FRACTIONATION OF THE LIPOPROTEINS OF THE FAT GLOBULE MEMBRANE FROM CREAM [1]

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SUMMARY

The membrane material obtained by freezing washed cream has been fractionated from suspensions in 2% sodium desoxycholate into five sedimentable fractions representing 75% of the total material and one soluble fraction (25%). These fractions were clearly differentiated by their appearance and sedimentation behavior. Fractions A and D contained most of the xanthine oxidase activity and were about 32% lipid. Fraction B contained most of the alkaline phosphatase activity and was about 65% lipid. The other fractions were over 70% lipid. In Fractions A, B, and D, the lipid was 15% phospholipid; in Fractions C and E, it was 5-6% phospholipid; and in the supernatant, it was 50% phospholipid.

The literature on the membrane of the bovine fat globule before 1955 has been reviewed comprehensively (11). Extensive fractionation and characterization studies have been carried out on the proteins of membrane material obtained after fat extraction [e.g., Herald and Brunner (7, 9)]. However, only limited work has been done on the lipoproteins as such. Enzymic and chemical analyses have been performed on milk microsomes, a small fraction of membrane material that sedimented in 15 min. at 50,000 × g but not at 10,000 × g (3, 4). Also, material obtained by churning unwashed cream, in which it is, therefore, difficult to determine the origin of the various fractions, has been fractionated with ammonium sulfate, and membrane material from washed cream was divided arbitrarily into two fractions by centrifugation (14). These fractions were analyzed only for alkaline phosphatase activity. The present paper reports the fractionation of the lipoproteins of most of the membrane material obtained from washed cream by freezing (8), and characterizes the fractions by enzyme content and chemical analyses.

MATERIALS AND METHODS

Fresh bulk milk, kept at a temperature higher than 30° C., was separated within 3 hr. after milking. The cream was washed and membrane material prepared essentially as described previously (8). The cream, approximately 50% fat, was frozen and stored at −10° C. in test tubes (1.4 cm. i.d.) for about 20 hr.; stored at −40° C. if not required immediately, never longer than 4 wk. After thawing the cream and diluting it fourfold with 0.01% gelatin solution, the membrane material was sedimented at 78,410 × g for 1 hr.

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Xanthine oxidase and alkaline phosphatase activities were measured as previously described (8). Nitrogen was estimated by micro-Kjeldahl (2) and total phosphorus by King's method (10). Lipids were extracted by the method of Bligh and Dyer (5); lipid phosphorus was estimated by a modified method (6) based on the hydrolysis technique of Allen (1) and on the color development of Polley (13), in which the perchloric acid was neutralized and the molybdate–trichloroacetic acid reagent was concentrated to enable the use of a larger volume of test solution.

Centrifugal forces are reported as average values from the Spinco Ultracentrifuge Technical Manual. Forces below 80,000 × g were obtained with a No. 30 head, higher ones with a No. 40, in a Spinco Model L centrifuge.

RESULTS

Fractionation procedure. Centrifugation of thawed cream yielded three layers [cf. (8)]: a small opaque pellet of the membrane material; an aqueous layer that contained only traces of enzyme activity or nitrogen; and an upper fatty layer. Some fluffy material was observed at the lower edge of the fatty layer; this material was easily resuspended in water and then sedimented at 78,410 × g for 1 hr. This sediment had xanthine oxidase and alkaline phosphatase activities, nitrogen and phosphorus, in proportions similar to those in the opaque pellet, but was not studied further.

During development of the fractionation procedure, preliminary tests indicated that the conditions used to redisperse the opaque pellet material (homogenization, ultrasonic treatment; suspension in sodium chloride, potassium chloride, or sucrose solutions, or in isobutanol) did not significantly affect subsequent fractionation by centrifugation. Fractionation was attempted by differential centrifugation of resuspensions made in the solvents mentioned above, and by means of an hydroxy apatite column, but the only method that gave satisfactory results was differential centrifugation of a suspension in at least 1%, and preferably 2%, sodium desoxycholate (DOC).

The procedure finally adopted (Figure 1) was carried out at 4% C., within three days from the thawing of the cream, as follows: Pellet material was obtained from about 1-1.5 liters of washed cream. The material from each 20 ml. of cream was homogenized in 1 ml. of 2% DOC, yielding a suspension containing about 5% total solids, and the suspension adjusted to pH 7.8. This suspension, centrifuged at 54,450 × g for 1 hr., yielded brownish pellets with a halo of slightly colored material. The lighter material was gently resuspended in the supernatant, and the mixture recentrifuged under the same conditions until no more brownish pellet sedimented (five times). All of the brownish pellets were suspended in fresh 2% DOC, combined and centrifuged (Fraction A).

Similar centrifuging-washing-recentrifuging procedures at succesively higher centrifugal forces yielded further fractions. Fraction B, the light-colored material described above, was sedimented from the combined supernatants from Fraction A at 78,410 × g for 1 hr. Fraction C, a white material, was obtained

by centrifuging the supernatant from B at 105,000 × g for 1 hr. The supernatant from Fraction C was finally centrifuged at 105,000 × g for 6 hr. to yield three fractions: D—a transparent, brown, firmly packed pellet; E—a loosely packed, creamy white sediment; and S—the supernatant.

All sedimented fractions were finally resuspended in a suitable volume of water for analysis.

Analysis of fractions. Five fractionations were carried out by the above procedure, and the average analytical results and standard deviations of the means are reported (Table 1). The standard deviations include, to a large extent, the variability introduced by the subjective steps involved in the fractionation procedure.

Fraction D was obtained only in the presence of DOC. In preliminary fractionations without DOC, all xanthine oxidase activity was found in the heaviest fraction, which must be the source of Fractions A and D. However, this separation was difficult to duplicate quantitatively, as indicated by the relatively large standard deviation for the xanthine oxidase activity in Fractions A and D ($\pm$ 8.7), in contrast with the value for the sum of A + D (85.1 $\pm$ 3.9%). Fractions A and D had the same lipid content (32%) and per cent phospholipid in the lipid fraction (15%). However, the specific activity of xanthine oxidase was much higher in D than in A. Fraction B, the major fraction, contained most of the alkaline phosphatase activity and had a high lipid content and a high phospholipid content. The two smallest fractions, C and E, were similar in appearance and composition and had the highest lipid content and lowest phospholipid content. The supernatant contained 25% of the membrane material, 29% of the lipid, and 50% of the phospholipid.

The neutral lipids of the fractions contained predominantly triglycerides, with free fatty acids, cholesterol, and monoglycerides in smaller amounts. The free and total fatty acids of the fractions obtained in one experiment were determined by gas-liquid chromatography on a Pye-Argon apparatus. The values are reported as mole per cent. The free fatty acid content of the fractions varied from 1 to 5% of the total lipids. The total fatty acids of the membrane material were lauric (2%), myristic (11%), palmitic (40%), oleic (14%), stearic (24%), with smaller amounts (approximately 1%) of volatile acids, C_{13} acids, palmitoleic and C_{15} acids. Fractions C and D contained 24 and 40% lauric acid, respectively. The final supernatant contained lauric (3%), myristic (3.5%), palmitic (12%), oleic (62%), and stearic (12%).

The phospholipid composition of the fractions of one fractionation was estimated by chromatography on silicic acid–impregnated paper and total phosphate estimation in spots identified by staining with Rhodamine G (12). In all fractions except D, 60-80% of the phospholipids was lecithin, with minor amounts of lysolecithin, sphingomyelin, lysocephalin, and phosphatidyl ethanolamine and serine. In Fraction D, 40% of the total phospholipid was lecithin, 30% phosphatidyl ethanolamine, and 20% phosphatidyl serine. The supernatant fraction contained slightly higher levels (15% total) of lysolecithin, lysocephalin, and sphingomyelin than did the other fractions.

No further characterization of the lipoprotein fractions by electrophoresis and ultracentrifugation was possible, because no suitable solvent was found. Most traditional lipoprotein solvents (i.e., sodium chloride, potassium chloride, isobutanol, sodium desoxycholate, doccanol, formic acid, urea) were tried without success.

DISCUSSION

The lipid composition and, particularly, the very low volatile fatty acids content of the membrane material studied indicate that it was not contaminated by milk fat.

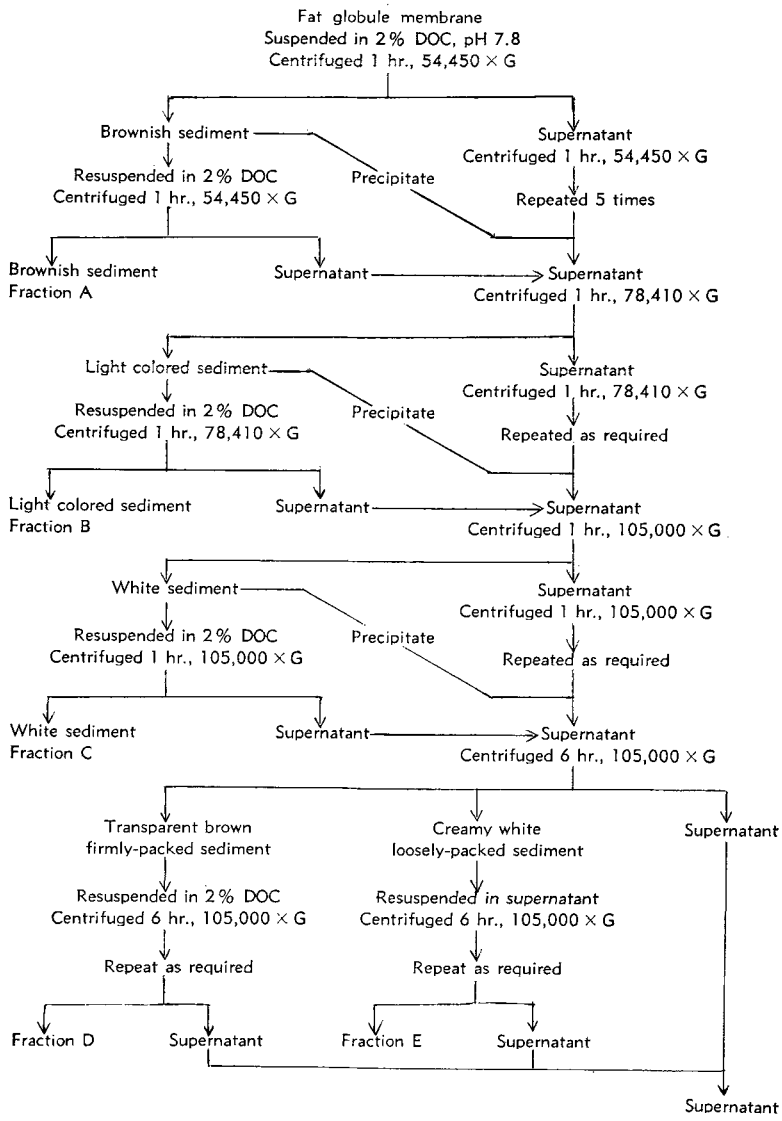

TABLE 1

Enzyme activity and composition of fractions from membrane material

Fractions	Amount of fraction,[a] % of original[d]	Xanthine oxidase activity[a]		Alkaline phosphatase activity[b]		Lipid[c] % composition[d]	Phospholipid[c] in lipid, %[g]
		% of original	Specific[e]	% of original	Specific[f]		
Original	100	100	135 ± 11	100	87 ± 30	60.2 ± 1.8	29.1 ± 5.6
A	24.9 ± 1.6	56.0 ± 8.7[h]	173 ± 24	12.7 ± 2.1	26 ± 9	32.5 ± 2.5	15.0 ± 2.7
B	35.7 ± 3.7	10.4 ± 2.3	86 ± 15	65.1 ± 4.9	343 ± 122	64.8 ± 16.4	14.7 ± 2.3
C	1.4 ± 0.3	1.1 ± 0.5	84 ± 26	1.1 ± 0.2	108 ± 34	76.1 ± 3.8	5.7 ± 1.3
D	4.5 ± 0.7	29.1 ± 8.7[h]	433 ± 83	3.3 ± 0.4	39 ± 3	32.4 ± 9.9	13.9 ± 6.4
E	3.5 ± 1.0	4.8 ± 2.2	155 ± 41	2.3 ± 0.8	84 ± 30	69.1 ± 0.5	5.1 ± 0.7
Supernatant	24.6 ± 4.6	3.4 ± 1.4	24 ± 8	3.2 ± 1.3	11 ± 2	70.2 ± 9.8	51.0 ± 20.0
Recovery	94.6	104.8		87.7			

[a] Averages ± standard deviation of the mean for five fractionations.
[b] Averages ± standard deviation of the mean for three fractionations.
[c] Averages ± standard deviation of the mean for two fractionations.
[d] Calculated from N × 6.25 and lipid weight, assuming that no other substance would significantly contribute.
[e] Micromoles of methylene blue reduced/hr/mg of nitrogen.
[f] Micromoles of phosphorus released/hr/mg of nitrogen.
[g] Assuming P × 25 = phospholipids.
[h] Per cent xanthine oxidase activity A + D = 85.1 ± 3.9.

Although obviously still impure, the fractions obtained in these tests were clearly differentiated by their appearance, their sedimentation behavior and their enzyme, total lipid, and phospholipid content. Sodium desoxycholate was necessary to separate the components. But even when suspended in 2% desoxycholate solution, about 75% of the membrane material was still sedimentable. Seventy per cent of the unsedimentable fraction was a lipid mixture containing 51% phospholipid.

The xanthine oxidase and alkaline phosphatase activities were closely associated with specific fractions, but neither was obtained free from the other. The lipid fractionation studies revealed a markedly different composition in Fraction D, which had a high lauric acid and phosphatidyl ethanolamine and serine content, and in the supernatant fraction (high phospholipid content), but again the fractionation was very incomplete. No relation between the composition of a fraction and its affinity for either of the enzymes is apparent.

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