Characterization and quantification of red cell lipids in normal man

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SUMMARY Human red cell lipids have been studied in a series of normal individuals. Cholesterol comprises 25% of total red cell lipid, and free fatty acid is present. Evidence is presented that the total amount of lipid phosphorus per average red cell is 1.40×10^{-11} mg and that most extraction procedures fail to extract 8% or more (some as much as 40–50%) of red cell phospholipid.

The average percentage distribution of the individual phospholipids was: choline glycerophosphatides 30%, sphingomyelin 24%, ethanolamine glycerophosphatides 26%, and serine glycerophosphatides 15%. Other minor phospholipid components were also identified. The normal range for total red cell plasmalogen was found to be $4.65–5.85 \times 10^{-11}$ μmole/cell. Evidence for the presence of a carbohydrate-containing lipid which was eluted from silicic acid columns with serine and inositol glycerophosphatides is also given.

The fatty acid distribution of each major phospholipid fraction is characteristic. Ethanolamine glycerophosphatides are high in 20:4, 22:5, and 22:6, while serine and inositol glycerophosphatides contain large amounts of 18:0 and 20:4. Lecithin is distinguished by 20–25 moles % 18:2 and sphingomyelin by high concentrations of 24:0 and 24:1.

A THOROUGH KNOWLEDGE of normal human red cell lipids is necessary to study and understand the composition and dynamic behavior of lipids in abnormal erythrocytes, and to achieve insight into the role of lipids in cell membranes.

Numerous studies have estimated red cell total phospholipid (1–9) and cholesterol (1, 4–7, 9, 10) (see reference 10 for review of the earlier studies on red cell cholesterol), and analyzed the distribution of some or all of the individual phospholipids (1–5, 7–9, 11–14); but the values established by different investigators have not always been in close agreement. The differences are partially explained by the variety of extraction and analytical procedures employed, not all of which have been definitively evaluated.

In addition, some of the red cell phospholipids have been characterized and shown to contain diverse fatty acid and vinyl ether moieties (3, 5, 12, 14). For purposes of comparison with abnormal cells and as a basis of hypothetical formulations of membrane structure, differences in previous data must be resolved and the normal mean and range for various measurements of red cell lipids more firmly established. The present study attempts to accomplish these ends by quantifying and characterizing total lipid and the phospholipid fractions[1] of red cells from a series of normal individuals, using extraction procedures whose efficiency has been established for the red cell.

METHODS

Donors were healthy males and females, 21–35 years of age, on ad lib. diets. All had normal hematocrit readings, hemoglobin levels, reticulocyte counts, and peripheral blood smears. Blood was drawn after an overnight fast into acid citrate dextrose (ACD, NIH formula A), 7 ml of whole blood to each milliliter of ACD. Plasma and red cells were separated by centrifugation at 4° within 1 hr after the blood was drawn. The cells were

* This work was done, in part, during the tenure of an Established Investigatorship of the American Heart Association.

[1] *Nomenclature.* Present separation methods, the use of which is exemplified in this paper, give rise to chromatographic fractions characterized by a single base but containing diacyl, vinyl ether, and saturated ether derivatives of glycerophosphate. The term *ethanolamine glycerophosphatides* (EGP) refers to such a chromatographic fraction based on ethanolamine which is known to contain ethanolamine diacyl glycerophosphatide (phosphatidyl ethanolamine) and may or may not prove, later in the investigation, to contain the corresponding plasmalogen (phosphatidal ethanolamine) and/or glyceryl ether derivative(s). Corresponding terms are used for serine-, inositol-, and choline-derivative mixtures (respectively abbreviated SGP, IGP, CGP).

then washed three times in cold 0.9% NaCl solutions (pH adjusted to 7.4 with sodium bicarbonate), and resuspended in 0.9% NaCl to a hematocrit reading of 35–55%. After mixing, aliquots were pipetted for electronic red cell counting,[2] hematocrit value, and lipid extraction. The results of lipid analyses are expressed on a per cell basis. The average mean corpuscular volume (MCV) was 100×10^{-12} ml (SD $\pm 1 \times 10^{-12}$), allowing calculation of the values per milliliter of red cells by multiplying the per cell value by 10^{10}.

Absolute methanol (MeOH), chloroform, and diethyl ether were reagent grade commercial products and were not redistilled. Hexane was redistilled from potassium permanganate and the fraction boiling at 68—70° was collected. Benzene was reagent grade and redistilled. Ethyl acetate was reagent grade and was redistilled after standing over potassium carbonate for 24 hr.

Extraction of Lipids

Analytical data were obtained on lipids extracted by three slightly different procedures (procedures I, II, and III), all carried out at room temperature. *Procedure I* was a slight modification of that described by Reed et al. (4). A given volume (*n* milliliters) of washed resuspended cells was added slowly to 5*n* ml of MeOH in a beaker with vigorous stirring. All lumps were then carefully broken and the mixture was allowed to stand for half an hour with occasional stirring at room temperature. A like volume (5*n* ml) of chloroform was then added with stirring, and after 10 min the solvents were filtered through Whatman No. 1 filter paper (previously washed with MeOH and $CHCl_3$) into a round-bottomed flask. The residue was returned to the beaker and reextracted in identical fashion twice more. The combined filtrates were evaporated to complete dryness in vacuo at 25–37° and then the lipid–protein residue was reextracted with 3–5 aliquots of fresh $CHCl_3$, totaling 10*n* ml, filtering each aliquot through solvent-washed Whatman No. 2 filter paper. The combined $CHCl_3$ filtrates were evaporated to dryness in vacuo at 25–37°, and the lipid was brought to volume in benzene and stored at −20°. In *Procedure II* the initial steps were the same as in Procedure I. After the three primary chloroform–methanol (C–M) extracts had been combined and evaporated to dryness,[3] the lipid–protein residue was reextracted with three aliquots of $CHCl_3$, totaling ten times the original volume of cell suspension, and filtered through solvent-washed Whatman No. 2 filter paper into a separatory funnel. MeOH and 0.1 M KCl were then added directly to the separatory funnel so that the final proportions of $CHCl_3$, MeOH, and KCl were 10:5:3 (all solvent ratios are v/v). After being shaken and cooled to 4° or lower, the mixture was warmed to room temperature. Clean separation occurred and the $CHCl_3$ phase was recovered. This contained 99–100% of the lipid phosphorus present in a similar unwashed extract. *Procedure III* differed from Procedure II only in that the residue remaining after evaporation of the three primary C–M extracts was reextracted with two aliquots of MeOH (totaling 5 times the original volume of cell suspension) after the reextraction with $CHCl_3$. KCl (0.1 M) was again added directly to the separatory funnel and the extraction completed as in procedure II. In Procedures II and III the $CNCl_3$ phase was dried in vacuo, then dissolved in benzene and stored at −20°. These procedures were compared with the methods of Folch et al. (16), Bligh and Dyer (17), and Hanahan, Watts, and Pappajohn (14).

The residues from these extraction procedures were analyzed for lipid as follows. The moist residue was added to the reextraction flask. Enough 6 N HCl was added to fill one-third of the flask and the mixture refluxed at 100° for 16–24 hr. After cooling, the mixture was extracted three times with diethyl ether. This extract was washed with water to remove HCl. Total fatty acids were quantified either by their infrared absorbance at 5.80–5.85 μ or by titration against 0.015 N ethanolic NaOH.

Separation of Lipids by Silicic Acid Chromatography

Small amounts of the total lipid extract were separated at 4° into phospholipid (for preparation of total phospholipid methyl esters) and nonphospholipid (for analysis of free fatty acid) on 0.3 g of silicic acid (Mallinckrodt 100–200 mesh) in a sintered glass funnel (medium porosity, 13 mm diam). The adsorbent had been washed by suction with 10 ml of C–M 1:1, then 10 ml of $CHCl_3$. Up to 15 mg of total lipid was applied in $CHCl_3$ and nonphospholipids were eluted with 10 ml of $CHCl_3$. Phospholipid was eluted with 20–40 ml of M–H_2O 97:3. Recovery of lipid phosphorus averaged 96% with lipid samples up to 15 mg. When the procedure was scaled up to handle larger amounts of lipid, passage of C–M 1:1 or 1:9 (4–5 column volumes) was required before elution with M–H_2O 97:3, otherwise recovery of lipid phosphorus was not satisfactory.

Separation of red cell phospholipids into several fractions was achieved on silicic acid columns prepared essentially as previously described (14), run at room temperature the day following extraction (Fig. 1). A load of 4–10 mg of lipid phosphorus was applied to

[2] It has been documented that red cell counts done by the conventional method may be subject to a systematic error in chamber loading. In one laboratory, electronic counting resulted in lower counts (hence higher MCV) than the conventional procedure (15). In this study it has been shown also that washed red cells from ACD blood have a larger MCV than those from blood collected in EDTA solution.

[3] Complete "dryness" was not necessary. Results were the same if the film appeared moist, but liquid droplets were avoided.

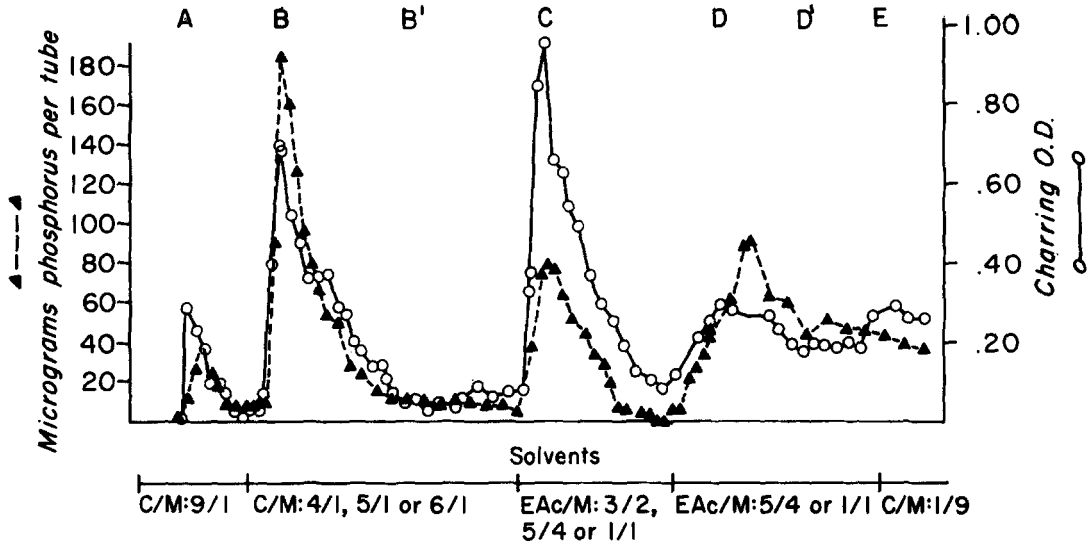

FIG. 1. Silicic acid chromatography of red cell phospholipids.
Load: 7 mg lipid P on column containing 14 g of silicic acid and 7 g of HyFlo Super Cel. Some columns gave better (but never complete) separation of the choline glycerophosphatides and sphingomyelin (peaks D and E) than that shown here. The majority of Fraction E was collected in bulk and is not shown. The first and last tubes of Fraction D′ were chosen after chromatographing an aliquot of every third, fourth or fifth tube (beginning at the top of peak D) by TLC or on glass fiber paper (see Methods). Fraction F is not shown.

columns containing 2 g of silicic acid and 1 g of Hyflo Super Cel (Johns Manville) for each milligram of lipid phosphorus. Nonphospholipids were removed with six or seven column volumes of $CHCl_3$. An initial small phospholipid peak (Fraction A) was eluted with 1.5 column volumes of C–M 8:1 or 9:1. The first major peak (Fraction B), which contained predominantly ethanolamine glycerophosphatides (EGP), was eluted with C–M 5:1, 6:1, or 7:1. Less contamination of this fraction with serine glycerophosphatides (SGP) was achieved by eluting with C–M 6:1 or 7:1, than when C–M 5:1 was used. After the passage of seven or eight column volumes of solvent, small amounts of phosphorus were still present in the eluate. Ethyl acetate–methanol (E–M) 3:2, 5:4, or 1:1 rapidly eluted a peak (Fraction C) which contained SGP together with inositol glycerophosphatides (IGP) and other minor components. After 3–4 column volumes, Fraction D was then eluted with the same solvent or by changing to a more polar one (E–M 1:1). The first two-thirds of this peak was pooled and was found to contain 83–100% choline glycerophosphatides (CGP). Since CGP and sphingomyelin were never completely separated on these columns, the remainder of this peak, the subsequent plateau, and the first $^1/_5$–$^1/_3$ of the subsequent peak were pooled as the mixture Fraction D′. The tube at which Fraction D′ was begun and terminated was determined for each column by monitoring the elution with glass fiber paper or thin-layer chromatography (TLC). When these indicated a predominance of sphingomyelin in the effluent, the solvent was changed to C–M 1:9 and 5–7 column volumes were collected in bulk. The remaining lipid (0.5–2% of lipid phosphorus) was eluted with 3–4 column volumes of M–H_2O 98:2, collected in bulk, and designated Fraction F (not shown in Fig. 1). The total recovery of lipid phosphorus from these columns averaged 95%.

The percentages of CGP and sphingomyelin in fractions D, D′, and E were calculated on the basis of nitrogen to phosphorus (N/P) and fatty acid ester/phosphorus (FAE/P) molar ratios and quantitative silicic acid paper chromatography (4). The latter technique was also used to quantify the phospholipids of fractions B, C, and F. In Fraction C, 5–10% of the phosphorus eluted from the silicic acid paper chromatograms was present in a small spot below IGP, and was classified as unknown. Fraction A was considered to be phosphatidic acid and/or polyglycerol phosphatide.

TLC and Paper Chromatography

Thin-layer chromatographic plates were prepared by applying a slurry of 27 g of Silica Gel G in 60 ml of water. Plates were activated at 120° for 2 hr and stored either in an oven at 37° or in a desiccator over silica gel. The following solvents were used: for phospholipids, C–M–H_2O (or 4 N NH_4OH) 70:35:4; for nonphospholipids, petroleum ether–diethyl ether–acetic acid 90:1:1 or 90:2:1. Silicated glass fiber paper was prepared as described by Muldrey et al. (18), and used primarily to separate phospholipids by development in a solvent system of benzene, pyridine, and water 100:100:10–15.

Quantitative silicic acid paper chromatography was carried out as described by Reed et al. (4) with phosphorus determined by a modification of Bartlett's (19) procedure employing perchloric acid digestion for 1 hr. Charring was carried out as previously described (19a).

Gas–Liquid Chromatography (GLC) of Fatty Acid Methyl Esters

Phospholipid fatty acids were methylated in H_2SO_4, MeOH, and benzene as described previously (20). The efficiency of this method for methanolyzing red cell total phospholipid was evaluated in three experiments. The recovery of methyl esters (including those derived from the fatty acid amide of sphingomyelin) was 105, 100, and 95% of theory.

GLC was performed on 4 or 6 ft columns using either a Barber-Colman Model 15 (hydrogen flame detector) or a Pye Argon gas chromatograph (argon ionization detector). With ethylene glycol succinate polyester (EGS) as stationary phase, column temperatures were 165–175° and with Apiezon L, 198–210°. In each case, flow rates were adjusted to achieve maximum column efficiency. Methyl esters of myristic, palmitic, stearic, oleic, linoleic, behenic, and arachidic acids (judged pure by TLC and GLC) were used to standardize the instruments. With the particular resistors employed, the argon detector gave a response proportional to the moles of methyl ester present for the first five esters (behenic and arachidic were not tested on the argon detector). Methyl arachidonate and longer chain methyl esters, for which pure standards were not available, were, therefore, also calculated by assuming the molar percentage of each ester to be proportional to its peak area. With the flame detector, the response was proportional to weight for all esters tested and the "corrected area" of each peak was obtained by dividing the measured area by the molecular weight of the methyl ester. In calculating methyl ester distribution, peaks known to represent dimethylacetals were disregarded.

Peaks were identified by determining carbon numbers on EGS and (for the less common methyl esters) by comparison with published data on relative retention times (5). These identifications were verified by chromatography on both EGS and Apiezon as proposed by James (21), and by rechromatography after hydrogenation.

Other Analytical Techniques

Fatty acid esters were estimated quantitatively by infrared absorption as previously described (20). Plasmalogen content was measured by Norton's (22) potentiometric procedure; and cholesterol, total weight, phosphorus, and iodine uptake were quantified by methods previously reported (14). Anthrone was used for qualitative detection of carbohydrates.

RESULTS

Evaluation of the Extraction Procedure

As shown in Table 1, procedure III has proved the most efficient in our hands. None of the "lipid phosphorus" was inorganic, as demonstrated by the failure of undigested samples to develop color, and by 95–100% recovery from silicic acid columns or paper chromato-

TABLE 1 COMPARISON OF LIPID PHOSPHORUS BY VARIOUS EXTRACTION PROCEDURES*
(UNITS: 10^{-11} MG PER CELL)

Type of Comparison	Expt. No.	Extraction method			
		Procedure II	Folch	Ethanol–Ether	Procedure III
Direct comparison†	1	1.14		1.11	
	2	1.20		0.69	
	3	1.01		0.86	
	4	1.18	1.06	0.95	
	5	1.18	0.66		
	6	1.14‡	1.07	0.88	
	7	1.09			1.31
	8	1.17			1.24
	9	1.16			1.21
Red cells from the same individuals extracted at different times§	1	1.04			1.22
	2	1.01			1.22
Mean and range of all extractions		1.15 (18) (1.01–1.25)			1.27 (18) (1.20–1.35)

* Total lipid values showed differences comparable to those in lipid phosphorus. All extractions were on 10 ml or more of red cell suspension.

† Separate aliquots of the same cell suspension were extracted and analyzed simultaneously.

‡ In experiment 6, procedure II was not compared directly. The value was obtained from an extraction done two weeks before.

§ Individuals whose red cells had given low values using procedure II were purposely selected.

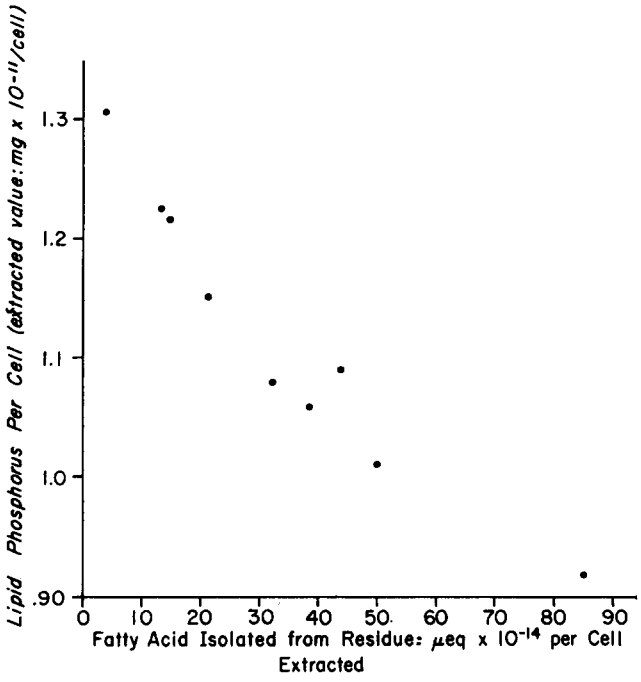

FIG. 2. Analysis of red cell extraction residues after acid hydrolysis. Each point represents the average of duplicate residue analyses. Extrapolation of the upper portion of the curve to the vertical axis gives an estimated value of 1.33×10^{-11} mg of lipid phosphorus per cell.

grams as phospholipid. When extracting less than 5–6 ml of red blood cell suspension, procedures I and II gave values comparable to procedure III, but for larger amounts, duplicates for the first two procedures were often in poor agreement and extraction of lipid was not quantitative. Procedure I, when used to extract 10 ml or more of red cell suspension, resulted in extracts contaminated with free amino acids (paper chromatography) and peptides (paper chromatography and infrared spectrophotometry). Lipid samples obtained by procedures II or III were free of such nonlipid material. The ethanol–ether extraction previously employed (14), and Bligh and Dyer's method (17) (not shown in table) were not quantitative for red cells, and the resulting extracts were contaminated with proteinaceous material. The procedure of Folch et al. (16) was evaluated three times. On one occasion the amount of lipid phosphorus extracted was very low. The other two times it was lower than values obtained on the same individual by procedure II and significantly lower than the average for procedure III. In addition, two of the Folch extracts contained chloroform-insoluble material, free amino acids, and peptides. In one Folch extraction, phosphorus comprised only 2% of the total extract. For these reasons, no further work with the Folch procedure was done.

Figure 2 plots the amount of lipid phosphorus extracted per cell against the amount of fatty acid extracted from the residue after strong acid hydrolysis. The two extractions yielding the highest phosphorus values were done using procedure III, the others with procedure II or I. On the basis of these data, it is evident that the absolute amount of phospholipid in normal red cells is more constant than might appear from perusal of the extraction data alone.

Analysis of Total Extractable Red Cell Lipid

Values for total lipid, lipid phosphorus, cholesterol, plasmalogen, fatty acid ester, and free fatty acid are presented in Table 2. Data obtained using extraction procedures I and II are compared with those on extracts obtained using procedure III.

Table 3 records the fatty acid distribution of total red cell phospholipid. Since the dimethylacetals formed from methylation of vinyl ether moieties were present in the mixtures, the stearate value is high because of the identical retention time of the 18:1 dimethylacetals. Other dimethylacetals of significant quantitative importance did not coincide with methyl ester peaks and were disregarded in the calculations. It is noteworthy that after hydrogenation, a peak with the proper retention time for methyl lignocerate remained on both the EGS and Apiezon chromatograms. To exclude the possibility that this might represent a long-chain nitrogenous base from sphingomyelin, the methyl esters were chromatographed on silicic acid. The chloroform eluate contained only 35% of the nitrogen contained in the total "methyl ester" preparation, but on subsequent GLC there was no reduction in the size of the peaks attributed to lignoceric and nervonic acids.[4]

Trace amounts of 26:0 and an unidentified unsaturated C_{26} acid were detected. These are not shown in Table 3.

Characterization and Percentage Distribution of Red Cell Phospholipids

The lipid used in these analyses was extracted by procedure II or III. The analytical data obtained on the phospholipid fractions separated by silicic acid chromatography are presented in Tables 4 and 5. (Because of insufficient material, Fraction F was not analyzed except by TLC and paper chromatography. It contained CGP, sphingomyelin, and lysolecithin.) In Fractions B, C, D, and E, one phospholipid greatly predominated, but in

[4] In one methanolysis experiment 41 μEq of N was present in the phospholipid sample used. In the resulting methyl esters, 1.5 μEq of N was found (despite fatty acid ester values indicating complete methanolysis), indicating at least partial removal of long chain bases or 3-OH-methyl sphingosine into the methyl ester mixture. In another experiment the "methyl esters" were chromatographed in the system described by Sambasivarao and McCluer (23) for long chain bases and a ninhydrin-positive spot with an R_F consistent with that given for 3-O-methyl sphingosine was found.

TABLE 2 COMPOSITION OF TOTAL RED CELL LIPID

	Extraction Procedures I and II			Extraction Procedure III		
	No. of Subjects	Mean	Range	No. of Subjects	Mean	Range
Total lipid per cell (10^{-10} mg)	18	4.76	4.00–5.28	9	4.88	4.60–5.30
Lipid phosphorus per cell (10^{-11} mg)	18	1.15	1.01–1.25	18	1.27	1.20–1.35
Cholesterol per cell (10^{-10} mg)	11	1.17	1.06–1.30	16	1.26	1.12–1.42
Plasmalogen per cell (10^{-11} mole)	13	4.94	4.17–6.00	10	5.24	4.65–5.85
Free fatty acid per cell (10^{-14} meq)				8	2.7	1.06–3.80
Fatty acid ester/phosphorus molar ratio	3	1.40	1.35–1.46	13	1.42	1.34–1.52

Fractions B, C, and D′, more than one component was invariably present. The relative distribution of each phospholipid, calculated as described in Methods, is given in Table 6. When these results were examined individually, no clear relationship between the amount of lipid phosphorus extracted and the distribution of the phospholipids was apparent. In two experiments, however, one aliquot of the same cell suspension was extracted by procedure II and another by procedure III, and the expected differences in total lipid phosphorus resulted (direct comparisons 5 and 6, Table 1). In both cases the percentage of SGP was lower in the less efficient extraction.

DISCUSSION

As stated by Erickson et al. (1), and reiterated by Reed et al. (4), chemical analyses of red cells are more meaningful if related to the individual cell. Differences in washing procedures (or, in the case of pathological cells, shape) can cause variability in the number of cells in a given packed volume, and although cell surface area (6) may eventually prove to be more significant where membrane lipid measurements are concerned, uncertainties of methodology and interpretation complicate this mode of reporting at the present time.

In order to interpret red cell lipid data properly, the efficiency of the extraction procedure used should be known. The analysis of residues after strong acid hydrolysis and the comparative extraction data presented in this study demonstrate that several methods fail to remove all of the lipid from red cells, but of the extractions evaluated, procedure III is least deficient in this respect. In this study none of the "lipid phosphorus" was inorganic, and it was recovered almost completely after chromatography. In other studies (3,4), "lipid phosphorus" recovery of a similar magnitude has been demonstrated following chromatography, suggesting that the "lipid phosphorus" is not significantly contaminated with nonlipid material. Finally, analysis of the residues has indicated that the absolute amount of phospholipid in normal red cells is more constant than might appear from perusal of the extraction values alone, and that this value approaches 1.35–1.40 $\times$ 10^{-11} mg of lipid phosphorus per cell. The figures of several other investigators (2–6) indicate recoveries of lipid which are probably[5] within 90% or better of this value, while those of others (1, 7, 9) are 80% or less. Cholesterol values reported by most of the same workers (1,4–7) are generally[5] more uniform. However, extraction procedure III in this study recovered higher amounts of cholesterol than procedure II, indicating that approximately 8% of the red cell cholesterol is bound tightly enough to the proteinaceous residue resulting from the initial phase of the extraction that it requires methanol to release it.

While we agree with Farquhar (5) that the majority of the plasmalogens are ethanolamine glycerophosphatides, the total quantity we have found (12–13% of total phospholipid) is lower than either Farquhar (18–23%) or Leopold and Büttner (24) (16–17%) have reported,

[5] Not all of these comparisons are direct since some authors have not reported their results on a per cell basis.

TABLE 3 FATTY ACID DISTRIBUTION OF RED CELL TOTAL PHOSPHOLIPID*

Fatty acid	Moles/100 Moles of Fatty Acid	
	Average†	SD
14:0	0.7	0.18
16:0	24.5	1.05
16:1	0.2	
18:0	19.0	1.16
18:1	16.4	1.07
18:2	11.2	1.27
20:3	1.5	0.48
20:4	15.1	1.14
20:5	0.2	
24:0	2.4‡	1.89
24:1	3.3‡	1.20
22:5	1.6	0.59
22:6	3.5	0.78

* All assays were done with the hydrogen flame detector.

† Less than 0.5 mole/100 moles of 12:0, 17-branched, 18:3, 22:0, and 22:5 (with shorter retention time than the one given) were seen. The determinations were done on red cell lipid from 6 males and 2 females.

‡ These two methyl esters were eluted together with an unsaturated C_{22} ester which was mixed with one or both peaks. The total values given for 24:0 and 24:1 include this unsaturated methyl ester.

but higher than Dawson et al. (11) have found. It is unlikely that the extraction procedure utilized in the present study degraded significant quantities of plasmalogen, because no definite evidence was found for the presence of lyso-ethanolamine glycerophosphatides in any of the phospholipid fractions or on chromatography of fresh lipid preparations on silicic acid paper. Also, since the phospholipid mixture contained 25% sphingomyelin, it would be expected to have a FAE/P molar ratio of 1.5 if the remainder consisted entirely of diacyl phospholipids. Subtraction of the mean plasmalogen/phosphorus molar ratio of 0.125 found in this study would reduce the expected FAE/P ratio in total phospholipid to 1.38, which corresponds well with the average value of 1.42 found for total lipid (the latter value includes a small fraction of nonphospholipid FAE). The different methods for measuring plasmalogen employed in each of the three studies may be responsible for the differences in plasmalogen found.

Our values for free fatty acid (1.06–3.80 $\times 10^{-11}$ μEq/cell) are similar to those of Soloff, Schwartz, and Baldwin (25), but much lower than those found by Mendelsohn (mean 7.50 $\times 10^{-11}$) (26). When Mendelsohn's procedures (27) were used in this laboratory, it was found that appreciable amounts of titratable acidic phospholipid (ethanolamine glycerophosphatides and

TABLE 4 ANALYTICAL AND CHROMATOGRAPHIC DATA ON PHOSPHOLIPID FRACTIONS*

Fraction→	A	B	B′	C	D	D′	E
Major component		EGP		SGP	CGP		SP
Phosphorus							
% of total recovered	1.5	29	3.6	13	20	12	21
% of total weight		3.76 (3.44–4.03)		2.62 (2.19–2.96)	3.64 (2.90–4.20)		3.86 (3.54–4.32)
N/P molar ratio	N present	1.01 (0.97–1.09)		1.21 (1.03–1.44)	1.01 (0.96–1.14)	(1.2–1.72)	1.88 (1.76–1.95)
Fatty acid ester/P molar ratio	4 (av. of 4)	1.55 (1.32–1.70)		2.03 (1.86–2.18)	1.98 (1.78–2.12)	(0.67–1.45)	0.23 (0.12–0.41)
Plasmalogen/P molar ratio		0.35 (0.25–0.50)		0.015 0–(0.05)	0.02 (0–0.05)		
Paper and thin-layer chromatography							
Major components	PA, SGP	EGP	IGP, EGP, SGP	SGP, IGP	CGP	CGP, SP	SP
Minor components	UK†	SGP		UK	SP,† SGP†		CGP
Nitrogen base detected after hydrolysis		Ethanolamine	Serine, Ethanolamine	Serine, Ethanolamine†	Choline	Choline	Choline
Anthrone reaction	Negative	Tr.	Positive	Strongly Positive	Tr.	Negative	Negative
Infrared absorbance							
		O.D. at indicated μ ÷ O.D. at 3.5 μ					
3.0–3.05				0.53 I	0.29 I		0.54 S
3.15–3.25		0.23 B					
3.50		1.0 D		1.0 D	1.0 D		1.0 D
5.77		1.0 S		1.0 S	1.3 S		0.11 S
6.03		0.25 I					
6.10				0.59 I	0.13 S		0.81 S
6.85		0.41 S		0.53 B	0.53 S		0.68 S
7.30		0.27 S		0.45 I	0.29 I		0.22 I
8.75		0.56 S		0.71 S	0.53 I		
9.2–9.7		0.97 B		1.0 B	1.3 B		1.1 B
10.35					0.63 S		0.87 S

Abbreviations: PA, phosphatidic acid; EGP, ethanolamine glycerophosphatides; IGP, inositol glycerophosphatides; SGP, serine glycerophosphatides; CGP, choline glycerophosphatides; SP, sphingomyelin; UK, unknown; B, broad; D, doublet; I, intermediate (between broad and sharp); and S, sharp.

* All mean values are computed from 7–10 individual analyses. The infrared data on fractions B and E were on samples representing 96% EGP and 93% SP respectively as judged from analytical data. The infrared data on Fraction C were from a sample containing 86% SGP and 8.5% IGP, and those on Fraction D from a sample containing 86% CGP. All infrared data given in the table were obtained using $CHCl_3$ as solvent. Comparable fractions from other column separations gave qualitatively similar patterns. Several purer samples of CGP, all run in CCl_4, showed no definite peak at 6.1μ.

† Only occasionally observed.

serine glycerophosphatides) were extracted from red cells in addition to the nonphospholipids. It was further shown that EGP gave five times as much color development, mole for mole, as fatty acids using Mendelsohn's dye assay procedure. It would appear, therefore, that Mendelsohn's results are not indicative of the true amount of free fatty acid occurring in normal human red cells.

In previous studies of total red cell phospholipid fatty acids by GLC, lignoceric and nervonic acid methyl esters were not reported. Both Farquhar (5) and Leibetseder (28) prolonged elution sufficiently to have detected 24:0 and 24:1 methyl esters on the polar phases used. Twelve or more hours are necessary for the elution of 24:0 and 24:1 esters from an 8-ft Apiezon column; it is not clear whether the previous workers allowed sufficient time to detect them on this phase. If they did not, the 24:0 and 24:1 methyl esters eluted from EGS might have been mistaken for C_{20} or C_{22} polyunsaturates. In a later study, Farquhar and Ahrens (29) do note the presence of lignoceric acid in red cell sphingomyelin, and both lignoceric and nervonic acids were identified earlier as components of red cell sphingomyelin by Yamakawa and co-workers, utilizing fractional distillation and reversed phase chromatography (30, 31). In fact, the values obtained in the latter study correspond closely to the averages reported in the present investigation for Fraction E. Other, less significant, differences in the values reported for fatty acid composition of total red cell phospholipids (5, 28, 32–34) are probably explained by variations in extraction and methanolysis techniques. Specifically, the lower values for 18:0 found in two investigations (5, 32) are undoubtedly a result of eliminating dimethylacetals from the sample before GLC.

In the separation of phospholipids by column chromatography, it was recognized that the technique employed was imperfect in that each fraction contained contaminants from other phospholipid classes. Nevertheless, it provided a reproducible, not too laborious method for recovering fractions homogeneous enough to allow characterization and quantification of the

TABLE 5 Distribution of Fatty Acids in Phospholipid Fractions Separated by Silicic Acid Chromatography (Moles/100 Moles of Fatty Acid)

Fatty Acid (as Methyl Ester)	Fraction B (7)* EGP		Fraction C (7)* SGP (+IGP)		Fraction D (6)* CGP		Fraction E (7)* Sphingomyelins	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
12:0							0.2	0–1.0
14:0	0.6	T–1.5		0–T		T–0.6	0.9	0–2.7
16:0	15.5	10.2–20.7	4.4	3.5–6.1	34.7	30.7–38.0	41.3	31.6–51.3
16:1	0.1	0–0.6		0–T		0–T	0.1	0–0.6
18:0	14.1	10.9–19.4	39.7	32.2–45.2	13.8	11.6–16.2	9.1	6.8–11.0
18:1	17.2	15.1–19.2	9.8	7.4–12.6	21.1	18.6–24.7	5.2	4.1–6.3
18:2	5.6	5.0–6.2	2.6	1.5–5.1	21.9	18.7–24.0	3.7	0.9–6.8
18:3	0.3	T–1.3						
20:0							1.2	0–3.6
20:3	1.0	T–1.8	2.6	0–6.9	1.0	T–2.0		
20:4	21.8	20.8–22.9	23.5	20.6–27.2	6.7	3.0–9.5	0.1	0–0.7
20:5	1.4	0.6–2.0						
22:0							8.0	5.2–9.9
22:4	7.8	6.3–9.9						
22:5	1.1	1.0–1.3	.5	T–1.2				
22:5	4.6	3.4–6.7	2.4	0.7–5.4				
22:6	8.9	7.4–11.3	7.0	4.4–11.4				
24:0			4.1†	1.2–8.7			15.0	12–19.2
24:1			3.7†	1.0–6.1			15.5	11.3–18.4
Other	Acid	Range	Acid	Range	Acid	Range	Acid	Range
	12:2	0–1.7	15:Br	0–T	12:2	T–0.3	17:0	0–T
			17:0	0–T	15:0	0–T		
			17:Br	0–T				

* Numbers in parenthesis represent number of individual samples analyzed. The predominant phospholipid in each fraction is given. In actual composition the samples used had the following composition (average and range): Fraction B, ethanolamine glycerophosphatides 86% (76–95), SGP 11% (5–17); Fraction C, SGP 78% (72–84), IGP 14% (6–21); Fraction D, CGP 97% (83–100), remainder SGP and traces of sphingomyelins; and Fraction E, sphingomyelins 91% (85–95), choline glycerophosphatides 7% (5–10). These distributions were determined by quantitative silicic acid paper chromatography for Fraction B and C and by paper chromatography, N/P ratios, and FAE/P ratios for Fractions D and E. See Discussion for comments.

† In Fraction C these two methyl esters were eluted with an unsaturated C_{22} ester which was mixed with one or both peaks. The total values given for 24:0 and 24:1 include this unsaturated methyl ester.

Abbreviations: T, trace; Br, branched.

individual phospholipids. Fraction A contained 1–3% of the total lipid phosphorus, but because of insufficient material few analytical data were obtained. The high average FAE/P ratio is consistent with the presence of polyglycerol phosphatide. However, this is not the entire explanation, for mono- and diglycerides, apparently not eluted with the other neutral lipids by $CHCl_3$, were observed by TLC, and nitrogen was present. On silicic acid paper this fraction had components with a mobility similar to phosphatidic acid and, in some cases, to SGP. GLC of fatty acids was not performed.

According to analytical data, TLC and paper chromatography, Fraction B was predominantly ethanolamine glycerophosphatides (EGP), but did contain 5–20% serine glycerophosphatides (SGP). Although 95% of the plasmalogen recovered from the column was in this fraction, the total quantity of plasmalogen recovered averaged only 80% of that applied. Subsequent to these studies we have carried out the column chromatography at 4° (5) and plasmalogen recovery has been between 90 and 100%.

Fraction C was the most complex of the phospholipid fractions. It contained 12–16% of the total phosphorus eluted from the column, and by thin-layer, paper, and glass fiber chromatography contained 72–84% SGP, 6–21% IGP, and additional lipids which have not yet been completely characterized. Its phosphorus content averaged 2.7% (range 2.0–3.66) and the N:P molar ratio was always greater than 1. It reacted strongly with anthrone reagent. The infrared spectrum was similar to that of Fraction B, but absorbance was greater in the 3.0μ region and a band was consistently observed at 6.10 μ. These data strongly suggest the presence of some other nitrogen-containing nonphospholipid component, possibly a "glycolipid". Other workers (35–37) using different methods of isolation have described and characterized a glycolipid in human red cells designated by Yamakawa as "globoside" (36). If all of the "extra" nitrogen found in this fraction is attributable to the ceramide-trihexose-galactosamine compound proposed by Yamakawa (37), it may comprise as much as 4% of the lipid extracted in the present investigation.

The analytical values for Fraction D indicate that 83–100% was choline glycerophosphatide (CGP). Although significant amounts of plasmalogen were not detected here, contrary to Farquhar's findings (5), a small amount was present in Fraction D′, which *also* contained CGP. It is likely, therefore, that the losses of plasmalogen during chromatography involved choline plasmalogen to a greater extent than ethanolamine plasmalogen.

Fraction E contained small amounts of CGP, but chemical analysis, infrared analysis, and chromatography indicated that the fraction consisted predominantly of sphingomyelin. The final fraction (F) comprised only 2–3% of the total lipid phosphorus eluted. TLC and paper chromatography showed it to be a mixture of lysolecithin, CGP, and sphingomyelin; it was the only fraction that hemolyzed human red cells suspended in saline. The presence of lysolecithin in red cells has been previously documented by Phillips and Roome (3), Reed et al. (4), and Farquhar (5). The previous failure of this laboratory to identify it (14) was probably due either to the extraction procedure employed, or to the aqueous wash found which was used to purify the lipids.

The percentage distribution of phospholipids found in this study corresponds well with that found by Reed et al. (4) and, less closely, Phillips and Roome (3). Others have reported a higher percentage of CGP and/or less SGP (5, 7, 11, 13), and some do not report a complete distribution of acidic phospholipids (3, 7, 8, 13). These discrepancies are probably explained by differences in the efficiency of extraction, inadequate characterization of the phospholipid classes, and breakdown of phospholipids during storage and chromatography (13, 14).

In this investigation quite different fatty acid patterns have been found for each of the four major phospholipid fractions eluted from the silicic acid columns (Table 5). Each fraction was predominantly one phospholipid; the footnote to Table 5 defines the degree of admixture within each fraction. Fractions B and C averaged 86

TABLE 6 PERCENTAGE DISTRIBUTION OF PHOSPHOLIPID IN THE HUMAN RED CELL*

		Glycerophosphatides						Total Phosphorus Recovered
	Sphingomyelin	Choline	Ethanolamine	Serine	Inositol	Lysolecithin	Other†	
Mean	23.8	29.5	25.7	15.0	2.2		3.3	95
SD	1.3	1.2	2.5	1.7	1.0		1.6	‡
Range	21.8–26.0	27.5–30.9	21.5–28.9	11.4–17.6	1.2–4.2	0–1.6	1.5–5.9	

* See text for basis of calculations; all samples were extracted by procedure II or III; and the determinations were carried out on red cell lipid from 8 males and 3 females, ages 22–34.

† Primarily phosphatidic acid or polyglycerol phosphatide, but includes other components (see text).

‡ On 9 of the 11 columns, recovery of lipid phosphorus was equal to or greater than 94%; in two others, 87.5 and 91%.

and 78% EGP and SGP respectively, with the remainder of Fraction B primarily SGP, and of Fraction C primarily IGP. It might reasonably be asked to what extent the values given truly represent the fatty acids esterified to the major phospholipid component of Fractions B and C. For the following reasons it would seem that they are quite representative. First, when the individual samples that were most nearly homogeneous were compared with those containing the highest percentage of contaminating phospholipid, no consistent differences were noted in the distribution of any of the component fatty acids. Second, in one experiment, pure samples of the acidic phosphatides were obtained by preparative TLC, and although they differed to some degree in their content of the minor constituent fatty acids, the principal fatty acids were comparable in distribution with those found in the column fractions. Third, the values obtained correspond to those of Farquhar, whose samples were probably more homogeneous (as judged by TLC; analytical values were not given).

Fractions D (CGP) and E (sphingomyelin) were more homogeneous in their phospholipid composition (footnote to Table 5). However, it should be recognized that the fatty acid analyses were carried out on samples comprising only 80–90% of the total CGP or sphingomyelin present in red cells (the remainder of each being in Fraction D′). Nevertheless, we have recently analyzed the fatty acids of total CGP and sphingomyelin isolated by TLC, and the results correspond closely to the mean values given in Table 5.

This study and Farquhar's reports (5, 29) have defined characteristic fatty acid patterns for each phospholipid class. All of the acidic glycerophosphatides contain high percentages of arachidonic acid and significant amounts of unsaturated C_{20} and C_{22}, but can be distinguished by their other fatty acid components (very high stearic acid in the mixture of SGP and IGP, with more evenly distributed saturated moieties in the ethanolamine glycerophosphatides). Fraction C also has a small percentage of lignoceric acid, again suggesting the presence of Yamakawa's "globoside". Choline glycerophosphatide is characterized by a high percentage of linoleic acid; and sphingomyelin is characterized by the predominance of palmitic, lignoceric, and nervonic acids.

Since phospholipid fatty acids (29) and lipid phosphorus (38) have been shown to exchange with comparable plasma moieties, the fatty acids detected in any of these classes may represent a composite of those derived from de novo synthesis and those which have exchanged from the plasma. Thus, it is difficult to draw conclusions regarding the pathway of phospholipid synthesis in erythroid precursors from these data alone.

In two recently reported studies (39, 40) it has been demonstrated that the total lipid content, lipid phosphorus content, cholesterol content, and phospholipid distribution of intact red cells is virtually identical with these parameters of lipid measurement in the red cell ghost. Thus, most of the lipid present in the intact red cell seems to reside in the "membrane", and analysis of lipids isolated from the intact cell actually describe red cell membrane lipid. It is hoped that such analyses will provide some insight into disturbances of membrane composition and metabolism in the hemolytic anemias, demyelinating diseases, and diseases of lipid storage.

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