Kowalsky, A. (1961), Abstracts, 140th National Meeting of the American Chemical Society, Chicago, Ill., Sept, p 13C.
Kowalsky, A., and Cohn, M. (1964), *Ann. Rev. Biochem. 33*, 481.
Pimentel, G. C., and McClellan, A. L. (1960), The Hydrogen Bond, San Francisco, Calif., Freeman.
Redfield, R. R. (1953), *Biochim. Biophys. Acta 10*, 344.
Rees, P. S., Tong, D. P., and Young, G. T. (1954), *J. Chem. Soc.*, 662.
Schellman, J. A., and Schellman, C. (1964), *Proteins 2*, 1.
Schwyzer, R. (1958), *Ciba Found. Symp. Amino Acids Peptides Antimetab. Activity*, 171.
Schwyzer, R., Carrion, J. P., Gorup, B., Nolting, H., and Aung, T.-K. (1964), *Helv. Chim. Acta 47*, 441.
Sheehan, J. C., and Hess, G. P. (1955), *J. Am. Chem. Soc. 77*, 1067.
Shields, J. E., McGregor, W. H., and Carpenter, F. H. (1961), *J. Org. Chem. 26*, 1491.
Swallow, D. L., Lockhart, I. M., and Abraham, E. P. (1958), *Biochem. J. 70*, 359.
Waley, S. G., and Watson, J. (1953), *Biochem. J. 55*, 328.
Woodward, R. B., Olofson, R. A., Mayer, H. (1961). *J. Am. Chem. Soc. 83*, 1010.

Solubilization and Characterization of a Lipoprotein from Erythrocyte Stroma*

Thomas E. Morgan† and Donald J. Hanahan

ABSTRACT: A soluble lipoprotein component containing 94% lipid and 6% protein has been isolated from human erythrocyte stroma by ultrasonic irradiation in a 10% 1-butanol solution followed by density gradient ultracentrifugation. The lipoprotein thus isolated contains 68–80% of the original stromal lipid but only 9–20% of the original stromal protein. Lipid distribution was that of the intact erythrocyte. In the analytical ultracentrifuge a single peak (S_f 6.2–12.6) was obtained and on electrophoresis the lipoprotein moved as an homogeneous band with a mobility comparable to that of plasma α_2-lipoprotein. The lipoprotein was characterized by *N*-terminal serine and glutamic acid and an amino acid composition that differed from that of other erythrocyte proteins. Lipid was removed by low-temperature ether–alcohol extraction and the residual protein was again characterized. On the basis of an equilibrium ultracentrifugal technique an average molecular weight of 163,000 was determined for the lipid-free protein.

An elucidation of the interactions between lipids and proteins in the mammalian cell membrane has presented a stimulating problem for many years. A fundamental difficulty in this area of study has been the proper selection of a system in which the membrane, free of other lipid–protein complexes, could be defined with confidence. To this end the human erythrocyte has been chosen as a reasonable model for investigation of lipid–protein involvement in cell membrane structure. The mature erythrocyte does not contain any subcellular structures, such as mitochondria, hence all of the cellular lipid is considered to be located within the membrane. Previous studies in this laboratory have shown that the hemoglobin-free stroma of erythrocytes quantitatively retain the cellular lipid; thus these stroma may be considered representative of the membrane (Dodge *et al.*, 1963).

Inasmuch as hemoglobin-free stroma have been shown to have structural and functional integrity (Dodge *et al.*, 1963; Post *et al.*, 1960) as well as constant composition, several methods have been used to gain information on the structural arrangement and interaction of lipids and proteins in the stroma. One approach has been to study the removal of lipid and protein from stroma by a variety of agents (Calvin *et al.*, 1964; Ponder, 1951; Andersen, 1963); another, to observe changes in stromal enzymatic function after certain treatments (Ewers *et al.*, 1963; Weed *et al.*, 1963); and yet another approach has been to attempt further characterization of the stromal protein after solubilization and the removal of lipid from these complexes (Maddy, 1964). Some insight into cell membrane structure has been gained in this manner, but the ques-

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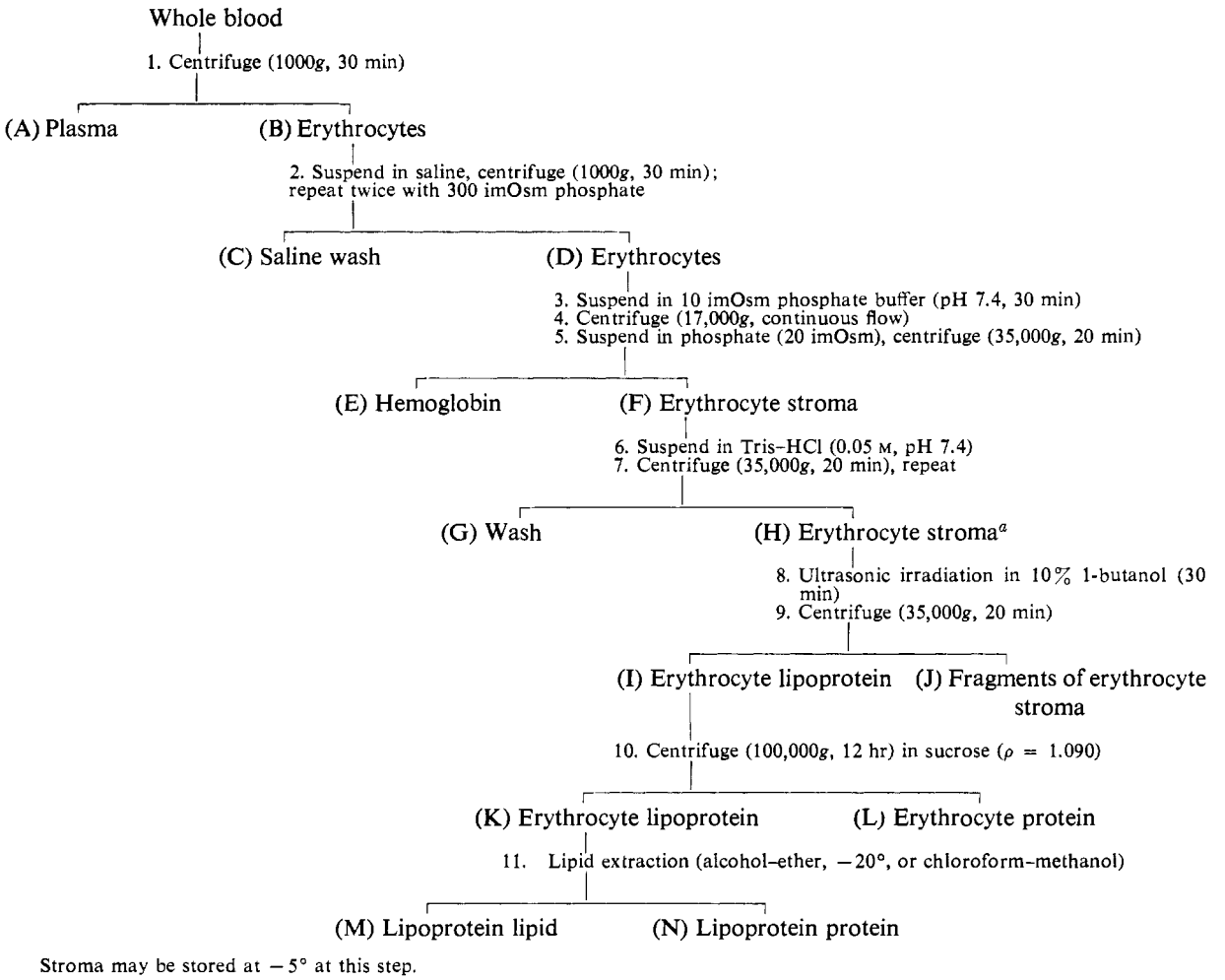

FIGURE 1: Procedures employed in the isolation and solubilization of components of human erythrocyte stroma.

tion as to whether all of the lipid and protein in the membrane is involved in a lipoprotein complex has remained unanswered. In the present study of the lipid–protein complexes of the stroma a physicochemical approach has been adopted in which the ability of 1-butanol to effect solubilization of erythrocyte lipids and proteins has been utilized. Morton (1954) showed that this reagent possesses special properties which make it valuable for the separation and isolation of lipoprotein enzymes. The use of 1-butanol coupled with ultrasonic irradiation of erythrocyte stroma released soluble products which have been partially characterized by physical and chemical techniques.

Experimental Procedures

Preparation of Erythrocyte Stroma. The method of preparation of stroma is outlined in Figure 1. Venous blood obtained from normal adult donors was collected in 10% acid citrate–dextrose (solution A, U.S.P. grade) and centrifuged at 1000*g* for 30 min at 4° (Figure 1, step 1). The plasma (A)[1] and buffy coat were removed by aspiration and all subsequent operations were performed at 0–4°. The cells (B) were washed once with two volumes of 0.9% saline, then twice with two volumes of 310 ideal milliosmolar (imOsm)[2] sodium phosphate buffer (pH 7.4), and then resuspended in one volume of isotonic phosphate (step 2). All buffers were prepared as described by Dodge *et al.* (1963). Hemoglobin-free erythrocyte stroma was prepared by suspending one volume of suspended cells (D) in 30 volumes of 10 imOsm phosphate buffer, pH 7.4, for 30 min (step 3). This mixture was centrifuged at 17,000*g* in a continuous-flow apparatus (Servall–Szent-Györgi and Blum) and the stroma were collected in 50-ml polypropylene cups (step 4). The stroma were washed repeatedly by resuspension in five volumes of 20 imOsm phosphate and recentrifugation at 17,000*g* for 20 min until less than 1% of the original hemoglobin remained (step 5). Finally, the stroma (F)

[1] Capital letters refer throughout to fractions designated in Figure 1.

[2] Abbreviations used: imOsm, ideal milliosmolar.

were diluted to one-fourth the original red cell volume with 20 imOsm phosphate, divided into 5-ml aliquots and stored at −20° until used.

Ultrasonic Irradiation of Hemoglobin-Free Stroma. Frozen stroma (F) were thawed at room temperature, centrifuged at 35,000*g* for 20 min, and resuspended in 0.05 M Tris–HCl, pH 7.4, 4° (Figure 1, steps 6–7). Centrifugation and resuspension were repeated to remove inorganic phosphate. 1-Butanol (Baker and Adamson, reagent grade) was added to make a 10% (v/v) mixture which was irradiated at 4° for 30 min using a 20-Kcycle Raytheon ultrasonic generator at 1.3–1.5-amp output (step 8). The resulting homogeneous, opalescent mixture was centrifuged at 35,000*g* for 20 min to remove a small amount of insoluble protein (J). Protein, phospholipid phosphorus, and cholesterol were measured on the supernatant and sediment. In some experiments sonic irradiation was interrupted before 30 min (at 2 and 5 min) and the supernatant was isolated or additional erythrocyte stroma added. Irradiation was then resumed for a total of 30 min. The supernatant solution was treated further by mixing with 34% sucrose (ρ = 1.305) to form a solution of density near 1.090. This solution was centrifuged for 10–12 hr at 100,000*g* in a Spinco Model L ultracentrifuge using a 39 SW swinging-bucket rotor (step 10). In certain instances a continuous sucrose density gradient was formed by the method of Britten and Roberts (1960) with centrifugation as above.

Lipid Extraction. Lipid was removed from erythrocyte stroma lipoprotein (K), prepared as described above, by a modification of the method of Scanu *et al.*, 1958 (step 11). One volume of lipoprotein (K) suspension was mixed with 25 volumese thanol–diethyl ether (3:1, v/v) at −20°. After standing for 2 hr, the mixture was filtered by gentle aspiration, and the precipitate transferred to an apparatus for continuous diethyl ether extraction at −20° for 48 hr.[3] At the end of this time the precipitated protein, virtually free of lipid, was dissolved in 0.05 M Tris–HCl, pH 7.4, for further studies. Lipids were also extracted in certain instances from stroma (H) or lipoprotein (K) by use of chloroform–methanol mixtures. Methanol, followed by chloroform and 0.1 M KCl, was added to stroma or lipoprotein to form an extraction mixture of chloroform–methanol–aqueous KCl (8:4:3, v/v). Emulsions were broken by centrifugation, the lower solvent phase was removed, and the upper layer was reextracted as before. The lower phases were pooled, dried by rotary evaporation *in vacuo*, and redissolved in chloroform for subsequent analysis. This lipid extraction method was shown to extract more than 95% of cholesterol and organic phosphorus present in the stroma or lipoprotein.

Analytical Procedures. Lipids were separated by column and thin layer chromatography and were identified and quantified using methods previously reported (Morgan *et al.*, 1963). Protein was determined by the method of Lowry *et al.* (1951) using crystalline serum albumin (Pentex Corp.) as a reference standard. Density of sucrose solutions was estimated by the refractive index method using the Abbe refractometer. Residual stromal hemoglobin was estimated by the method described by Dodge *et al.* (1963).

Stromal proteins separated by preparative ultracentrifugation were partially characterized by *N*-terminal amino acid analysis (Fraenkel-Conrat *et al.*, 1954), total amino acid analysis (Spackman *et al.*, 1958), electrophoretic mobility on cellulose acetate and on starch and 3% acrylamide gels (Smithies, 1955; Ornstein and Davis, 1964),[4] and ultracentrifugal analysis for sedimentation constant (Schachman, 1959). Lipids were extracted as described above and the protein was oxidized with performic acid, treated with fluorodinitrobenzene, and hydrolyzed for 4 or 24 hr in HCl. *N*-Terminal residues were recovered, chromatographed on paper, and eluted for spectrophotometric quantitation. Other lipid-free samples were hydrolyzed (see Table V) and chromatographed using the Spinco amino acid analyzer for total amino acid determination. These analyses were performed on the supernatant lipoprotein (K), sedimented proteins (L) isolated by preparative ultracentrifugation, and on the lipoprotein before (K) and after (N) lipid extraction at low temperature. A Spinco Model E analytical ultracentrifuge with Schlieren or absorption optics was used for determination of sedimentation constants. Correction factors obtained from Svedberg and Pedersen (1940) or International Critical Tables (1929) were applied to adjust the observed values to a NaCl solution, density 1.026 at 26°, for direct comparison with reported plasma lipoprotein values.[5] The molecular weight of erythrocyte lipoprotein (N) was estimated by the approach-to-equilibrium method of Schachman (1959) using absorption optics and fringe analysis.

Results

Solubilization Studies. Initial experiments were designed to determine the optimum conditions for solubilization of erythrocyte stromal protein. An im-

[3] The apparatus described by Scanu *et al.* (1958) was modified so that the cold box was replaced by a methanol–Dry Ice bath at −20°.

[4] Electrophoresis on cellulose acetate was performed with the Microzone and Analytrol apparatus (Beckman Instrument Co., Palo Alto, Calif.) according to the manufacturers recommendations. Horizontal starch and acrylamide electrophoresis was performed using a Canalco apparatus (Canal Industrial Corp., Bethesda, Md.).

[5] The notation S_f is used to designate negative S values. The following correction formula was applied

$$S_{26^\circ,\mathrm{NaCl},1.063} = S_{T^\circ,\mathrm{suc},d} \times \left[\left(\frac{\eta_{T^\circ}}{\eta_{26^\circ}}\right)_{\mathrm{suc}}\left(\frac{\eta_{\mathrm{NaCl}}}{\eta_{\mathrm{suc}}}\right)^{T^\circ}\left(\frac{\eta_d}{\eta_{1.063}}\right)^{T^\circ}_{\mathrm{suc}}\left(\frac{1-\bar{V}(1.063)}{1-\bar{V}(d)}\right)\right]$$

where T^0, d = observed temperature and density; suc = sucrose solution in 0.05 M Tris–HCl, pH 7.4; η = viscosity (in millipoise); and $\bar{V}$ = partial specific volume (assumed value of 0.950).

TABLE I: Effect of Physical Methods on Release of Protein from Erythrocyte Stroma.

Method	% of Total Protein in Supernatant[a]
Freezing-thawing with 10% 1-butanol	6
Vigorous mixing with 10% 1-butanol, 15 min	13
Ultrasonic irradiation, no butanol, 15 min, 4°	27–80
Ultrasonic irradiation, with 10% 1-butanol, 15 min, 4°	78–91

[a] Aliquots (20 mg) of hemoglobin-free erythrocyte stroma (F) were treated as shown. The amount of protein remaining in the supernatant after centrifugation at 35,000*g*, 20 min (Figure 1, step 9), was determined and shown as per cent of total protein treated.

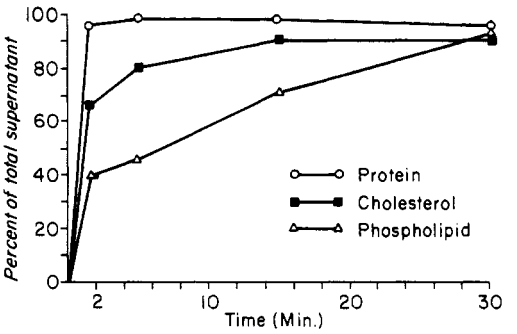

FIGURE 2: Effect of time of ultrasonic irradiation on solubilization of erythrocyte stromal protein, cholesterol, and phospholipid. Stroma (H) was suspended in 0.05 M Tris–HCl, pH 7.4, containing 10% 1-butanol (v/v) at 4°. Each point represents an average of three determinations of protein, cholesterol, or phosphorus remaining in supernatant after centrifugation at 35,000*g* for 20 min.

portant aspect of this problem was the adoption of an operational definition of solubility; soluble lipid and protein was considered to be that remaining in the supernatant of a mixture after centrifugation at 35,000*g* for 20 min at 4°.

Stromal suspensions (H) in 0.05 M Tris–HCl (1:4 v/v) were exposed to freezing and thawing, vigorous mixing (15 min on a Clay-Adams cyclomixer) with 10% 1-butanol and ultrasonic irradiation for 15 min with and without added 10% 1-butanol. The results of the various treatments (given in Table I) indicate that ultrasonic irradiation in the presence of 1-butanol was most effective in solubilizing erythrocyte protein. Irradiation in the absence of 1-butanol gave highly variable results. Consequently, the influence of time of ultrasonic irradiation on the solubilization process was followed; the results are presented in Figure 2. During the initial phases of sonic irradiation (2–5 min) a supernatant lipoprotein could be isolated by centrifugation which contained relatively less lipid and more protein than the final product. Interruption of irradiation, isolation of lipoprotein, and resumption of irradiation for a total of 30 min did not alter these lipid–protein ratios. When erythrocyte stroma was added during the interruption the final lipoprotein contained less lipid as expected. On the basis of these results, a 30-min irradiation at 4° in a mixture of Tris–HCl and 10% 1-butanol was used in all subsequent experiments.

The erythrocyte lipoprotein (I) prepared by ultrasonic irradiation could be further fractionated into a supernatant lipid-rich fraction (K) and a sedimenting protein-rich fraction (L). This fractionation was accomplished by preparative ultracentrifugation in a medium of density greater than 1.063. The fractions thus obtained were found to have marked differences in composition and physical properties. The lipid and protein com

TABLE II: Composition of Erythrocyte Stromal Fractions after Ultrasonic Irradiation and Preparative Ultracentrifugation.[a]

Expt	Starting Wt of Stroma (H) (mg)	Fractions	Total Wt (mg)	% of Lipid	% of Protein	% of Total Lipid by Wt: Triglyceride	Cholesterol	Phospholipid
1	72.1	Supernatant (K)	41.9	95	5	8	33	59
		Sediment (L)	26.0	45	55	17	32	50
2	82.0	Supernatant (K)	41.1	95	5	3	34	62
		Sediment (L)	40.0	45	55	10	45	45
3	71.7	Supernatant (K)	39.7	91	9	8	27	69
		Sediment (L)	24.1	38	62	11	25	64

[a] Preparation of erythrocyte fractions K and L from stromal fraction H as shown in Figure 1, steps 1–11.

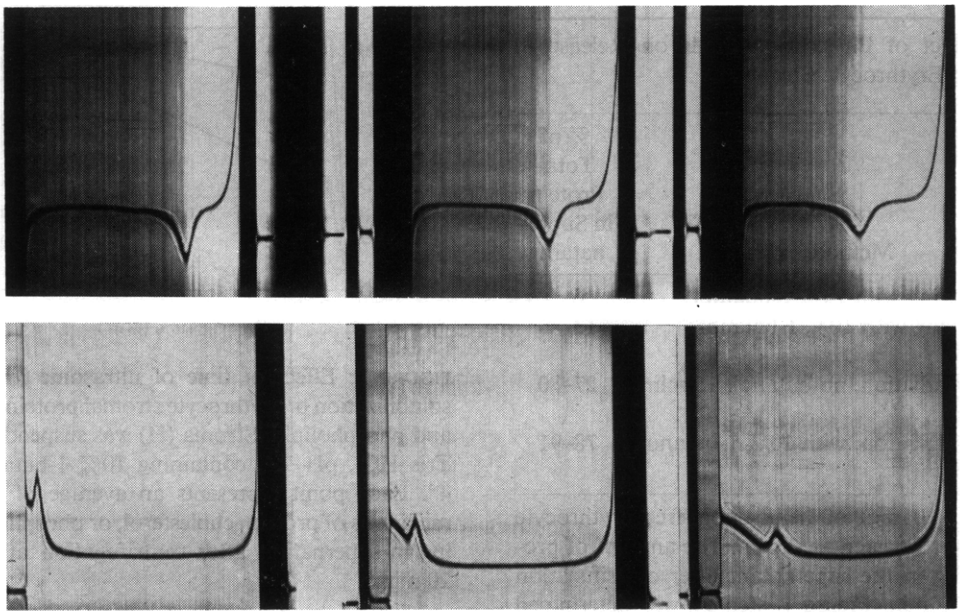

FIGURE 3: Typical ultracentrifugal behavior of erythrocyte stromal lipoprotein (K) before (upper series) and after (lower series) low-temperature lipid extraction. In the upper series, protein concentration 1.5 mg/ml, ρ = 1.091, exposures at 0, 4, 8 min, S_f 12.6; in the lower series protein was 1.1 mg/ml, ρ = 1.010, exposures at 0, 16, 32 min. Observations were at 59,350 rpm, 20°, in 0.05 M Tris–HCl with sucrose in Spinco Model E ultracentrifuge with schlieren optics.

TABLE III: Comparison of Erythrocyte and Serum Lipoproteins.

	% of Total Lipid[a]							
	TG	PL	Cholesterol	% of Lipid[a]	% of Protein[a]	Density (g/ml)	Sedimentation Coefficient (S_f)	Electrophoretic Mobility
Intact erythrocyte (D)[b]	10	62	28	35–45	55–65	...	...	..
Erythrocyte stroma after irradiation (H)[b]	2–6	39–63	35–58	71–82	18–29	...	...	..
Erythrocyte lipoprotein (K)[b]	3–8	59–69	27–34	91–95	5–9	1.060–1.070	6.2–12.6	α_2
Serum lipoproteins[c]								
High density	17	44	34	40–65	35–60	1.063–1.200	1	α_1
Low density	14	25	60	75–80	20–25	1.006–1.063	0–20	β_1
	55	20	13	90	10	<1.006	20–400	β_1
	85–90	6–9	3	98	2	...	400	..

[a] Per cent by weight. [b] Preparations were carried out as indicated in Figure 1. Figures are ranges obtained in three or more duplicate determinations. [c] Data from Lindgren and Nichols (1960).

position of the supernatant (K) and sediment (L) are shown in Table II. The difference in composition of the supernatant and sedimenting fractions is emphasized by the calculation that the supernatant lipoprotein (K) contains 68–80% of the original stromal lipid, but only 9–20% of the original stromal protein.

Characteristics of Stromal Lipoprotein. The studies described below were concerned with the nature of the supernatant lipoprotein (K), its composition, physical properties and specific lipid and protein moieties.

PHYSICAL CHARACTERISTICS. The supernatant lipoprotein (K) obtained as described above was dialyzed *vs.* 0.05 M Tris–HCl, pH 7.4, for 12 hr at 4° to remove sucrose and 1-butanol. Subsequently, in the analytical ultracentrifuge an inverted peak separated at low speed (39,460 rpm) and rose to the top of the cell (Figure 3).

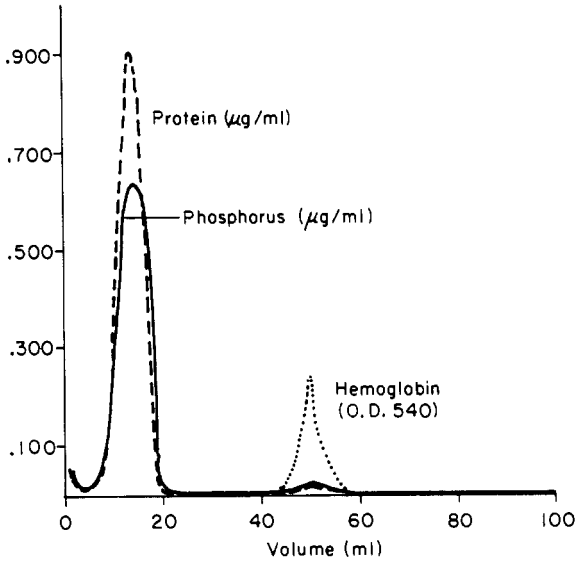

FIGURE 4: Chromatographic behavior of erythrocyte lipoprotein (K) on Sephadex G-200. The sample (10.8 mg in 3.0 ml of 0.5 M Tris–HCl, pH 7.4) was applied to a 1.8 × 23 cm column (total volume 60 ml) and eluted at 23° with 0.5 M Tris–HCl, pH 7.4. Protein and phosphorus were determined by methods given by Morgan *et al.*, 1963. Elution of hemoglobin marker added after completion of lipoprotein chromatography is shown.

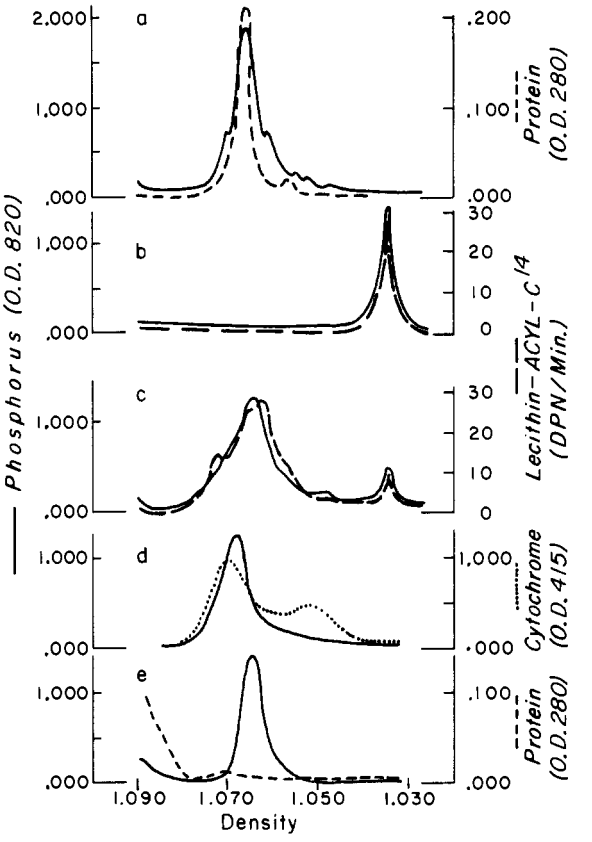

FIGURE 5: Sucrose density gradient centrifugation of erythrocyte lipoprotein. Gradients were formed by the method of Britten and Roberts (1960) with approximately 10 mg of lipoprotein (K) in a total volume of 5.0 ml and centrifuged 16 hr at 100,000*g*, 4°. Samples were removed by puncture of the bottom of the centrifuge tube. Representative diagrams are shown after centrifugation of lipoprotein (K) alone (a), of lecithin-acyl-^{14}C (1 μmole, 0.1 μc) alone (b), and of lipoprotein (K) and lecithin-acyl-^{14}C irradiated together (c). In (d) cytochrome C (1 mg) was irradiated and centrifuged with erythrocyte lipoprotein. Results of irradiation and centrifugation of sedimentable erythrocyte lipoprotein (L) and lipoprotein total lipid extract (M) are shown in (e). For further details see text.

TABLE IV: Erythrocyte Phospholipid Distribution.

	% of Total Phospholipid[a]	
	Intact Erythrocyte (D)	Erythrocyte Lipoprotein (K)
Phosphatidylethanolamine	21	26
Phosphatidylcholine	32	28
Phosphatidylinositol-sphingomyelin	40	40
Phosphatidylserine	8	10

[a] Chloroform–methanol extracts were chromatographed on thin layers of silica gel G, the components identified and phosphorus determined by the method of Doizaki and Zieve (1963).

Values for $S_{f1.063,NaCl,26°}$ on three separate lipoprotein preparations were 11.0, 12.6, and 6.2. A small amount of protein (approximately 10% of each sample) sedimented very rapidly and compacted at the bottom on the cell.

The lipoprotein (K) behaved as expected on Sephadex G-200 column chromatography wherein a single peak containing all the applied lipid and protein was eluted in the void volume (Figure 4). Furthermore a single, uniform peak was obtained by density gradient centrifugation (Figure 5a). Electrophoretic mobility on cellulose acetate (Figure 6) and 3% acrylamide gel showed it to be comparable to plasma α_2-lipoprotein. The major protein component moved as a broad homogeneous band but a small amount of protein remained at the point of application to the cellulose acetate and was presumably denatured. On starch gel electrophoresis (borate buffer, pH 8) the lipoprotein (K) remained at the origin suggesting that the size of the complex was too large to allow it to enter the gel.

CHEMICAL NATURE OF STROMAL LIPIDS AND PROTEINS. Total lipids extracted with chloroform–methanol from

TABLE V: Amino Acid Composition of Erythrocyte Stromal Proteins.[a]

Amino Acid	Erythrocyte Protein (L)		Lipoprotein (K)		Lipoprotein after Lipid Extraction (N)	
	Residues	g/100 g[b]	Residues	g/100 g	Residues	g/100 g
Lysine (inc. ε-DNP-lysine)	24	4.18	20	4.98	20	5.08
Histidine	1	0.18	1	0.26	1	0.27
Arginine	19	3.93	25	7.42	24	7.27
Aspartic	75	11.81	43	9.75	43	9.95
Threonine	47	6.70	32	6.38	36	7.39
Serine	50	6.26	36	6.36	38	6.90
Glutamic	110	19.19	64	16.04	78	19.96
Proline	37	5.02	25	4.90	26	5.20
Glycine	51	4.56	36	4.61	36	4.70
Alanine	61	6.41	43	6.52	40	6.20
Cystine/2	1	0.14	0	0.00	1	0.21
Valine	41	5.72	33	6.58	31	6.31
Isoleucine	36	5.62	25	5.58	23	5.24
Leucine	78	12.18	64	14.30	48	10.95
Phenylalanine	11	2.07	16	4.50	13	3.73
Cysteic acid	6	1.11	2	0.64	6	1.77
Tyrosine	23	4.99	0	0.00	0	0.00
Meth-sulfone	6	1.10	0	0.00	5	1.74
Methionine	0	0.00	1	0.25	0	0.00
Minimum molecular weight[c]	84,800		59,300		58,100	

[a] Results are averages of two determinations after 24-hr hydrolysis in constant-boiling HCl at 105°; results after 10-hr hydrolysis were comparable. All samples were subjected to prior performic oxidation. [b] Amino acid (g)/100 g of protein. [c] Approximate values based on unit amino acid residues (histidine, half-cystine, methionine) as shown.

whole irradiated stroma (H) and from supernatant lipoprotein (K) were generally similar in composition to those found in intact erythrocytes (Table III) with somewhat higher cholesterol content in irradiated stroma (H). Also, phospholipid distribution in supernatant lipoprotein (K) was similar to that of the intact erythrocyte (Table IV). Results of amino acid analysis are given in Table V. Glutamic acid and serine were *N*-terminal; the latter possibly being derived from residual phosphatidyl serine.

Nature of Protein Obtained from Stromal Lipoprotein by Low-Temperature Extraction of Lipid. Extraction of stromal lipoprotein with ethanol–diethyl ether (3:1, v/v) at −20° for 2 hr removed 98% of the cholesterol and 88% of the phospholipid phosphorus. Extraction was continued for an additional 48 hr with diethyl ether at −20°. At the end of this extraction period 100% of the cholesterol and 95% of the phospholipid had been removed. The protein residue was redissolved in 0.05 M Tris–HCl buffer, pH 7.4, forming a slightly opalescent solution. Ultracentrifugation showed a major component which sedimented with $s_{20,w}$ = 3.5 and 2.8 S (Figure 3). A small amount of material remained at the top of the cell and did not sediment after 80 min at 59,450 rpm. This may have been residual lipoprotein or a protein of low density. Recentrifugation of lipoprotein (K) at 100,000*g* for 12 hr in sucrose at density 1.070 followed by dialysis of the supernatant against 0.05 M Tris–HCl removed this minor component. The weighted-average molecular weight of the lipid-free protein component was 163,000 as determined by the sedimentation-equilibrium procedure of Schachman (1959). Meniscus, z-average, and bottom-cell values were 151,000, 163,000, and 192,000, respectively. Electrophoretic mobility on starch gel, 3% acrylamide, and cellulose acetate (Figure 6) was greater than that of α_1-lipoprotein of plasma and very nearly equal to that of albumin. Glutamic acid was *N*-terminal; no *N*-terminal serine was found. Amounts of dinitrophenyl(DNP)-amino acids obtained were too small to permit accurate quantitation (0.03–0.05 μmole/sample) but corresponded to the yield expected for proteins of 50–100,000 molecular weight. Results of amino acid analyses are given in Table V.

Studies on Interaction of Lipids and Proteins of Erythrocyte Stroma. The interaction of lipids and protein derived from erythrocyte stroma (H) by ultrasonic irradiation was studied further by a series of experiments using sucrose gradient density centrifugation. As shown previously, the supernatant lipoprotein (K) has an isopycnic density of 1.060–1.070 (Figure 5a). Under the same conditions the isopycnic density of phospholipids extracted from erythrocyte stroma is 1.025–1.045 (Figure 5b). In order to study the association of erythrocyte stroma protein and lipid, a mixture

of 4.5 ml of buffered suspension of hemoglobin-free erythrocyte stroma (H), 0.5 ml of 1-butanol, and 20 μl of lecithin-acyl-^{14}C (1 μM, containing 1.7 μc[6]) was irradiated for 30 min as described above. The mixture was then handled as described for erythrocyte stroma (steps 8–10) and the supernatant lipoprotein centrifuged in a sucrose density gradient. The resulting pattern, shown in Figure 5c, indicated that labeled phospholipid had associated with the lipoprotein or that exchange of phospholipid had occurred. That isotopic exchange alone could not account for these results is suggested by the fact that approximately equimolar amounts of labeled and nonlabeled lecithin were present yet more than 90% of the labeled phospholipid appeared in the lipoprotein peak within 30 min at 0–4°.

The possible association of stromal protein and lipid with exogenous lipophilic protein during ultrasonic irradiation was then studied by repeating the experiment using cytochrome *c* (9.5 μmoles) instead of lecithin (Figure 5d). Cytochrome *c* (crystalline beef heart, Sigma) was chosen because of its known affinity for lipid and the ease of determination of the reduced form by its absorption band at a wavelength of 415 mμ. In the absence of erythrocyte stroma its isopycnic density was approximately 1.050. When cytochrome *c* was irradiated in the presence of erythrocyte stroma and 1-butanol the lipoprotein and cytochrome were found together with a density about 1.080.

Inasmuch as these results suggested a nonspecific association of lipid and protein might exist in the supernatant lipoprotein (K) the affinity of sedimentable protein (L) for lipid was tested in the following representative experiment. Stroma (H, 6.1 mg of protein) was irradiated in 10% 1-butanol as above [Figure 1 (8)] and the supernatant lipoprotein (K) was removed by preparative centrifugation in a sucrose solution (ρ = 1.090). The sedimented erythrocyte protein (L) contained 4.9 mg of protein and contained much smaller amounts of lipid. This protein was resuspended in 1.8 ml of Tris–HCl buffer (0.05 M, pH 7.4), mixed with 0.2 ml of 1-butanol containing 10 μmoles of total lipid phosphorus extracted from another aliquot of erythrocyte stroma and subjected to ultrasonic irradiation for 30 min. A sucrose density gradient was prepared as before and, after centrifugation, analyzed for phosphorus and protein (Figure 5e). The results show clearly that sedimented erythrocyte protein (L) has little or no affinity for erythrocyte lipids and suggest that the lipid-complexing properties of supernatant and sediment proteins are quite different.

Discussion

In this study it has been shown that a soluble lipoprotein component containing 94% lipid and 6% protein can be obtained from the hemoglobin-free stroma of human erythrocytes by ultrasonic irradiation in a 10% 1-butanol solution. This lipoprotein has been

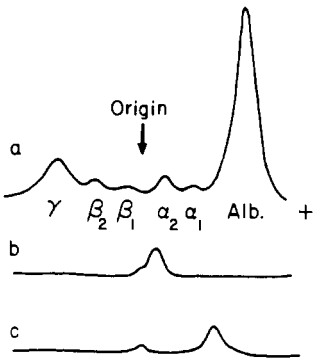

FIGURE 6: Electrophoresis on cellulose acetate of (a) serum proteins, (b) erythrocyte lipoprotein (K), and (c) lipoprotein protein (N). Electrophoresis was carried out for 20 min at 23°, 250 vdc, barbital buffer, pH 8.6 in a Spinco Microzone apparatus. Analysis was made after staining with Ponceau-S on the Analytrol apparatus.

obtained repeatedly from the erythrocyte stroma of several normal adult humans and forms a homogeneous, opalescent sol which is stable for several days at 0–5°. After its isolation by preparative ultracentrifugation, it shows a single peak on analytical ultracentrifugation (S_f 6.2–12.6), on starch block and cellulose acetate electrophoresis (mobility about that of plasma α_2-lipoprotein), and possesses the same lipid composition as the intact erythrocyte. The protein was further characterized by the presence of *N*-terminal glutamic acid and an amino acid composition which, though approximate, was reproducibly different from that of other erythrocyte proteins. Lipid was removed by low temperature ether–alcohol extraction and the residual freely soluble protein was characterized by a set of analyses similar to those cited above. In addition, molecular weight for the lipid-free protein was estimated by an approach-to-equilibrium ultracentrifugal technique.

Membrane solubilization by ultrasonic irradiation may be the result of disruption of the protein–lipid substructure with reassociation of the liberated lipid with lipophilic protein. It is likely that the lipids of the membrane are dissociated by the action of 1-butanol and ultrasonic irradiation in the manner suggested by Morton (1954). Maddy has applied this principle to bovine erythrocyte stroma and has shown that the dissociation of lipid and protein can be accomplished in 1-butanol with the relatively small energy input of stirring (Maddy, 1964). The probable course of events in the present experiments is the same as outlined by both Morton and Maddy as is indicated by the following factors. First, the composition of supernatant material varies with the time of irradiation in butanol (Figure 2). These data suggest that liberation and reassociation of lipid–protein complexes is occurring as sonic irradiation proceeds. When sonic irradiation was interrupted at 2, 5, and 15 min, followed by centrifugal isolation of lipoprotein and additional irradiation for a

[6] Prepared according to the method of Hanahan *et al.* (1964).

total of 30 min, no change in the initial protein, cholesterol, or phospholipid composition was noted. Addition of lipoprotein to nonirradiated stroma gave a final product whose composition reflected the starting mixture. Second, the lipids of the solubilized lipoprotein (I) were found to be similar to those of intact erythrocytes (B) and hemoglobin-free erythrocyte stroma (F). However, this soluble lipoprotein contained only about 15% of the total erythrocyte stromal protein and differed from the total protein in several respects, *e.g.*, density, electrophoretic mobility, and amino acid composition. Thus, the protein of the soluble lipid–protein complex but not the lipid, appears to possess some degree of specificity. Further evidence supporting the suggestion that the lipid–protein substructure is disrupted and subsequently reassociated is given by the results of the series of experiments presented in Figure 5. Lipid added to the erythrocyte stroma before ultrasonic irradiation is incorporated into the lipoprotein or exchanges with lipoprotein phospholipid and, similarly, a lipophilic protein such as cytochrome *c* is incorporated under similar conditions. On the other hand, ultrasonic irradiation of a mixture of erythrocyte total lipid and nonlipophilic stromal protein (L) using the same procedure did not result in the production of a lipid–protein complex. Therefore, it is possible that any erythrocyte lipid freed by ultrasonic irradiation associates or reassociates with a specific lipophilic stromal protein. The resulting lowered density of the protein then permits flotation and isolation of the lipoprotein complex for subsequent analyses. Alternatively, partial dissociation of stromal lipoproteins may occur during ultrasonic irradiation so that proteins normally associated with lipophilic proteins by protein–protein interaction may be excluded from this lipoprotein complex. Similarly, lipid enrichment of the lipoprotein can occur. Notwithstanding lack of knowledge of the mechanism, reproducible results are obtained by this method.

Hagerman and Gould (1951) have demonstrated that serum lipoprotein lipids exchange with erythrocyte lipids *in vivo* and *in vitro*. Therefore it is of interest to compare the data on the nature of the erythrocyte lipoprotein (K) with those of the well-characterized serum lipoproteins. The properties and analyses of these lipoproteins are presented in Table III. Considering both the erythrocyte lipoprotein (K) and the lipid-free protein (N) derived from it, the erythrocyte lipoprotein (K) appears to be most similar to serum high-density lipoprotein (HDL_2-HDL_3) especially with regard to density, electrophoretic mobility of the protein after lipid removal, and amino acid composition. Thus, both lipoproteins have densities greater than 1.060, comparable electrophoretic mobility of the protein moieties (Scanu *et al.*, 1958), and absence of methionine, tryptophan, and half-cystine (Shore, 1957). Certain differences also exist, however, especially with regard to protein and lipid composition, sedimentation coefficient, and *N*-terminal amino acid content. Thus, the protein content of erythrocyte lipoprotein is much lower than that of serum high-density lipoprotein coupled with a correspondingly higher S_f value. As regards lipid composition the erythrocyte lipoprotein complex contains less cholesterol and more phospholipid (with correspondingly less protein) than serum lipoproteins of comparable density and electrophoretic mobility. These facts suggest that erythrocyte lipid composition may be determined, at least in part, by specific associative properties of lipophilic proteins peculiar to the red cell. This suggestion also fits the observation that although erythrocyte lipid composition is more or less dependent on serum lipid composition the erythrocyte lipids do not correspond exactly to serum patterns.

The sedimentation coefficient for a lipoprotein is determined by both lipid and protein composition and, therefore, erythrocyte lipoprotein (K) is, not surprisingly, more nearly comparable with serum low-density lipoprotein (β_1-lipoprotein). It should be noted also that the *N*-terminal acid of erythrocyte lipoprotein (K) is glutamic acid (also found in low-density lipoprotein) rather than aspartic acid as reported for serum high-density lipoprotein (Shore, 1957). Unfortunately, total amino acid analyses of low-density lipoprotein are not available for comparison with erythrocyte lipoprotein.

These comparisons are not intended to suggest that the protein components of serum and erythrocyte lipoproteins are identical but rather to suggest in which respects they may be similar. On the basis of the present results it is possible to compare the lipid-free protein of erythrocyte lipoprotein (N) with a comparable protein from serum α_1-lipoprotein as reported by Scanu *et al.* (1958). The erythrocyte protein contained 5% residual phospholipid after 50-hr extraction as compared with 0.6% residual lipid in α_1-lipoprotein after 24-hr extraction as reported by Scanu *et al.* (1958). There was no residual cholesterol in our preparation. Solubility was similar to that reported by Scanu but some instability of the lipid-free erythrocyte protein has been noted after dialysis for removal of low molecular weight contaminants. Thus, an average *S* of 3.2 was observed immediately after lipid extraction. Subsequent dialysis of this preparation for 24 hr yielded a product with an average *S* of 5.9 and an estimated molecular weight of 163,000. These observations suggested that molecular aggregation and dimerization occurred during dialysis in which case the estimated molecular weight of the 3.2-*S* unit would be approximately 80,000 as compared with a minimum molecular weight of 58,000 calculated from amino acid analysis.

It must be emphasized that the approach adopted in these studies represents an attempt to study the association of well-defined membrane lipids with protein by a physicochemical approach. The success of this method and the significance of the results achieved by its use must remain conjectural. For example, emphasis has been placed on the specific nature of the protein obtained but the possible association of a protein with no affinity for lipid to the lipid–protein complex by protein–protein interaction cannot be excluded. Nevertheless, this method may provide a new approach to the study of the structure of the membrane and hopefully, may shed light on such problems as the exchangeability

of membrane lipids, lipid–protein interaction, and the changes attendant on aging of erythrocytes.

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References

Andersen, V. (1963), *Clin. Chem. Acta 8*, 454.
Britten, R. J., and Roberts, R. B. (1960), *Science 131*, 32.
Calvin, M., Evans, R. S., Behrendt, V., and Calvin, G. (1964), *Proc. Soc. Exptl. Biol. Med. 61*, 416.
Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963), *Arch. Biochem. Biophys. 100*, 119.
Doizaki, W. M., and Zieve, L. (1963), *Proc. Soc. Exptl. Biol. Med. 113*, 91.
Ewers, A. C., Haskell, C. M., and Fineberg, R. A. (1963), *J. Cell. Comp. Physiol. 61*, 195.
Fraenkel-Conrat, H., Harris, U. I., and Levy, A. L. (1954), *Methods Biochem. Analy. 2*, 359.
Hagerman, J. S., and Gould, R. G. (1951), *Proc. Soc. Exptl. Biol. Med. 78*, 329.
Hanahan, D. J., Ziro, S., and Pappajohn, D. J. (1964), *in* Proceedings of the International Symposium on Lipid Transport, Meng, H. C., Ed., Springfield, Ill., Thomas.
International Critical Tables (1929), Vol. 5, New York, N. Y., McGraw-Hill.
Lindgren, F. T., and Nichols, A. V. (1960), *in* The Plasma Proteins, Vol. 2, Putnam, F. W., Ed., New York, N. Y., Academic.
Lowry, O. H., Rosebrough, N. V., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Maddy, A. H. (1964), *Biochim. Biophys. Acta 88*, 448.
Morgan, T. E., Tinker, D. O., and Hanahan, D. J. (1963), *Arch. Biochem. Biophys. 103*, 54.
Morton, R. K. (1954), *Biochem. J. 57*, 595.
Ornstein, L., and Davis, B. J. (1964), Eastman Kodak Technical Bulletin, Rochester, N. Y.
Ponder, E. (1951), *Blood 6*, 350.
Post, R. L., Merritt, C. R., Kinsolving, C. R., and Albright, C. D. (1960), *J. Biol. Chem. 232*, 1796.
Scanu, A., Lewis, L. A., and Bumpus, F. M. (1958), *Arch. Biochem. Biophys. 74*, 390.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic.
Shore, B. (1957), *Arch. Biochem. Biophys. 71*, 1.
Smithies, O. (1955), *Biochem. J. 61*, 629.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford.
Weed, R. I., Reed, C. F., and Berg, G. (1963), *J. Clin. Invest. 42*, 581.