein from skimmilk. Approximately 60% of the alkaline phosphatase of whole milk is found in skimmilk (7). The distribution of xanthine oxidase is roughly the same (2). Assays for these enzymes served to guide the isolation of a fraction from skimmilk for comparison with the cream preparation. The method followed in part the purification of the alkaline phosphatase of milk (20)—precipitation of the casein with 1.38 *M* ammonium sulfate and separation of the whey fraction that precipitates with 2.3 *M* ammonium sulfate. This precipitate was stored as a damp salt cake, which was dissolved in water for further study. Alkaline phosphatase in this form is quite stable. Xanthine oxidase shows some loss on storage.

The pellets obtained on ultracentrifugation (see later) of solutions of the 2.3 *M* ammonium sulfate precipitates from the whey are more comparable to the cream preparations in levels of enzyme activities, particularly alkaline phosphatase (immune globulins, etc. present in the 2.3 *M* fraction remain in the supernatant fluid on ultracentrifugation), than is the starting material. Accordingly, the properties of the ultracentrifuge pellet of the 2.3 *M* whey precipitate are compared with the washed cream buttermilk fraction. The pellet contained ap-

proximately 20 units of alkaline phosphatase per 1 mg. freeze-dried solids. These whey pellets have shown the same relative enzyme stabilities as the cream preparations. Nine per cent of the pellet material dried from the frozen state was extracted with acetone and ether. Extraction of the residue with *n*-butanol (1.7% of the extract being phosphorus) removed an additional 18%. The residue after extraction contained 10.7% nitrogen and 0.24% phosphorus. This fraction, too, contained less than 0.05% nucleic acid phosphorus.

TABLE 2
*Distribution of xanthine oxidase and alkaline phosphatase
in cream, skimmilk, and fractions of the latter*

	Xanthine oxidase (units/1 ml.)	Alkaline phosphatase (units/1 ml.)
Cream ^a	143	400
Skimmilk ^b	23	88
Whey ^c	18	28
2.3 M precipitate ^d	10	22

^a Obtained by gravity separation (approx. 25% fat). This cream was of a different source than the cream used in the buttermilk studies shown in Table 1. The xanthine oxidase activity was regularly lower.

^b All assay values except the cream were calculated in terms of the volume of skimmilk. The relative volumes of skimmilk and cream were 6.5:1.

^c Casein removed with 1.4 M ammonium sulfate.

^d Precipitated from whey by raising the ammonium sulfate concentration to 2.3 M.

The data in Table 2 show that there was considerable loss of alkaline phosphatase when casein was removed from skimmilk with ammonium sulfate (1.4 M). NaCl (36 g. per 100 ml.) was equally detrimental. Some of the missing alkaline phosphatase was found in the casein fraction (about 25% of the total), but there was a destruction of phosphatase as well. Morton (12) found a number of other salts equally detrimental to the alkaline phosphatase. This behavior of alkaline phosphatase is strange, since in later stages of purification (20) ammonium sulfate is a satisfactory precipitant. Xanthine oxidase, on the other hand, was recovered almost quantitatively in this fractionation. About 60% of the alkaline phosphatase was recovered in the whey when rennin (18, 20) or pepsin (12) was used to remove the casein. In one experiment, crystalline pepsin was used (0.5 mg. per 250 ml. skimmilk at pH 6.5 and 35° C.) for this purpose, with the thought that only the clotting of casein would occur with no proteolysis of other proteins. The recovery of xanthine oxidase and alkaline phosphatase in the whey was 68% and 58%, respectively. On centrifuging this material, however, only 10% of the enzymes was obtained in the pellet. Presumably, the pepsin had caused some dissociation of the lipoprotein complex. Crude proteolytic enzyme preparations have frequently been used for this purpose in the purification of alkaline phosphatases (20) and xanthine oxidase (1, 2, 8). Recent experiments have shown that precipitation of the casein with acetic acid at pH 4.7 was more satisfactory for recovery of the alkaline phosphatase in the whey than the use of ammonium sulfate. (Immediately after precipitation and filtration of the casein, the whey was adjusted to pH 7.0). Approximately 50% of the activity of both enzymes was recovered in the whey.

Ultracentrifugation of the lipoprotein from cream and skimmilk. The results obtained when the washed cream buttermilk fraction was subjected to ultracentrifugation for ½ hour at 40,000 r.p.m. ($105,400 \times G$) are shown by the following typical example. The ammonium sulfate precipitate of the washed cream buttermilk was dispersed in water to a concentration of 0.26% protein. The solution contained 228 units of phosphatase and 60 units of xanthine oxidase per milligram of protein. Eleven and one-half ml. were placed in each tube. After centrifugation, a top milky fraction of 1.8 ml. was removed with a supported syringe and needle, the intermediate fraction of 9.7 ml. was decanted, and the pellet was dispersed in 2.6 ml. of 0.1 *M* ethanolamine buffer, pH 9.6 (18). The distribution of enzymes is shown in Table 3. Considerably more of the xanthine

TABLE 3
Distribution of enzymes on centrifugation of the lipoprotein from cream and skimmilk

	Xanthine oxidase				Alkaline phosphatase		
	Vol. (ml.)	(units/1 ml.)	(units/1 mg. protein)	Recovery (%)	(units/1 ml.)	(units/1 mg. protein)	Recovery (%)
Lipoprotein from cream							
Original	11.5	155	60	100	592	228	100
Top	1.8	7.5	—	7.6	717	—	19.0
Intermediate	9.7	23	5.2	12.8	222	148	31.6
Pellet	2.6	472	108	69.0	824	187	31.5
Lipoprotein from skimmilk							
Original	11.5	130	9.2	100	239	17	100
Supernatant	11.5	95	—	73.0	155	—	64.8
Pellet	2.0	78	15.6	10.4	540	108	39.3

oxidase was obtained in the pellet than the alkaline phosphatase with some increase in purity of the former but not of the latter. Much of the top milky layer is probably unchurned cream or butterfat since, after drying, 95% of this top fraction was extracted with acetone and ether. The amount of this top fraction is small in terms of solids.

In some experiments the buttermilk fraction was prepared by washing the cream with magnesium chloride (0.1%). The magnesium salt had to be dialyzed from the final cream for churning to proceed satisfactorily. Some experiments suggested that the recovery of enzymes was improved, but in general the results were variable. In the ultracentrifuge it was regularly found, however, that material prepared in this way deposited considerably more of the enzymes in the pellet. The recovery of the alkaline phosphatase in the pellet was 65%; the xanthine oxidase recovery was 80%.

A typical ultracentrifuge experiment with the skimmilk lipoprotein fraction is illustrated also in Table 3. The fraction was concentrated by ammonium sulfate precipitation, and the solution contained 1.41% protein. With this material no top layer was obtained. In this instance the alkaline phosphatase recovery in the pellet was about the same as the buttermilk. Since the alkaline phosphatase protein was a small part of the total, this brings about an increase in the

purity to the approximate level of the washed cream buttermilk fraction. When the pellet was recentrifuged, over 80% of the alkaline phosphatase was found in the new pellet. Xanthine oxidase sedimented very poorly. On centrifuging acid whey, 67% alkaline phosphatase and 37% xanthine oxidase were recovered in the pellet—somewhat more than the amounts obtained when the 2.3 M ammonium sulfate whey fraction was centrifuged.

*Filtration with Filter-Cel.*² Treatment of the milk fractions with Filter-Cel provides information that may be used to characterize the different fractions. Filtration of the skimmilk lipoprotein preparation with 1% Filter-Cel mixed with it removed approximately 40% alkaline phosphatase and 20% xanthine oxidase. The removal of alkaline phosphatase by Filter-Cel is due to a rapid adsorption (19, 20), and this is probably true also of xanthine oxidase. Use of the ultracentrifuge on Filter-Cel-treated solutions of the skimmilk fraction indicated that the alkaline phosphatase in the pellet fraction was decreased to 12%, compared with 39.3% before treatment (Table 3). There was little loss of xanthine oxidase on filtration and no reduction in the pellet fraction in subsequent ultracentrifugation. The ability of Filter-Cel to remove the enzymes appears to parallel the association of the enzymes with large, easily sedimentable complexes. It was noted in the pepsin whey experiment that not only were the enzymes poorly sedimented but also the solution could be filtered with 1% Filter-Cel with only a 10% reduction in the enzymatic activities. Filtration of the cream lipoprotein preparations with 1% Filter-Cel leads to a 90% removal of both enzyme activities, indicating that both enzymes are associated in these preparations with relatively large complexes. This is supported by the ultracentrifuge results.

DISCUSSION

The milk microsomes are about 100 m μ in cross section and contain a number of enzymes in addition to xanthine oxidase and alkaline phosphatase (11). Among the other enzymes present in the milk microsome, Morton reported (11, 13) diaphorase, DPN-cytochrome C-reductase, and a hemochromogen, probably cytochrome C. Acid phosphatase, esterase, adenosine-5'-phosphatase and uricase also have been reported (15) in the microsome fraction from other tissues. The microsomes contain protein, phospholipide, and nucleic acid. They can be pictured as a parcel of enzymes cemented together by phospholipide and nucleic acid. Electrophoresis of a fat-globule membrane preparation has shown as many as three components (5). Several stages of dissociation of this complex appear to be a definite possibility. Morton (11, 13) has summarized the evidence for his conclusion that the unique membrane component of the fat-globule is the complex microsome. Size, composition, and other properties support this conclusion. Morton concluded also (14) that the low nucleic acid content of this fraction in milk compared with the microsome fraction from the mammary gland probably results from the action of a milk enzyme.

² The mention of products does not imply endorsement or recommendation by the Department of Agriculture over other products of a similar nature not mentioned.

Formerly it was believed that the microsome was a unit substance. Recent evidence (15), however, indicates that microsomes have a range of sizes and different distribution of enzymes. Each complex unit probably contains a number of enzyme proteins. By suitable dissociative methods, each enzyme protein, as well as other components, can be obtained in the free form and purified. The present studies show that xanthine oxidase and alkaline phosphatase activities are not parallel throughout the fractionation of the easily sedimented components, suggesting that these enzymes might occur in different complexes, or in different proportions in the same complex, with only slightly different physical properties. The preparations from ammonium sulfate and pepsin wheys indicate also that the alkaline phosphatase component is more strongly adsorbed to casein than is the xanthine oxidase component under a variety of conditions. An ultracentrifuge study (13) of skimmilk does not show this microsome fraction, for it is diluted out by the much larger amount of the casein complex. This study of the skimmilk fraction suggests that although the alkaline phosphatase occurs in the same form as in the cream fractions, xanthine oxidase occurs as a smaller, much less easily sedimented unit. Purification studies have indicated that the xanthine oxidase unit is much more easily dissociated (1, 2, 8) to give the free protein than is the alkaline phosphatase (20). The association of these enzymes with the fat-globule membrane gives added interest to studies of their distribution between cream and skimmilk under the influence of temperature changes, agitation, etc.

Unfortunately, the enzymes cannot serve to follow the microsome fraction during the heat treatment of milk because of their inactivation. It is proposed to characterize this fraction by its easily sedimentable nature, as well as by some other property, such as the colored cytochrome C content, to distinguish it from casein. Sedimentation studies (4) of the fat-globule membrane, after extraction with lipid solvents, did not show any easily sedimentable components. A similar experiment in the present series showed that only 8% of the total nitrogen of an extracted preparation was obtained in a pellet fraction. This suggests that the phospholipide is the major cementing component of the membrane complex. Distribution of the phospholipide with various heat treatments of the membrane complex will be of interest.

SUMMARY

The fat-globule lipoprotein membrane of milk has been prepared from cream by washing and churning. Physical and chemical properties of this fraction have been correlated with xanthine oxidase and alkaline phosphatase activities. The membrane fraction has been compared with a related fraction obtained from skimmilk in behavior in the ultracentrifuge, in filtrability, chemical composition, and enzyme content.

REFERENCES

- (1) AVIS, P. G., BERGEL, F., AND BRAY, R. C. Cellular Constituents. The Chemistry of Xanthine Oxidase. Part I. The Preparation of a Crystalline Xanthine Oxidase from Cow's Milk. *J. Chem. Soc.*, p. 1100. 1955.

- (2) BALL, E. G. Xanthine Oxidase: Purification and Properties. *J. Biol. Chem.*, 128: 51. 1939.
- (3) BRUNNER, J. R., DUNCAN, C. W., AND TROUT, G. M. The Fat-Globule Membrane of Non-homogenized and Homogenized Milk. I. The Isolation and Amino Acid Composition of the Fat-Membrane Proteins. *Food Research*, 18: 454. 1953.
- (4) BRUNNER, J. R., DUNCAN, C. W., TROUT, G. M., AND MACKENZIE, M. The Fat-Globule Membrane of Nonhomogenized and Homogenized Milk. III. Differences in the Sedimentation Diagrams of the Fat-Membrane Proteins. *Food Research*, 18: 469. 1953.
- (5) BRUNNER, J. R., LILLEVIK, H. A., TROUT, G. M., AND DUNCAN, C. W. The Fat-Globule Membrane of Nonhomogenized and Homogenized Milk. II. Differences in the Electrophoretic Patterns of the Fat-Membrane Proteins. *Food Research*, 18: 463. 1953.
- (6) HARE, J. H., SCHWARTZ, D. P., AND WEESE, S. J. The Amino Acid Composition of the Fat-Globule Membrane Protein of Milk. *J. Dairy Sci.*, 35: 615. 1952.
- (7) HETRICK, J. H., AND TRACY, P. H. Effect of High-Temperature Short-Time Heat Treatments on Some Properties of Milk. I. Inactivation of the Phosphatase Enzyme. *J. Dairy Sci.*, 31: 867. 1948.
- (8) HORECKER, B. L., AND HEPPEL, L. A. The Reduction of Cytochrome C by Xanthine Oxidase. *J. Biol. Chem.*, 178: 683. 1949.
- (9) JENNESS, R., AND PALMER, L. S. Substances Adsorbed on the Fat-Globules in Cream and Their Relation to Churning. V. Composition of the "Membrane" and Distribution of the Adsorbed Substances in Churning. *J. Dairy Sci.*, 28: 611. 1945.
- (10) MORTON, R. K. Separation and Purification of Enzymes Associated with Insoluble Particles. *Nature*, 166: 1092. 1950.
- (11) MORTON, R. K. Microsomal Particles of Normal Cow's Milk. *Nature*, 171: 734. 1953.
- (12) MORTON, R. K. Alkaline Phosphatase of Milk. I. Association of the Enzyme with a Particulate Lipoprotein Complex. *Biochem. J.*, 55: 786. 1953.
- (13) MORTON, R. K. The Lipoprotein Particles in Cow's Milk. *Biochem. J.*, 57: 231. 1954.
- (14) MORTON, R. K. Personal communication to C. A. Zittle.
- (15) NOVIKOFF, A. B., PODBER, E., RYAN, J., AND NOE, E. Biochemical Heterogeneity of the Cytoplasmic Particles Isolated from Rat Liver Homogenate. *J. Histochem. and Cytochem.*, 1: 27. 1953.
- (16) RIMPILA, C. E., AND PALMER, L. S. Substances Adsorbed on the Fat-Globules in Cream and Their Relation to Churning. VI. Factors Influencing the Composition of the Adsorption "Membrane." *J. Dairy Sci.*, 18: 827. 1935.
- (17) ZITTLE, C. A., CUSTER, J. H., AND DELLAMONICA, E. S. Determination of Xanthine Oxidase in Milk with Triphenyl Tetrazolium Chloride. *J. Dairy Sci.*, 39: 522. 1956.
- (18) ZITTLE, C. A., AND DELLAMONICA, E. S. Effects of Borate and Other Ions on the Alkaline Phosphatase of Bovine Milk and Intestinal Mucosa. *Arch. Biochem.*, 26: 112. 1950.
- (19) ZITTLE, C. A., AND DELLAMONICA, E. S. Adsorption of Bovine Alkaline Phosphatase on Diatomaceous Silica. *Proc. Soc. Exptl. Biol. Med.*, 76: 193. 1951.
- (20) ZITTLE, C. A., AND DELLAMONICA, E. S. Use of Butanol in the Purification of the Alkaline Phosphatase of Bovine Milk. *Arch. Biochem. and Biophys.*, 35: 321. 1952.