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Some chemical characteristics of the lipids of human and bovine erythrocytes and plasma*

Donald J. Hanahan, Ruth M. Watts, and Douglas Pappajohn

Department of Biochemistry, University of Washington, Seattle 5, Washington

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SUMMARY

The results of a study on the chemical nature of the natural lipids and phospholipids in bovine and human erythrocytes and blood plasma are presented. The fatty acid distribution in the various lipids is reported and certain aspects of the general findings are discussed.

Turner *et al.* (1, 2) reported the separation of human and bovine erythrocyte phospholipids by paper and column chromatography. In these studies a decided difference was noted in the lipid pattern in the two species, but only limited information was offered on the chemical nature of the individual lipid components. Phillips has described the fractionation of human serum phospholipids (3), and the isolation and assay of plasma lipoproteins (4). Hirsch and Ahrens (5) have described the chromatographic fractionation of the entire lipid fraction of human plasma. In the course of a study of the possible role of phospholipids in cation transport, Kirschner (6), through the use of pyridine, fractionated the phospholipids of swine erythrocytes into four major components, but no detailed chemical analyses were reported.

Although the above observations have been of considerable value, it appears worthwhile to acquire additional knowledge on the chemical nature of the blood lipids. Accordingly, this paper presents information on the fractionation and characteristics of the lipids found in the erythrocytes and plasma of man and cow.

EXPERIMENTAL

Materials. All solvents were reagent grade. Chloroform contained 0.75% ethanol as a preservative. In instances of apparent decomposition, this solvent was washed with water and alkali, and distilled over barium oxide. When necessary, diethyl ether (anhydrous, Merck) was distilled over sodium for removal of peroxides; otherwise it was used as received. Commercial hexane, obtained from Phillips Petroleum Company (Bartlesville, Oklahoma), was redistilled from potassium permanganate (dissolved in a small amount of acetone) and the fraction distilling at 67.5° to 69.5° was collected. Silicic acid (Mallinckrodt) reagent grade, 100 mesh for chromatographic analysis, was dried for 12 hours at 110° before use. This latter point is particularly important in the chromatography of the neutral lipids, in which "anhydrous" conditions are necessary for the best separations. The Hyflo Super-Cel was obtained from Johns Mansville. The long-chain fatty acids, for chromatography standards and used in some of the synthetic procedures, were obtained from the California Foundation for Biochemical Research (Los Angeles, California), the Hormel Foundation (Austin, Minnesota), and Applied Science Laboratories (State College, Pennsylvania). Triolein from the California Foundation for Biochemical Research showed a fatty acid ester to glycerol (molar) ratio of 2.97, and revealed only oleic acid by gas-liquid chromatography. Both cholesterol palmitate and cholesterol oleate were synthesized (7), and purified by silicic-acid chromatography and recrystallization from chloroform-acetone. Snake venom (*Crotalus adamanteus*) was obtained from the Ross Allen Reptile Institute (Silver Springs, Florida).

Methods. Many of the analytical procedures, e.g., P, N, choline, have been described previously (8, 9,

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The following abbreviations are used: C, chloroform; M, methanol; DNP, dinitrophenyl-; H, hexane; B, benzene; and E, diethyl ether.

10). Glycerol was identified and determined by a paper chromatographic technique (11), a method utilizing periodate oxidation followed by formaldehyde assay (11), and, in certain instances, by isolation as the tribenzoate (12). However, it should be noted that the hydrolytic cleavage of glycerol-containing lipids should be effected in 1 to 2 N KOH in ethanol, or in 1 to 2 N aqueous HCl. Glycerol is labile in 4 to 6 N HCl and may be lost upon concentration in acid solutions at atmospheric pressure or *in vacuo*. The colorimetric assay for fatty acid ester was that of Goddu *et al.* (13), as modified by Lands (14). Total cholesterol was estimated by a colorimetric procedure (15). Plasmalogens (vinyl ethers) were determined essentially by a methanolic iodine procedure as outlined by Siggia and Edsberg (16), and Rapport and Franzl (17). In order to extend this assay system to levels of 0.1 to 1.0 μmoles, a spectrophotometric procedure, which employed the spectral absorption peak of iodine in methanol at 360 mμ, was used.[1] Although another absorption peak at 292 mμ could have been employed, ordinary glass tubes transmit more light at the higher wave length and interference from impurities is less. In the estimation of the iodine number of lipid fractions, the Yasuda procedure (18, 19) was applied to all sterol fractions, and the Wijs technique to all other lipid fractions.

The hydrolytic release of fatty acids from glycerides and glycerol-containing phospholipids was accomplished by reflux of the lipids in 0.5 N alcoholic KOH for 4 to 6 hours, followed by removal of alcohol *in vacuo*, acidification of the aqueous mixture, and extraction with diethyl ether. In the case of the sphingosine-containing fractions, the fatty acids were obtained as methyl esters through reflux in methanolic-sulfuric acid as described by Brady and Koval (20), or as the free acids by reflux in 6 N HCl for 8 to 10 hours. The position of the fatty acids on lecithins was determined through the action of the phospholipase A in *Crotalus adamanteus* venom (21), which effects hydrolytic cleavage of the β ester (C-2) fatty acid (22).

Ash and cation analyses were performed by Laucks Testing Laboratories (Seattle, Washington). Infrared spectra were obtained with a Perkin-Elmer Model 21 instrument (NaCl optics), with samples in $CHCl_3$ solution or as Nujol mulls. All optical rotation values were obtained with a Rudolph polarimeter.

The fatty acids were analyzed by gas-liquid chromatography of their methyl esters. These esters were prepared by interesterification in anhydrous methanolic HCl or by treatment of the free acids with diazomethane (23). The two procedures gave comparable distribution patterns for the fatty acids. The esters, in hexane, were chromatographed on a 4-foot column of 15% succinate-ethylene glycol polymer (prepared in this laboratory by Dr. J. N. A. Ridyard) on Celite® (Johns Mansville, 80 to 100 mesh) using a Pye Argon Chromatograph (Cambridge, England). At 175°, with an Argon flow rate of 30 ml per minute, stearic acid and oleic acid methyl esters were separated in 10 minutes (separation factor, 1.2). At the 10 times setting (assumed to be approximately 1×10^{-7} amperes) and at 1000 volts, the detector was linear in response over a tenfold range to moles of individual long-chain fatty acid esters. This range was from 3.3×10^{-3} to 3.3×10^{-2} moles (1 to 10 μg) of long-chain fatty acid esters.

Carbohydrates were detected by the Molisch or anthrone reaction. Free amino acids in the intact lipid samples were determined by chromatography on Whatman No. 1 paper with C-M 4/1 (v/v) water (0.5%), and subsequent spraying with ninhydrin (24). The paper chromatographic technique of Westley *et al.* (25) was employed to check for the presence of nonlipid peptides. The detection of certain lipids and contaminating nonlipid material was accomplished by chromatography on silicate-impregnated glass fiber filter paper in a pyridine-benzene-water system (26)[2].

Isolation of Lipids. The bovine blood samples were collected in 0.2% sodium citrate and 2% glucose at pH 7.4 to 7.5. The human blood was collected in 0.264% sodium citrate, 0.090% citric acid, and 0.294% dextrose. Normally, the samples were processed within 2 hours after collection.[3] No attempt was made to select the age and sex of the donor, but obviously lipemic blood was not used in these experiments.[4]

[1] Dr. J. N. A. Ridyard, unpublished observations.

[2] The authors are indebted to Dr. J. G. Hamilton for details of this technique prior to publication.

[3] In a few instances the human blood samples were stored for 24 hours at 4°. On rare occasions it was necessary to store the erythrocytes in the frozen state overnight. Upon thawing at 35°, extensive hemolysis was evident and only one-tenth the volume of water was added and the procedure continued as described. No apparent difference in behavior of lipids isolated from these samples, as compared to the immediately processed material, was noted.

[4] In two instances human blood collected in heparin was studied. The results of fractionation showed considerable alterations of the phospholipid fraction of the erythrocyte. This was especially evident with the more acidic type of phospholipid, i.e., phosphatidylethanolamine, which tended to form lyso derivatives. Consequently, blood collected in heparin was not considered a reliable source for "native" phospholipids from the erythrocyte and was not used in any of the experiments described here.

The whole blood was centrifuged at 700 × *g* and 4° in 600 ml plastic cups for 20 minutes. The supernatant fluid plus buffy coat was removed by gentle aspiration and the cells brought back to the original volume of blood with 0.9% NaCl, pH 7.4 to 7.5. After careful stirring this mixture was recentrifuged as above. One additional saline wash was employed and the volume of the packed cells was measured. The washings and plasma[5] were combined and recentrifuged at 700 × *g* for 20 minutes at 4° to remove any residual red cells and buffy coat. It would appear unlikely that a significant number of platelets was present in the isolated erythrocyte fraction. However, since platelets are present in the isolated plasma, the results of Rouser *et al.* (27, 28) and of Marcus and Spaet (29) would suggest that the major portion of the plasma phosphatidylethanolamine (and phosphatidylserine) must have been derived from this source.

Lipids were isolated from both plasma and red cell hemolysate by the same techniques. To the packed cells one-fourth volume of distilled water was added with stirring and the mixture was allowed to stand for 10 minutes at 25°. The hemolysate (or plasma) was then poured slowly with constant stirring into three times its volume of 95% ethanol and allowed to stand in the dark at room temperature. After 2 hours a volume of diethyl ether equal to that of the hemolysate was added and the mixture stirred again. One hour later the mixture was filtered through Eaton and Dikeman No. 615 filter paper. The moist filter cake was resuspended in an amount of 95% ethanol:diethyl ether 3/1(v/v) sufficient to cover the residue, and allowed to stand in the dark at room temperature for 2 to 3 hours. After filtration the combined filtrates were concentrated at 35° to 37° in a cyclone evaporator until less than 2% of the original solvent remained. This concentrate was then extracted twice with twice its volume of diethyl ether, and the ether-soluble extract was washed at least three times with one-tenth volume of water. If two phases failed to form, a small amount of ethanol was used to break the emulsion. With successive water washes a considerable amount of dark red pigment (but no fatty acid ester) was removed from the lipid extract, and the final extract usually had a light straw color. In actual practice it was found that this washing was at least as effective in the removal of free amino acids, nonlipid sugar, mineral ions (such as NaCl), and traces of urea, as the procedure of Folch *et al.* (30). The final ether extract was evaporated to dryness at 30° to 35° in a rotary evaporator. The residue was dissolved in chloroform and stored at 4°. Usually this chloroform fraction was analyzed immediately for plasmalogens, unsaturation, and total phosphorus. Because of the pronounced solubility of certain of the phospholipids in acetone at temperatures as low as —25°, a fractionation of the simple lipids and phospholipids by solvent treatment alone was not feasible.

Chromatographic Procedures. In part, these techniques have been described previously (31, 10). A quantitative separation of neutral lipids and phospholipids in blood was achieved by the technique of Borgström (32). The total lipid fraction was placed on a silicic-acid column in chloroform and eluted with this solvent until no more neutral lipid was removed. Subsequently the phospholipids were eluted with 10 column-volumes of methanol or C-M 1/9(v/v). In most instances the flow rate was 2.5 to 3.5 ml per minute, but a faster and equally suitable separation can be effected by the inclusion of 1 part Hyflo Super Cel per 2 parts silicic acid in the column. Such columns were also used for the fractionation of phospholipids. The load was 15 to 20 mg neutral lipid and 0.3 to 0.5 mg phospholipid phosphorus per g of silicic acid, with a height-to-diameter ratio of 7 to 10. The same considerations apply to the separation of neutral lipids, which were separated essentially as described by Barron and Hanahan (31). It is important that certain of the solvents, particularly the benzene, be dried over silicic acid prior to use. Hexane is preferred to pentane or low boiling petroleum ether to avoid bubbling of the column. Fractionation was checked by cholesterol and colorimetric fatty acid ester assays and by infrared spectrophotometry according to Freeman *et al.* (33). The latter was particularly valuable in the determination of cholesterol esters and glycerides with an absorption band at 5.78 μ, especially since the colorimetric test for cholesterol esters presented some difficulties (34). Triolein, cholesterol palmitate, and cholesterol oleate all showed absorbancies proportional to concentration at this wave length. The unesterified cholesterol fraction, as well as any components eluted later, was examined in the infrared for the presence of fatty acid esters.

[5] To date, 10 to 12 units of human blood and nearly 200 liters (in 10- or 20-liter batches) of bovine blood have been processed. The results indicate a good reproducibility of extraction and fractionation. In certain instances blood from humans with a previous history of jaundice or malaria was used, but no apparent differences from "normal" were observed.

RESULTS

Erythrocyte Lipids.[6] The total lipids were separated into neutral lipids and phospholipids by silicic-acid chromatography, and each fraction was weighed, as well as assayed, by infrared examination and phosphorus determination. The recoveries ranged from 96% to 98% and in three typical samples gave the following neutral lipid-to-phospholipid ratios (wt/wt): *Human*, 0.57 to 0.67; *Cow*, 0.50 to 0.58. A separation of the "stroma" by centrifugation at pH 5.0 at 3000 × *g* for 30 minutes, and isolation of its lipids showed that 90% to 92% of the total lipid of the intact cell resided therein, and subsequent fractionation showed the same distribution of lipids as found in the intact cell.[7] A rather similar distribution pattern was noted by Ponder (35).

The fractionation of the neutral lipids of the human erythrocytes is illustrated in Figure 1. An almost identical separation pattern was obtained with the neutral lipids of bovine erythrocytes. The fatty acid composition of typical fractions obtained from these two sources is given in Table 1. In both species unesterified cholesterol was the major component (80%).

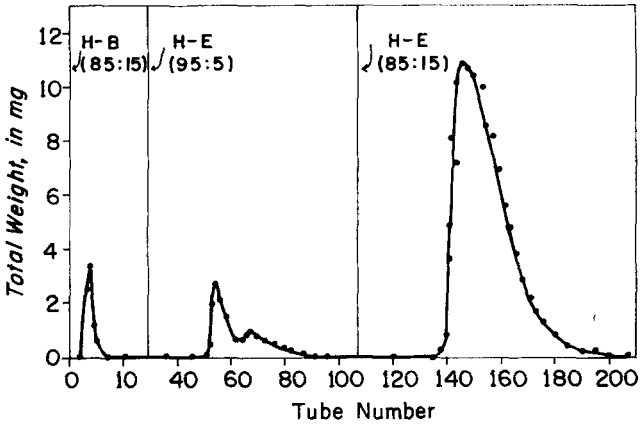

FIG. 1. A typical chromatographic separation of neutral lipids of human erythrocytes. In this instance, 290 mg total neutral lipids were chromatographed on a 20 g silicic-acid column (height to diameter = 7.5), flow rate, 1.8 ml/minute. Volume of eluate in each tube was 4.3 ml.

In cow erythrocytes the entire weight of the fraction eluted with hexane-benzene 85/15(v/v) represented cholesterol esters, but in human cells only one-half of the total weight was cholesterol ester. The "contaminant," which did not contain any long-chain fatty acid, may be a hydrocarbon. No further information on this fraction is available.

In both species the main fatty acid ester components were the long-chain esters of cholesterol, which represented 4% of the total neutral lipids. In part, this was ascertained by a 12-hour reflux with 2 N alcoholic KOH and subsequent isolation of cholesterol in 90% to 95% yields and of long-chain acid in 95% yields; cholesterol to fatty acid ratios (molar)[8] ranged from 0.98 to 1.05. It is of interest that these fatty acid esters were considerably more unsaturated than the triglycerides (see below) and showed the typical fatty acid pattern described in Table 1. The sterol, which was isolated from hydrolysis of the intact ester, had the same properties as the free cholesterol in the H-E 85:15 fraction and was considered to be identical.

An examination of the hexane-diethyl ether 95/5 (v/v) fraction from the two sources revealed it to contain triglycerides and accounted for nearly 10% of the total neutral lipid, a conclusion justified by fatty acid ester assay and by the infrared spectrum (36). Long-chain fatty acids were present in 95% to 97% of theoretical yield; no evidence was obtained for the presence of any long-chain alkyl glyceryl ethers. Less than 0.5% of this fraction was composed of free fatty acids. While the iodine number of the triglycerides varied from 5 to 35, over 80% of the preparations had values in the 5-to-10 range, and there is no doubt that this component was consistently much more saturated than the cholesterol esters. This is supported by data on the composition of the fatty acids from a typical run (Table 1).

In each species the only substance detected in the hexane-diethyl ether 85/15(v/v) fraction was free cholesterol; 90% of this material was recovered from methanol recrystallization and showed the following characteristics: m.p. 151°-152° (uncorrected; melting point block); $[\alpha]_D^{25}$ -40.0° (6% in C-M 1:1). Authentic cholesterol melted at 151°-152° (uncorrected; melting point block) $[\alpha]_D^{25}$ -39.0° (6% in C-M 1:1). A mixture of the unknown and pure cholesterol showed no alteration in melting point. The infrared spectra and X-ray diffraction patterns of the unknown and authentic cholesterol showed them to be identical.

Diethyl ether was used to remove any remaining nonphosphorus-containing fatty acid esters from the column. In each source approximately 2% of the total

[6] The results described here and the data given in the various tables represent the average (or composite) values of at least six different samples of blood. In general, deviations in composition between samples were no greater than ±10% of the values reported here.

[7] J. Dodge, unpublished observation.

[8] All subsequent composition ratios will be given as molar ratios.

TABLE 1. FATTY ACID COMPOSITION OF NEUTRAL LIPIDS OF BOVINE AND HUMAN ERYTHROCYTES AND PLASMA

Fatty Acids	Specific Distribution in Mole Percentage										
	Saturates *					Unsaturates *					
	14:0	16:0	18:0	20:0	22:0	14:1	16:1	18:1	18:2	20:4	22:?
Bovine Erythrocytes											
Cholesterol esters	1	9	1	2	†	1	4	9	61	6	6
Triglycerides	†	62	12	†	†	†	†	20	7		
Bovine Plasma											
Cholesterol esters	2	6	1			2	6	10	67	4	
Triglycerides		30	19			2	6	24	14	2	
Human Erythrocytes											
Cholesterol esters	9	11	4	†			†	13	63	†	
Triglycerides		31	13			1	5	30	12	2	
Human Plasma											
Cholesterol esters		17	4				6	22	51		
Triglycerides		22	6	†			4	41	17	10	†

* Numbers following the colons refer to the degree of unsaturation considered to be present in a particular fraction, i.e., 18:1 would be comparable to a monoenoic acid such as oleic acid, 18:2, a dienoic acid, such as linoleic acid, and so on.
† Trace.

neutral lipid of the erythrocytes was found in this eluate. Although the amounts available were very limited, infrared spectra, fatty acid ester assays by the hydroxamic acid reaction and by infrared, and total weight determination indicated a mixture of di- and monoglycerides. No further analyses were made on this fraction.

The phospholipids of the erythrocytes were fractionated by elution from a single column of silicic acid into at least five components. It was evident that the major difference in distribution of phospholipids of the two species occurred in the choline-containing types. In the bovine erythrocyte, sphingomyelin was the predominant form and accounted for nearly 95% of the choline-containing compounds; however, in the human source, lecithin was the main form. Nonetheless, it is interesting to note that the total choline-containing phospholipids in both sources comprised 55% to 60% of the total phospholipids.

The fractionation of bovine erythrocyte phospholipids is illustrated by the curve presented in Figure 2 and the data given in Table 2. Plasmalogen assay on the original, fresh sample (before application to the column) and on isolated fractions showed only trace amounts to be present. The C-M 6:1 fraction contained a fast-moving, highly pigmented phosphorus-containing substance, (A), similar in properties to a "polyglycerylphospholipid" (37), a slower moving major component, (B), and a minor, trailing fraction (B′). Fraction A was present to the extent of 0.5% to 1% of the phosphorus applied and was not examined in detail. Fraction B represented nearly 28% of the total phospholipids, and possessed the characteristics of a diacyl glycerylphosphorylethanolamine (Table 2). Fraction B contained 98% of ethanolamine and less than 2% of a serine-containing phospholipid (38). Ethanolamine and serine were also identified by paper chromatography and, in one instance, ethanolamine was isolated in 60% yields from an acid hydrolysate, as a DNP-derivative (m.p., 90°-91°; authentic DNP-ethanolamine, m.p., 90°-91°). Inasmuch as the optical rotation value, $[\alpha]_D^{25}$ +6.2°, of the B fraction was similar to that of a synthetic phosphatidylethanolamine of known configuration (39), it appears to be of the L-α-type. An infrared spectrum of this fraction gave the anticipated absorption peaks and in particular showed the absence of an absorption band at 10.35 μ.[9]

[9] Lecithin and sphingomyelin show a characteristic absorption band at 10.35 μ, which is absent in compounds such as phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and derivatives. This is in agreement with reports by other investigators (39, 40 to 43).

A very low content of mineral ions (<1.0% ash) was found. The fatty acid pattern of fraction B, shown in Table 2, illustrates the high content of unsaturated fatty acids.

TABLE 2. COMPOSITION OF THE PHOSPHOLIPIDS OF BOVINE ERYTHROCYTES *

	C-M 6:1 (B)	C-M 1:1 (C) †	‡ (E)
P, per cent	3.90	3.27	3.8
N, per cent	1.78	1.87	3.47
Choline, per cent	None	None	14.7
N/P, molar ratio	1.00	1.26	2.01
Choline/P molar ratio	—	—	0.99
Iodine number	77.5	53.2	39.2
$[\alpha]_D^{25}$	+6.2° §	Not run	+8.9° §
Inositol/P molar ratio	0.0	0.23	0
Fatty acid/P molar ratio	2.01	2.09	1.04
Fatty acid ester/P molar ratio	(1.15-1.70 ‖)	1.92	0.01
Fatty Acids in Mole Percentage			
Saturates			
14:0	trace	None	
16:0	4	9	
18:0	9	33	
Unsaturates			See Table 7
16:1	trace	None	
18:1	59	37	
18:2	21	13	
20:4	6	7	

* These fractions were obtained by silicic-acid chromatography as described in the text and shown in Fig. 2. These data represent a composite of all fractionations completed to date. In general, the deviations in composition were ±10% of the values shown; however, relationships within and between fractions were the same as illustrated here.

† This phospholipid fraction was the only one that had a significant ash content, with sodium representing 90% of the cation.

‡ Small fraction, D (see Fig. 2), in the immediate forefront of this fraction was lecithin. For a description of its properties see text.

§ 10% in C-M 10:1.

‖ In general, the hydroxamic acid color test for fatty acid esters failed to give reproducible results with this fraction. Consequently, the lipid was hydrolyzed and the fatty acids were isolated, weighed, and the neutral equivalent determined. An average molecular weight of 279 was obtained and no evidence for the presence of any long-chain aldehydes was found.

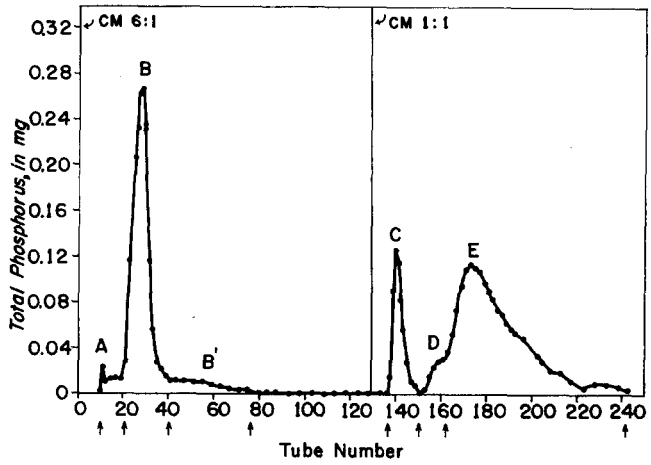

FIG. 2. The chromatographic fractionation of bovine erythrocyte phospholipids on a silicic-acid column. A total of 7.7 mg lipid phosphorus (including the neutral as well as the phospholipids) was placed on 15 g silicic acid plus 7.7 g Hyflo Super-Cel (height to diameter = 10), and the neutral lipids eluted with chloroform. Subsequently, the phospholipids were eluted with the solvents as indicated in the above figure; flow rate 2.4 ml/minute; tube volume, 3.9 ml. Between the points shown by the arrows the total fraction was combined and analyzed. Total recovery of phosphorus was 98% of that applied to the column.

A minor component, B′, differed chromatographically from B. Although an exhaustive examination of B′ was not possible, it had the following characteristics: P, 3.95; N, 1.74; N/P, 1.00; I_2 number, 65.4; fatty acid ester/P, 1.60 (by hydroxamate reaction only). A basic difference was noted in the degree of unsaturation in the fatty acids, B′ containing 20 moles per cent of saturates and B 11 moles per cent.

The C-M 1:1 (C) fraction, representing 7% to 8% of the total phospholipids, was composed of at least three components. It appears to contain a serine component (possibly a phosphatidyl peptide), a phosphatidylinositol (monophosphoinositide), and an unknown substance or substances. This was suggested from the results of the usual analyses, paper chromatography, and rechromatography on silicic-acid columns. Infrared examination showed a typical phosphoglyceride pattern, with no evidence for the presence of the usual sphingosine-containing compounds, and no covalent phosphate band at 10.35 μ, but evidence for an amide band at 6.06 to 6.10 μ. It is interesting to note the high content of stearic acid (Table 2) as compared to the other fractions. This may be due to the presence of an inositol phospholipid, which contains high proportions of this acid (9).

Sphingomyelin comprised 95% of the incompletely resolved C-M 1:1 E (and D) fraction, with no more than 5% as lecithin. Occasionally this latter phospho-

glyceride was present to a much lesser extent or not demonstrable, and may represent a metabolically mobile component of the cow erythrocyte. As shown in Figure 2, the lecithin (D) was not sharply separated from the sphingomyelin on silicic-acid columns, but analytical data showed that this component was indeed lecithin. It was isolated, free of the other fractions, and showed the following molar ratios of fatty acid/P, 1.98; N/P, 1.01; choline/P, 0.95, and an infrared spectrum typical of a lecithin (42).

The isolation of sphingomyelin from bovine red cells in reasonably high purity and yield prompted a study of a more rapid preparation in bulk amounts. The total lipids of bovine erythrocytes in chloroform were applied to a silicic-acid column and the neutral lipids eluted in the same solvent. Further elution with C-M 1:9 developed two well-separated phospholipid peaks containing over 98% of the applied phosphorus. The first phospholipid fraction (C-M 1:9) was shown to contain the noncholine-containing substances, and the second fraction (C-M 1:9 A) contained all the choline-containing phospholipids.[10] The C-M 1:9 A fraction was evaporated *in vacuo* at 30° and dissolved in a minimum amount of warm methanol, and to the clear solution were added 10 to 15 volumes of ethyl acetate. The turbid mixture was placed at 4° overnight. The white precipitate was filtered, washed with a small amount of cold ethyl acetate, and redissolved in a minimum amount of methanol. The above procedures were repeated and the final precipitate dried over P_2O_5 *in vacuo* at room temperature for 12 to 14 hours. The amorphous powdery material consisted of 85% to 90% of the initial lipid phosphorus in the C-M 1:9 fraction and was stable to atmospheric oxidation. It exhibited the following characteristics: P, 3.81; N, 3.44; choline, 14.5; N/P, 1.98; choline/P, 0.98; fatty acid ester (by hydroxamate reaction and by infrared examination), none detectable; iodine number, 38; fatty acid/P, 1.02. An infrared examination of this preparation (2% in $CHCl_3$, 1 mm cell) showed the following absorption peaks: 3.04, 6.06, 6.98, 8.2, 9.2, 9.55, 10.35, and 13.9μ. Methanolysis (for 24 hours) of this sphingomyelin preparation by the technique of Brady and Koval (20) yielded 95% of the fatty acids as methyl esters and 65% to 90% of the expected phosphorus-free "sphingosine" fraction. The methyl esters were analyzed by gas-liquid chromatography with results shown in Table 7.

Upon examination of the "sphingosine" fraction, the following data were obtained: N, 4.79 (theory for sphingosine, 4.66); iodine number, 88.3 (theory, 84.7). Paper chromatography in a pyridine system (20) showed only one ninhydrin reacting spot at the solvent front; an infrared spectrum (in $CHCl_3$) showed significant peaks at 2.9, 3.35, 3.40, 6.28, 6.79, 9.75, and 10.28μ. Preparation of a triacetyl derivative of the "sphingosine" fraction gave, in 65% yield, a product melting at 103°-104° with 101°-102° reported for triacetylsphingosine (44,45). In addition to sphingosine, there is apparently another long-chain nitrogen base of unknown structure present in this sphingolipid source.[11]

The chromatographic separation of the phospholipids of human erythrocytes is shown in Figure 3, and

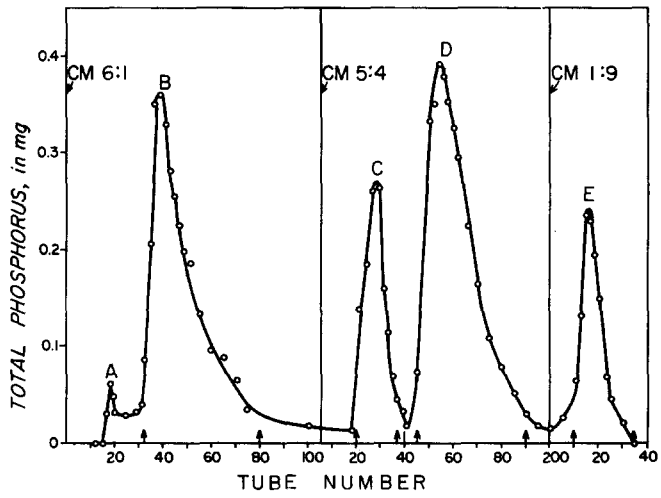

FIG. 3. The chromatographic fractionation of human erythrocyte phospholipids on a silicic-acid column. A total of 35 mg lipid phosphorus (including the neutral lipids as well as the phospholipids) was placed on 70 g silicic acid plus 35 g Hyflo Super-Cel (height to diameter = 8) and the neutral lipids eluted with chloroform. Subsequently the phospholipids were eluted with the indicated solvents; flow rate, 2 ml/minute; tube volume, 8.2 ml. Between the points shown by the arrows the total fractions were combined and analyzed. Total recovery of phosphorus was 96% of that applied to the column.

data on the chemical nature of the isolated fractions are recorded in Table 3. In addition to lecithin and sphingomyelin, phosphatidylethanolamine (C-M 6:1 B) was found in the cell and amounted to 30% of the total phospholipid. As found with the bovine phosphatidylethanolamine component, the human fraction con-

[10] In a private communication Dr. C. C. Sweeley has recommended the following solvent system for the rapid preparation of sphingomyelin (from cow erythrocytes) via silicic-acid chromatography: chloroform, for removal of the neutral lipids, then C-M 3:1 for removal of the noncholine-containing phospholipids. Finally, elution with C-M 1:4 will remove two peaks, (*a*) a small fast-moving component, and (*b*) a slower moving component which contains the sphingomyelin (plus lecithin). This latter fraction can then be treated as indicated in the text.

[11] Dr. C. C. Sweeley, private communication; see also Reference 46.

TABLE 3. COMPOSITION OF THE PHOSPHOLIPIDS OF HUMAN ERYTHROCYTES *

	C-M 6:1 (B) †	C-M 5:4 (C)	C-M 5:4 (D)	C-M 1:9 (E)
P, per cent	3.45	2.66	3.46	3.60
N, per cent	1.48	1.62	1.57	3.20
Choline, per cent	None	None	13.6	13.9
N/P, molar ratio	0.98	1.34	0.99	1.95
Choline/P, molar ratio	—	—	0.95	0.95
Inositol/P, molar ratio	None	0.14	None	None
Plasmalogen/P, molar ratio	0.028	0.01	None	None
$[\alpha]_D^{25}$	Not run	Not run	+6.1°	Not run
Iodine number	106	66.5	64.0	39.0
Fatty acid/P, molar ratio	1.95 ‡	1.90	2.01	1.03
Fatty acid ester/P, molar ratio	(1.2-1.9) ‡	Not run	1.98	0.01

* These fractions were obtained by silicic-acid chromatography as described in the text and shown in Figure 3.

† Fatty acids (mole percentage) 14:0, 16:0, and 18:0—trace, 15% and 14% respectively; 14:1, 16:1, 18:1, 18:2, and 20:4—4%, 16%, 19%, 9%, and 20%, respectively. The fatty acid composition of D and E are given in Tables 6 and 7.

‡ See footnote †, Table 2.

tained less than 2% of a serine-containing phospholipid. Among the other more minor phospholipids found in this source were a "polyglycerolphospholipid"-type compound (C-M 6:1 A) to the extent of 1%. This was a fatty acid-containing pigmented material with a low nitrogen content and fatty acid to phosphorus 1.61. There also was an inositol-containing fraction (C-M 1:1 C) present to the extent of nearly 8% of the total phosphorus-containing compounds. This latter fraction, though quite impure, showed some interesting characteristics. In particular, it gave high nitrogen-to-phosphorus ratios of 1.35 to 1.40, and no free amino acids, sugars, or long-chain nitrogen bases were detected. On the basis of the inositol content and the assumption of a monophosphoinositide without nitrogen, the other phospholipids must contain at least two nitrogen bases. An infrared spectrum of this fraction showed a typical fatty acid ester (phosphoglyceride) pattern, with no absorption at 10.35 μ but with amide band absorption at 6.06 μ. A quantitative assay for serine and ethanolamine (38) and paper chromatography showed the presence of serine with only traces of ethanolamine.

Plasma Lipids.[12] Inasmuch as the fractionation of the neutral lipids proceeded reproducibly and in a manner similar to that described previously (31), no graphic description is presented. In each instance the identifiable lipid components eluted by the following solvent system were (H-B 85:15) "hydrocarbon" and cholesterol esters, (H-E 95:5) triglycerides plus free fatty acids, (H-E 85:15) free cholesterol, and (ether, 100%) traces of partial glycerides.

A typical separation of human plasma phospholipids is depicted in Figure 4 and the chemical composition of the individual fractions is presented in Table 4. As

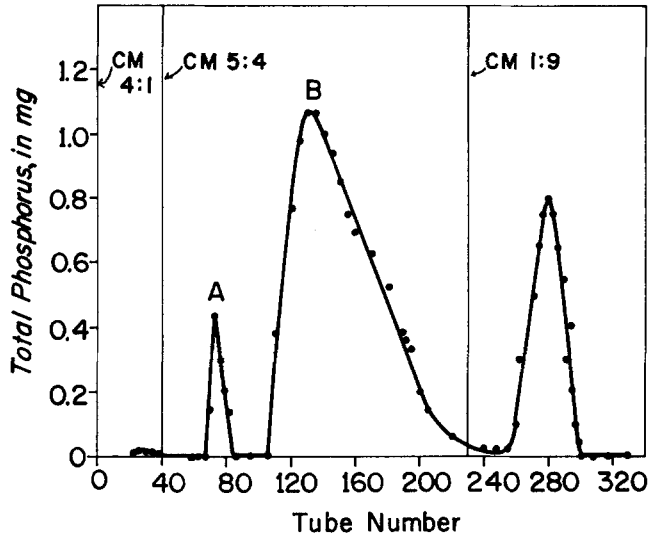

FIG. 4. A typical chromatographic separation of the phospholipids of human plasma. In this instance 93 mg phospholipid phosphorus was chromatographed on 160 g silicic acid plus 80 g Hyflo Super-Cel (height to diameter = 8); flow rate, 3.0 ml/minute. Volume of eluate in each tube was 10 ml. Recovery was 97% of that applied to the column.

is evident from these data, the major phospholipid component is phosphatidylcholine (lecithin) (eluted by C-M 5:4) with sphingomyelin as the lesser component (eluted by C-M 1:9). On the basis of its optical rotation value (and conversion by enzymatic degradation or dilute alkali treatment to L-α-glycerylphosphorylcholine), it is evident that the phosphoglyceride possessed the L-α-configuration. The phosphatidylcholine isolated from human and bovine plasma was nearly colorless and quite stable to atmospheric oxidation. Through the use of phospholipase (lecithinase) A of snake venom, these compounds were degraded and re-

[12] See footnote 6.

TABLE 4. COMPOSITION OF THE PHOSPHOLIPIDS OF HUMAN AND BOVINE PLASMA*

	Bovine Plasma		Human Plasma †	
	C-M 5:4 B	C-M 1:9	C-M 5:4 B	C-M 1:9
P, per cent	3.74	3.79	3.96	3.21
N, per cent	1.65	3.52	1.85	2.91
Choline, per cent	14.2	14.1	14.9	12.4
N/P, molar ratio	0.97	2.05	1.03	1.95
Choline/P, molar ratio	0.97	0.95	0.97	0.99
$[\alpha]_D^{24}$ ‡	+5.9	+7.1	+6.1	+7.9
Fatty acids/P, § molar ratio	1.97	1.04	2.00	0.98
Fatty acid ester/P, molar ratio	2.03	0.01	2.01	0.01

* These lipids were obtained by silicic-acid chromatography as described in the text and illustrated in Figure 4.

† In elution with C-M 4:1 (See Fig. 4), only a small amount of lipid P (~0.5%) and some color was removed; subsequent elution with C-M 5:4 removed first a highly pigmented fraction containing mainly an ethanolamine phospholipid (varying from 0.5% to 5% of the total) and later a elution with C-M 5:4 B, which contained only lecithin. A final elution with C-M 1:9 removed the sphingomyelin fraction. A subsequent elution with 100% methanol did not remove any additional phosphorus. The total recovery of phosphorus was 95% of that applied to the column.

‡ 15% in C-M 10:1.

§ The fatty acid composition of the lecithins (C-M 5:4 B) and the sphingomyelin (C-M 1:9) is given in Tables 5 and 7, respectively.

sultant free fatty acids (from the β-ester [C-2]) and lysolecithins (the α'ester [C-1] fatty acids) analyzed for type of fatty acids as well as other characteristics. These results, shown in Table 5, support a specific positioning of the fatty acids in plasma lecithins. As further proof, the lecithin (from bovine plasma) was subjected to the action of phospholipase (lecithinase) D (present in *Cl. perfringens toxin*), which causes the formation of diglyceride and phosphorylcholine (47, 48). The diglyceride was isolated in 95% yield and showed the following characteristics: $[\alpha]_D^{25}$ -2.3° (6% in $CHCl_3$); fatty acid/glycerol, 2.03. This was considered to be a D-α diglyceride. When this compound was incubated with lipase (49), primarily saturated fatty acids (92% saturates; 8% unsaturates) were liberated. This is to be expected if the saturated acids are located in the 1 position of the diglyceride (49, 22).

In both species the sphingomyelins were alike (Table 4). The exact position of the phosphorylcholine unit is not certain but it is probably located on carbon 1 of the sphingosine chain (50, 43). Methanolysis of the intact compound gave the methyl esters of the fatty acids (ester to phosphorus, 1.03) in good yields and nearly 70% of a "sphingosine" fraction. Paper chromatography of the latter component from both species in the pyridine system of Brady and Koval (20) showed only one fast-moving component, with no indication of any dihydrosphingomyelin. Although it has been reported that lysolecithin is present in plasma (3, 51), it was not found in the samples examined here.[13] Moreover, no evidence was obtained for the presence of any significant amount of plasmalogens, inositol lipids, or cerebrosides by direct chemical, infrared, or paper chromatographic assay.

DISCUSSION

The results presented here demonstrate that erythrocytes from man and cow exhibit an almost identical neutral-lipid-to-phospholipid ratio. Although not in exact agreement with the recent results of James *et al.* (52), there is no doubt that of the neutral lipids of the human erythrocyte, free cholesterol is the major substance present. The phospholipids, on the other hand, showed more substantial differences. While the noncholine-containing phospholipids were reasonably similar in chemical characteristics and amounts, the choline-containing phospholipids of the two species were significantly different. In a recent article, Rowe (53) presented evidence on the biosynthesis of phospholipids in human red cells. However, as limited data were presented on the chemical nature of the fractions obtained in his chromatographic separation, no comparison can be made with the information presented here.

The plasma of both species had a comparable distribution of lipids and in amounts similar in part to those found by other investigators (3, 5, 41, 51). Although Marinetti *et al.* (51) and Phillips (3) have reported the presence of lysolecithin in human serum, this compound was not detected in several different batches of human and bovine plasma examined here. In a similar manner, Nelson and Freeman (41, 54) have not found lysolecithin in human serum. Perhaps

[13] Evidently lysolecithin can be lost in the washing procedure described here. In two plasma samples (results not reported here), approximately 2% of the total phospholipid phosphorus of the plasma was found in the water washes as a lysolecithin-like compound. However, none of the other phospholipid phosphorus was present as a lysolecithin.

technical differences in the isolation of the lipids or possible solvent activated enzymatic degradation could explain these differences.[14]

Although the location of the fatty acids on the lecithins presented a rather distinct positional effect, the results with the phosphatidylethanolamine fractions were quite different. Surprisingly, this erythrocyte fraction contained a very high amount of unsaturated fatty acids. It is important to note that Rouser *et al.* (27, 28) have reported a decidedly high content of unsaturated fatty acids in the phosphatidylethanolamine fraction of platelets. Unfortunately, it was not possible to initiate phospholipase A action on phosphatidylethanolamine fraction in an effective manner; hence, no positioning results could be obtained. On the basis of the results on fatty acid distribution, it would appear unlikely that phosphatidylethanolamine would have a metabolic relation with phosphatidylcholine, but further study must be conducted to ascertain the true significance of these observations.

While the inositol-containing fraction (C; Figs. 2 and 3) of the erythrocytes was obviously not homogeneous, it did contain a high content of stearic acid (Table 2). A similar high content of stearic acid was noted in the phosphatidylinositol fraction of liver, and metabolic studies showed this fraction to have a high specificity for this saturated fatty acid (9). Finally, the sphingosine-containing fraction presented a distinct fatty acid pattern. On the basis of the data presented in Table 7, there is no doubt that this phospholipid contained a large amount of saturated fatty acids. While in general these results were expected, the large amount of palmitic acid was rather surprising. At present there is no adequate explanation for the specificity of the sphingolipids for saturated fatty acids.

The neutral lipid components showed an interesting pattern of fatty acid distribution. In the human erythrocyte and plasma cholesterol ester fractions, there was a high level of unsaturated fatty acids, with linoleic acid as the major type, and palmitic and stearic acids as the saturated ones. An examination of the fatty acid content of the triglycerides of the human erythrocyte showed it to contain 44 mole per cent of saturated fatty acids compared to 15 mole per cent in the cholesterol ester fractions or 28 mole per cent in a

TABLE 5. CHEMICAL NATURE AND LOCATION OF FATTY ACIDS OF HUMAN AND BOVINE PLASMA LECITHINS

Fraction	Relative Distribution in Mole Per Cent		Specific Distribution in Mole Per Cent					
	Saturates	Unsaturates	Saturates 16:0	Saturates 18:0	Unsaturates 16:1	Unsaturates 18:1	Unsaturates 18:2	Unsaturates 20:4
Human Plasma Lecithin, intact	47	53	32	15	3	13	22	15
Lysolecithin *	93	7	63	30	2	1	3	
Fatty acids, † liberated	1	98	1	1	1	20	50	28
Bovine Plasma Lecithin, intact	46	54	21	25	20	18	9	7
Lysolecithin *	94	6	43	51	4	2		
Fatty acids, † liberated	2	98	1	1	30	36	20	12

* The lysolecithins possessed the following characteristics: *Human*, P, 5.65; N/P, 1.00; fatty acid ester/P, 1.02; iodine number, 10; $[\alpha]_D^{26}$ — 2.78° (9% in C-M 10:1); *Cow*, P, 5.70; choline P, 1.00; fatty acid/P, 1.01; iodine number, 7.0; $[\alpha]_D^{24}$ —2.72° (10% in C-M 10:1).

† The liberated fatty acids had the following characteristics: *Human*, neutral equivalent, 282.6; iodine number, 202; P, none detectable. *Cow*, neutral equivalent, 279.0; iodine number, 176; P, none detectable.

TABLE 6. CHEMICAL NATURE AND LOCATION OF FATTY ACIDS OF HUMAN ERYTHROCYTE LECITHINS

Fraction	Relative Distribution in Mole Per Cent		Specific Distribution in Mole Per Cent					
	Saturates	Unsaturates	Saturates 16:0	Saturates 18:0	Unsaturates 18:1	Unsaturates 18:2	Unsaturates 20:4	Unsaturates 22:?
Lecithin, intact	48	52	34	14	17	26	7	2
Lysolecithin *	96	3	68	28	1	2	—	—
Fatty acids, † liberated	2	98	1	1	34	50	11	3

* The lysolecithins had the following characteristics: P, 5.52; choline/P, molar ratio, 0.97; fatty acid/P, molar ratio, 1.01; iodine number, 4; $[\alpha]_D^{25}$ — 2.72° (10% in C-M 10:1).

† The liberated fatty acids had the following characteristics: neutral equivalent, 289; iodine number, 171; P, none detectable.

[14] See footnote 13.

TABLE 7. FATTY ACID COMPOSITION OF SPHINGOMYELIN ISOLATED FROM HUMAN AND BOVINE BLOOD

Source	Specific Distribution in Mole Per Cent										
	Saturates					Unsaturates					
	16:0	18:0	20:0	22:0	24:0	16:1	18:1	18:2	20:4	22:?	24:?
Human											
Erythrocytes	52	12	2	16		6	6		5		
Plasma	48	18	*	18	9		2	4	3	*	
Bovine											
Erythrocytes	40	4	*	11	31	1	1			2	10
Plasma	77	13				5	1	4			

* Trace.

comparable triglyceride fraction from the plasma. However, the plasma lipid fatty acids may reflect variations in nutritional and dietary states. When the bovine erythrocyte and plasma cholesterol esters were examined for types of fatty acids, 61 to 67 mole per cent of linoleic acid was found together with smaller amounts of oleic and arachidonic acids. The saturated fatty acids represented only 10% of the total fatty acids in these fractions. On the other hand, the triglycerides from the bovine erythrocytes presented a completely different distribution pattern, with the saturated fatty acids as the predominant types. In general, the results on the fatty acids found in the plasma lipids of the cow are in agreement with the observations of Garton *et al.* (55, 56).

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