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Isolation and Physicochemical Characterization of a Lipoprotein Fraction from Bovine Milk*

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ABSTRACT: An ultracentrifugally and electrophoretically homogeneous low-density lipoprotein fraction was isolated from disrupted microsomes from bovine milk. Microsomes were obtained by sedimentation (14,000*g*) from buttermilk and subsequently disrupted by passage through a high-pressure cell at 8000 psi. Adjustment of the density of the medium with NaBr permitted isolation of the low-density lipoprotein by flotation at 104,000*g*. The lipoprotein floated in salt solution at a density of 1.063 g/cm^3, and analysis of the flotation patterns obtained with the ultracentrifuge yielded a flotation constant, $S_f^0 = 26.2$, with a k value of 39.5 ml/g describing the concentration dependence of the flotation coefficients, S_f. Further analysis of the flotation data yielded the values 1.0496 ± 0.0015 g/cm^3 for the density of the lipoprotein in its hydrated form, 232 A for the average molecular diameter, and 3.9 × 10^6 for the approximate average molecular weight of the lipoprotein. Analysis by moving-boundary electrophoresis yielded the values $\mu = -4.6 \times 10^{-5}$ cm^2 sec^{-1} v^{-1} in phosphate buffer (pH 6.60, $\mu = 0.1$) and $\mu = -5.48 \times 10^{-5}$ cm^2 sec^{-1} v^{-1} in Veronal buffer (pH 8.6, $\mu = 0.1$). The gross chemical composition of the lipoprotein was 12.87% protein, 87.13% total lipid, 52.02% phospholipid, and 35.11% neutral lipid. The lipoprotein was unique in its high phospholipid content and extremely low cholesterol value. The fatty acid distribution of the lipoprotein was typical of fats from animal sources and did contain small amounts of short-chain fatty acids. The density of the lipoprotein in its anhydrous form, as calculated from the compositional data, was 0.983 g/cm^3.

Milk fat exists as a microscopic, immiscible emulsion of liquid fat in an aqueous phase of milk plasma at body temperature. Because of the surface forces inherent in this system, the fat takes the form of finely dispersed spheres stabilized by a third phase oriented at the fat–plasma interface, commonly referred to as the fat globule membrane. The structure of these fat globules, the identity of the materials that stabilize this natural emulsion, and the nature of the stabilizing forces have received much attention in many laboratories. Work in this area of research has been extensively reviewed by King (1955) and more recently by Brunner (1965).

In freshly secreted milk the lipoprotein complex of the fat globule membrane represents the principal protein–lipid interaction product. However, many studies have been reported on various fractions of the fat globule membrane complex in terms of their physical characteristics as well as chemical composition. Fractionations were accomplished by differential centrifugation (Alexander and Lusena, 1961), treatment with detergents (Alexander and Lusena, 1961; Harwalkar and Brunner, 1965; Hayashi *et al.*, 1965; Hayashi and Smith, 1965), or precipitation by controlling ionic strength and pH (Herald and Brunner, 1957). The fractions mentioned in the literature are often heterogeneous (Brunner and Thompson, 1961; Jackson *et al.*, 1962; Ramachandran and Whitney, 1960; Thompson, 1960) and usually have not been clearly defined by ultracentrifugation because they are insoluble in the solvents commonly employed for study with the ultracentrifuge.

This paper reports on the isolation of a soluble, homogeneous, low-density lipoprotein fraction from the fat globule membrane complex of bovine milk by the application of techniques usually employed for the investigation of serum and cellular lipoprotein complexes. The physicochemical characterization of the lipoprotein by chemical analysis, flotation in

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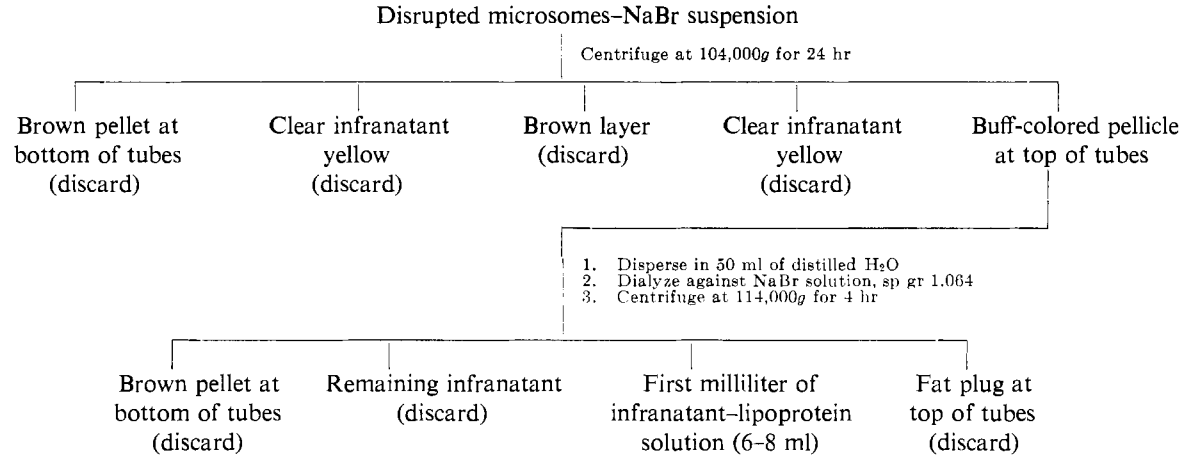

FIGURE 1: Scheme for isolation of the lipoprotein by flotation techniques.

the analytical ultracentrifuge, and moving-boundary electrophoresis is presented.

Experimental Section

Cooled bulk milk was obtained in 50-gal lots from the dairy herd at the Agricultural Research Center (Beltsville, Md.). The raw whole milk was warmed to 37° and separated with an air-tight DeLaval cream separator.[1] The cream was washed twice by dispersing in 25 gal of distilled water and reseparating. After cooling to 10° for 3 hr, the washed cream was churned with a stainless-steel-clad vane-type churn, equipped with a wooden paddle. The buttermilk was collected and the butter granules were washed with distilled water. Subsequently the buttermilk, together with the butter washings, were dialyzed against 25 gal of distilled water for at least 16 hr at 1–2°.

Ultracentrifugal Fractionation. Milk microsomes were isolated by sedimentation from the buttermilk at 14,000*g*, according to the procedure of Morton (1954), and dispersed in an aqueous NaBr solution of sp gr 1.32. The fat globule membrane was disrupted, according to the method of Marsh (1963), by extrusion through an Aminco-French pressure cell at 8000 psi with the needle valve opened sufficiently to allow the fluid to flow out slowly. The lipoprotein was subsequently isolated from the disrupted membrane by a differential preparative ultracentrifugal technique as outlined in Figure 1. The disrupted microsomes–NaBr suspension was centrifuged at 104,000*g* in the no. 40 rotor of the Spinco Model L ultracentrifuge for 24 hr, yielding several fractions of different densities. The pellicles which floated to the top of the tubes were then dispersed in distilled water and dialyzed against an aqueous NaBr solution of sp gr 1.064 at 1–2°. The lipoprotein was subsequently isolated by centrifuging at 114,000*g* in the no. 40.3 rotor of the Spinco Model L ultracentrifuge for 4 hr. During this centrifugation a small fat plug collected at the top of the centrifuge tubes while the lipoprotein was concentrated into the first clear milliliter of solution below the fat plug.

Lipoprotein solutions were then dialyzed against distilled water or NaBr solutions of appropriate specific gravity as required for further study. Specific gravities were measured with a Westphal balance.

Ultracentrifugal Analysis. The flotation rates (*F* values) of the lipoprotein in NaBr solutions of different densities were determined from the schlieren patterns obtained upon analytical ultracentrifugation at 52,640 rpm in the Spinco Model E analytical ultracentrifuge. The centrifugations were performed at 26°. Standard 4° single-sector cells with 12-mm aluminum centerpiece sections were used in the An-D rotor. Measurements of the schlieren patterns on the photographic plates were made with a Gaertner M2001 PH coordinate plate and film comparator. The flotation coefficient, S_f, was determined as outlined by Schachman (1957). The flotation coefficient at infinite dilutions, S_f^0, was calculated from values obtained at four different protein concentrations.

Concentration Dependence of S_f (Determination of k Value). The k value was calculated from the equation

$$S_f^0 = \frac{S_f}{1 - kc} \qquad (1)$$

where S_f and S_f^0 are flotation coefficients, c is the lipoprotein concentration in grams per milliliter, and k is the factor for concentration dependence in milliliters per gram (Shore, 1957). The k value was determined in the lipoprotein concentration range of 0.003–0.012 g/ml.

Calculation of Density. The density of the lipoprotein in its anhydrous form was calculated from its chemical

[1] Reference to certain products or companies does not imply an endorsement by the Department of Agriculture over others not mentioned.

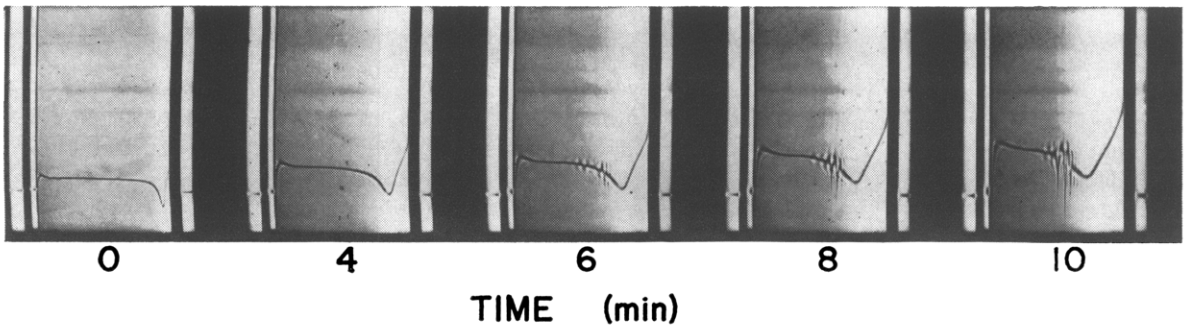

FIGURE 2: Ultracentrifugal schlieren pattern of the lipoprotein in NaBr solution of density 1.063. Time in minutes after rotor attained a speed of 52,640 rpm.

composition using the following approximate densities as suggested by Martin *et al.* (1964): protein, 1.348 g/cm^3; phospholipid, 0.968 g/cm^3; and neutral lipid, 0.913 g/cm^3.

The density of the hydrated lipoprotein was determined from measurements of the flotation rates, F, in solvents of different densities. Aliquots of the lipoprotein solution at a given concentration were dialyzed against NaBr solutions of different densities. Densities of the solvent, after dialysis, were measured by pycnometry at 26° and viscosities of the solvent were measured with an Ostwald viscometer at 26°. Values for $(\eta/\eta_0)F$ were then plotted against the solvent density to determine the density of zero flotation which was taken as the density of the hydrated lipoprotein.

Estimation of Diameter. The diameter of the lipoprotein molecule was estimated, assuming spherical particles, according to the equation (Oncley and Gurd, 1953)

$$S_f^0 = (1.063 - \rho)d^2/165 \qquad (2)$$

where ρ is the lipoprotein density in grams per cubic centimeter and d is the lipoprotein diameter in Angstroms.

Estimation of Molecular Weight. An approximate average molecular weight was calculated according to the equation (Oncley, 1963)

$$M = 0.317d^3\rho \qquad (3)$$

where ρ is the lipoprotein density in grams per cubic centimeter and d is the lipoprotein diameter in Angstroms.

Electrophoresis. Moving-boundary (free) electrophoresis was carried out in an Aminco portable electrophoresis apparatus in phosphate (pH 6.6, $\mu = 0.1$) and in Veronal (pH 8.6, $\mu = 0.1$) buffer systems.

Lipid Analysis. Total lipid content was determined gravimetrically by a microadaptation of the Roese-Gottlieb method (Mojonnier and Troy, 1922). Lipid phosphorus and total phosphorus values were measured colorimetrically by the method of Chen *et al.* (1956). The phospholipid analyses were performed either on the dried Roese-Gottlieb extracts or on samples of the lipoprotein solution extracted with alcohol–ether as suggested by Chen *et al.* (1956). The factor 25 was used to convert lipid phosphorus to phospholipid values. Cholesterol was determined according to the microfluorometric method of Albers and Lowry (1955), modified by using the Aminco-Bowman spectrophotofluorometer with a microcell of 0.1-ml capacity. Analyses were performed on the Roese-Gottlieb extracts dissolved in 1,1,2-trichloroethane. The fatty acid distribution was determined by gas–liquid partition chromatography of methyl esters of the fatty acids prepared from the Roese-Gottlieb extracts, according to the method of James (1960). A Micro-Tek 2000-R gas chromatograph equipped with dual columns and dual flame ionization detectors was used for the chromatographic analyses. A pair of matched stainless-steel columns (6 ft long and 0.25 in. in diameter and packed with 10% Apiezon L on siliconized Gas Chrom Cl) was used.

Protein Analysis. Nitrogen content of the lipoprotein solutions was determined by micro-Kjeldahl analysis using the A.O.A.C. (1945) method. Protein content was then estimated by multiplying the nitrogen values by the factor 6.25 after applying a suitable correction for the phospholipid nitrogen.

Results

Purification Procedure. The adopted isolation method yielded reproducibly homogeneous preparations of the lipoprotein. Evidence for homogeneity was obtained from the presence of a single inverted peak in the schlieren patterns obtained upon analytical ultracentrifugation in sodium bromide solutions of adjusted densities (see Figure 2). Calculation of the flotation coefficients of several preparations yielded reproducible values. The homogeneity of the lipoprotein was also confirmed by the presence of a single peak in the moving-boundary electrophoresis patterns. Uniformity of the lipoprotein preparation was also demonstrated by the results of chemical analysis.

In preliminary experiments designed to compare

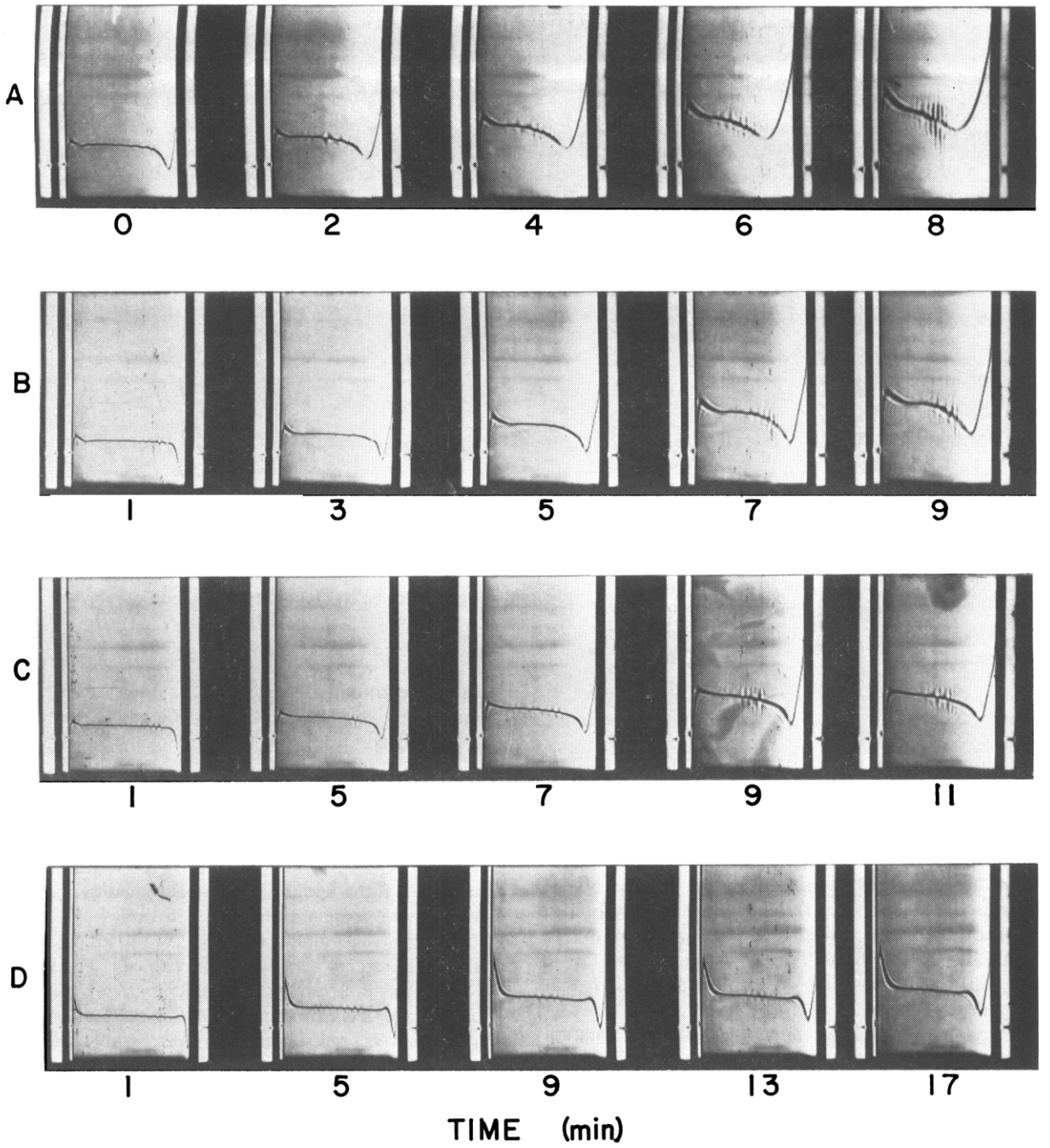

FIGURE 3: Schlieren patterns for the flotation of the lipoprotein in NaBr of different densities. Ultracentrifugal conditions were (A) 1.0691, (B) 1.0614, (C) 1.0560, and (D) 1.0533. Time in minutes after rotor attained a speed of 52,640 rpm.

isolation methods, ultracentrifugal analysis was used to test for the lipoprotein. Preparations were judged on the basis of the presence of an inverted peak in the ultracentrifugal patterns, the concentration of the lipoprotein as inferred from the peak depth, and the calculated flotation values. Using such criteria, it was determined that the centrifugal isolation of the lipoprotein from the disrupted microsomes was best performed in two steps, *i.e.*, centrifuging at 104,000*g* in a solvent of density 1.32 g/cm^3 followed by centrifugation in a solvent of density 1.06 g/cm^3, rather than centrifuging directly in a solvent of density 1.06 g/cm^3. The lipoprotein was definitely concentrated into the first milliliter of the solution in the centrifuge tubes upon the final flotation, as analytical ultracentrifugation of the infranatant below this first milliliter yielded

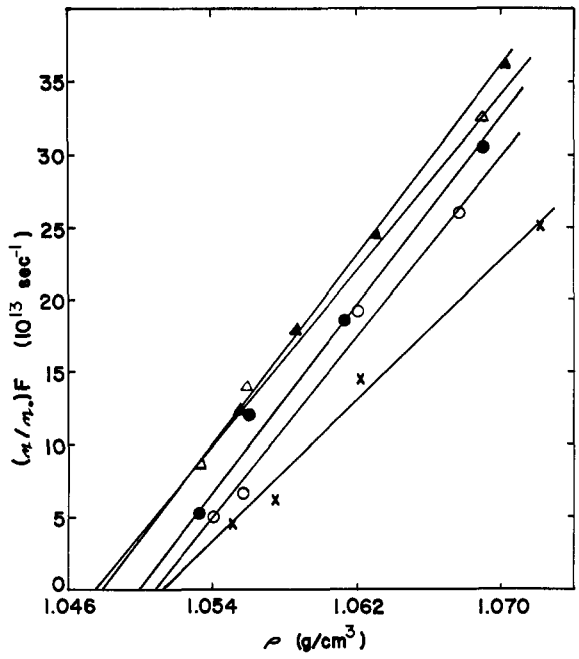

FIGURE 4: F vs. ρ plot for the determination of the density of the hydrated lipoprotein. Each set of data corresponds to an individual preparation of the lipoprotein.

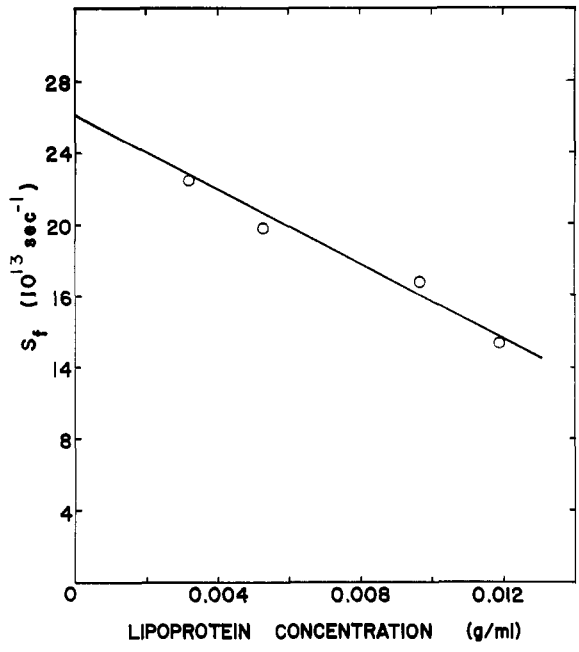

FIGURE 5: Graphical representation of the concentration dependence of the flotation coefficient, S_f.

no inverted peak but only a U-shaped salt pattern, whereas the first milliliter did contain the lipoprotein.

Similar evidence was obtained to justify the use of the Aminco-French pressure cell to disrupt the microsomes prior to isolating the lipoprotein. When the centrifugations were performed without prior passage of the microsome suspension through the Aminco-French cell no lipoprotein was isolated. Passage of the microsome suspension through the pressure cell at 16,000 psi did not increase the lipoprotein yield over that obtained at 8000 psi.

Physicochemical Characterization. A typical flotation pattern for the lipoprotein in NaBr solution at a density of 1.063 g/cm^3 is shown in Figure 2. The calculated flotation value was S_f = 23.36. The flotation patterns for the lipoprotein as obtained in NaBr solutions of different densities are shown in Figure 3. When the density of the solvent was decreased, the flotation rate of the lipoprotein was diminished as should be expected. Calculation of the flotation values and subsequent determination of density values for the hydrated lipoprotein, however, indicated an unexpected sharp decrease in the flotation rate as the NaBr concentration was lowered. The average value for five separate determinations of the density of the lipoprotein in its hydrated form, as taken from the $(\eta/\eta_0)F$ *vs.* ρ plots shown in Figure 4, was 1.0496 ± 0.0015 g/cm^3.

The flotation coefficient at infinite dilution for the lipoprotein, as determined from the data in Figure 5, was S_f^0 = 26.2 and the value of k in eq 1 describing the concentration dependence of S_f was 39.5 ml/g. The density of the lipoprotein in its anhydrous form, as calculated from its chemical composition, was 0.983 g/cm^3. Using this density value in eq 2 a value of 232 A was obtained for the average diameter of the lipoprotein molecule. The approximate average molecular weight of the lipoprotein as calculated from eq 3 was 3.9 × 10^6 mol wt units. The electrophoretic mobilities of the lipoprotein as determined by moving-boundary electrophoresis are listed in Table I.

The chemical composition of several preparations of the lipoprotein is presented in Table II. The values listed in this table are given as milligram per milliliter for the particular components in solutions of varying lipoprotein concentrations; however, examination of the data reveals uniform lipid:nitrogen and lipid:phosphorus:nitrogen ratios, as shown in Table III. The average gross lipid and protein composition of the lipoprotein was 12.87% protein, 87.13% total lipid, 52.02% phospholipid, and 35.11% neutral lipid. The lipoprotein isolated in this study is unique in

TABLE I: Electrophoretic Mobilities Calculated for Bovine Milk Lipoprotein ($\mu \times 10^5$ cm^2 sec^{-1} v^{-1}).

Buffer (pH)	Ascending Pattern	Descending Pattern
Phosphate (6.60)	−5.66	−4.59
Phosphate (6.34)	−4.31	−4.12
Veronal (8.6)	−5.77	−5.48

TABLE II: Chemical Composition of the Lipoprotein (milligrams per milliliter).

Sample	Nitrogen	Protein Nitrogen	Protein (protein N × 6.25)	Total Lipid	Lipid Phosphorus	(lipid-P × 25)	Cholesterol
A	0.351	0.239	1.49		0.249	6.2	
B	0.272	0.183	1.14		0.196	4.9	
C	0.231	0.159	0.99	6.7	0.160	5.0	0.000[a]
D	0.168	0.114	0.71	5.9			0.000[a]
E	0.221	0.112	0.70	8.3	0.238	6.0	0.009

[a] No measurable cholesterol with the method used.

that it is high in phospholipid content and unusually low in cholesterol. The distribution of the fatty acids present in the Roese–Gottlieb extracts from the lipoprotein, as determined by vapor phase chromatography of their methyl esters, is presented in Table IV.

Discussion

A single water-soluble, low-density lipoprotein, whose homogeneity was proven by ultracentrifugation and electrophoresis, has been isolated from the microsome fraction of bovine milk. It is well known that microsomes obtained from all cells contain lipid–protein complexes, and the mechanical rupture of these organelles by high pressure has been widely used to liberate lipoproteins. It is, therefore, quite reasonable that a lipoprotein could be isolated from disrupted milk microsomes by the methods used in this investigation.

The identification of the lipoprotein was primarily accomplished through measurements of flotation rates with the analytical ultracentrifuge. The chemical composition of the lipoprotein, as presented in Table II, is appropriate for a molecular entity possessing the described physical properties, *i.e.*, an anhydrous density of 0.983 g/cm^3 as calculated from these chemical data is entirely suitable for a lipoprotein with S_f^0 = 26.2. These data are consistent with the correlation between densities and S_f values given in reviews by Gurd (1960), Freeman *et al.* (1963), and Oncley and Gurd (1953).

Measurement of the density of the hydrated lipoprotein, however, led to anomalous results. The value of 1.0496 g/cm^3 reported here is much too high to correspond to a macromolecular species with the calculated S_f^0 value and with the reported compositional data. Hydration of the lipoprotein will increase its density value over that of the anhydrous form;

TABLE III: Uniformity of Lipid Nitrogen and Phospholipid:Nitrogen Ratios.

Sample	Total Nitrogen (mg/ml)	Total Lipid (mg/ml)	Phospholipid (mg/ml)	Lipid:N Ratio	P-Lipid:N Ratio
1	0.351		6.3		17.9
2	0.272		4.9		18.0
3	0.231	6.7	4.0	29.0	17.3
4	0.168	5.9		35.1	
5	0.221	8.3	5.9	37.6	26.7
6	0.299	10.0		33.4	
7	0.185	6.0		32.4	
8	0.194	7.2		37.1	

TABLE IV: Fatty Acid Composition of the Lipoprotein (mole per cent).

Fatty Acid[a]	Lipoprotein Samples A	B	C
18:0	16.14	16.46	14.62
18:1	13.57	15.84	11.89
18:2, 3	1.24	1.53	0.92
17:0	1.08	1.04	0.82
17:0, Br	1.18	1.22	0.87
16:0	43.95	44.76	39.45
16:0 Br, 16:1	1.29	1.29	1.24
15:0	1.91	1.53	2.04
15:0 Br	0.46	0.49	0.57
14:0	15.40	12.61	17.13
14:1	0.08	0.03	0.07
13:0	0.10	0.03	0.17
13:0 Br	0.18	0.16	Trace
12:0	2.78	1.94	3.95
10:0	0.23	0.21	1.42
9:0	0.03	0.03	0.40
8:0	0.12	0.18	1.56
6:0	0.21	0.28	1.22
4:0	0.05	0.39	1.66

[a] Structures obtained *via* gas–liquid partition chromatography are designated using the nomenclature proposed by Farquhar *et al.* (1959).

however, an increment from 0.983 to 1.05 g/cm^3 cannot be attributed to hydration effects alone. It is postulated that as the salt concentration of the solution was increased aggregation of the lipoprotein occurred. Therefore, when the flotation rate was measured in solutions of higher densities (higher NaBr concentrations), we were dealing with a higher molecular weight lipoprotein, and the flotation rate should be greater than would be expected on the basis of increasing the solvent density alone. At the lower salt concentrations, however, where the lipoprotein dissociates, the flotation rate was greatly reduced.

This hypothesis of aggregation is confirmed by the nature of the flotation patterns obtained at various salt concentrations. Examination of patterns shown in Figure 3 reveals that at elevated salt concentrations, convective flow spikes were present. When the salt concentration was reduced these convective flow spikes became less apparent, and at the lowest salt concentration studied the schlieren pattern was almost completely devoid of such convection peaks. The pattern obtained at the lowest salt concentration actually indicated the dissociation of the lipoprotein into separate floating and sedimenting components.

Schachman (1959) has discussed such convection phenomena in terms of either mechanical effects arising in the equipment used for the ultracentrifugation or the effects of aggregation phenomena involving the macromolecules studied (Schachman and Harrington, 1954). The convection observed in this investigation is attributed to an aggregation phenomenon inherent in the lipoprotein preparation and not to mechanical disturbances as convection phenomena have not been observed with other systems studied with the same instrument.

Therefore, on the basis of the sharp decrease in the flotation rate at lower NaBr concentrations and the nature of the flotation patterns themselves it is concluded that the lipoprotein isolated from bovine milk undergoes aggregation under appropriate conditions of salt concentration. The values reported in this paper for molecular diameter and approximate average molecular weight will then correspond to those parameters for the lipoprotein in the associated state.

Jackson *et al.* (1962) have also reported on the presence of aggregation phenomena in the ultracentrifugation of the mucoprotein of the fat–plasma interface of cow's milk on the basis of convective flow in the ultracentrifuge cell. Hayashi *et al.* (1965) have recently reported on the physical properties of some deoxycholate released lipoproteins from milk fat globule membranes. On the basis of solubility data they also showed that the aggregation of the lipoprotein particles is a function of ionic strength of the medium.

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