Composition of phospholipids and of phospholipid fatty acids and aldehydes in human red cells

JAMES T. DODGE* and GERALD B. PHILLIPS‡

Department of Medicine, College of Physicians and Surgeons, Columbia University, New York 10032

ABSTRACT Improved methods for lipid analysis that have been developed recently were employed to reevaluate the phospholipid composition, the fatty acid and fatty aldehyde composition of the total phospholipid, and the fatty acid composition of the individual phospholipids of normal human red cells.

Thirty-three fatty acids and five fatty aldehydes were estimated and tentatively identified in the total phospholipid of normal human red cells. Additional minor components were evident.

The major individual phospholipids were isolated by silicic acid thin-layer chromatography and quantified. The fatty acid compositions of phosphatidyl ethanolamine, phosphatidyl serine, lecithin, and sphingomyelin were determined. Each of these phospholipids showed a distinctive and characteristic fatty acid pattern.

KEY WORDS phospholipids · fatty acids · fatty aldehydes · human red cells · phosphatidyl ethanolamine · phosphatidyl serine · lecithin · sphingomyelin · autoxidation

Previous studies of the lipid composition of normal human red cells by means of chromatographic techniques (1–24) have shown inconsistencies which may be explained in part by differences in analytical methods or, in certain instances, by the artifactual autoxidation of unsaturated fatty acid constituents during isolation, storage, or analysis (25). Recently, a number of advances in lipid technology have been introduced that appear to merit application to a reevaluation of human red cell lipid composition. Techniques have been developed for the apparently complete separation of individual phospholipids by TLC (26, 27) in sufficient quantity to allow fatty acid analyses on the isolated phospholipid moieties by GLC. The development of organosilicone polyester stationary phases for GLC, which have greater thermal stability and lower volatility than ordinary polyesters (28, 29), have permitted scrutiny of the long-chain fatty acids from 20 to 26 carbon atoms in length. Overlapping peaks on GLC have been resolved by separation of dimethyl acetals from the methyl esters by TLC (30), and by fractionation of the methyl esters according to their degree of unsaturation via TLC of the mercuric acetate adducts (31) prior to GLC analysis. Using the antioxidant 2,6-di-*tert*-butyl-*p*-cresol (BHT) in the solvents (32) apparently has eliminated the troublesome problem of autoxidation in the red cell lipid extracts (25) and results in more reproducible analyses of phospholipids as well as of polyunsaturated fatty acids.

In the present study, these improved techniques were used to determine the concentration of the individual phospholipids, the fatty acid and aldehyde composition of the total phospholipid, and the fatty acid composition of the individual phospholipids of normal human red cells.

MATERIALS AND METHODS

20 ml of venous blood was obtained from each of seven male and three female, apparently healthy, fasting subjects between 20 and 40 yr of age. A brief dietary history revealed that none of the subjects was on a special diet

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; BHT, 2,6-di-*tert*-butyl-*p*-cresol. In the abbreviation of the fatty acids, the first two digits state the number of carbon atoms, the third digit states the number of double bonds, and the digit after the omega states the number of carbon atoms from the methyl end of the acyl chain to the middle of the nearest double bond.

* This work was done during the tenure of an Advanced Research Fellowship of the American Heart Association.

‡ Recipient of Lederle Medical Faculty Award. Present address: The Roosevelt Hospital, 428 West 59th Street, New York 10019. Reprint requests should be sent to Dr. G. B. Phillips at this address.

Downloaded from www.jlr.org by guest, on June 20, 2012

except one who intentionally consumed larger amounts of unsaturated fat than the others. The blood was anticoagulated with 0.15 ml of a solution containing 30 g of EDTA per 100 ml (Vacutainer, Becton-Dickinson & Co., Rutherford, N.J.), chilled at 0°C, and processed immediately. The red cells were isolated by centrifugation at 4°C and washed three times with an equal volume of a solution containing 0.15 M NaCl and 10^{-5} M Na_2EDTA that was titrated to pH 7.4 with $NaHCO_3$; the buffy coat was discarded. The washed red cells were resuspended in the saline–EDTA solution to a hematocrit value of approximately 50%, and duplicate aliquots were taken as follows: 1.0 ml for hematocrit determination (33); 50 µl for red cell counts by an electronic cell counter (Coulter Counter, Coulter Electronics Inc., Hialeah, Fla.) with a standardized human red cell suspension (Erythro-Trol, Dade Reagents Inc., Miami, Fla.) used as a control for each set of determinations; and 6.0 ml for extraction of red cell lipids.

Extraction of Lipids

The aliquots for lipid extraction were transferred to 40 ml centrifuge tubes and centrifuged at 3000 *g* for 10 min at 4°C, and the supernatant was discarded. The red cell lipid was isolated quantitatively by three extractions with methanol–chloroform 1:1 at room temperature in the presence of the antioxidant BHT (Ionol CP, Shell Chemical Corp, New York), and the pooled extracts were washed with 0.05 M KCl by the method of Folch, Lees, and Sloane Stanley (34) as follows. For the first extraction, 15 ml of methanol containing 5 mg of BHT per 100 ml was added to the packed red cells in each of the centrifuge tubes, which were then allowed to stand for 30 min with occasional stirring to break up the sediment; 15 ml of chloroform was then added, and the mixture again allowed to stand for 30 min with occasional stirring to break up clumps. The tubes were centrifuged for 5 min at 1500 *g* and the supernatant fractions decanted into separatory funnels fitted with Teflon stopcocks. The second and third extractions were performed similarly with 15 ml of the methanol–BHT added to the residue followed by 15 ml of chloroform, except that the extracts stood for only 10 min, with occasional stirring, after each addition. After centrifugation, the supernatant fractions were pooled in a separatory funnel, then 48 ml of chloroform and 28 ml of 0.05 M KCl were added and mixed; the mixture was allowed to stand overnight in darkness at 4°C for phase separation. After being warmed to room temperature, the lower of the two clear phases was collected and evaporated to dryness in vacuo at 40°C in a rotary vacuum evaporator. The lipid was transferred quantitatively to a 10 ml volumetric flask with chloroform and stored at −22°C. Red cells from three of the subjects were extracted by the "procedure III" method of Ways and Hanahan (18). A sample of red cell residue from lipid extraction by each method was hydrolyzed with 2 N HCl, the hydrolysate was extracted with pentane, and the pentane extract treated with the methyl ester procedure (see below); no evidence for a fatty acid methyl ester spot could be detected by TLC.

Determinations of Cholesterol and Phosphorus

The concentration of free cholesterol in the lipid extract was determined in triplicate as follows. The lipid extract was chromatographed on a 0.5 mm layer of Silica Gel HR (Brinkmann Instruments, Inc., Westbury, N.Y.) in hexane–diethyl ether–glacial acetic acid 80:20:1, the TLC plate was stained by spraying with 2,7-dichlorofluorescein solution (see below), the free cholesterol spot was scraped into a conical centrifuge tube and extracted once with 2.0 ml and three times with 1.0 ml of chloroform, the extract was evaporated to dryness in vacuo at 40°C in a rotary vacuum evaporator, and the cholesterol was estimated by the ferric chloride method of Mann (35) without saponification. A free cholesterol standard, which was prepared from a commercial certified reagent grade material by isolation through the dibromide derivative (36, 37), was taken through the chromatographic procedure and estimated with each set of determinations. The values for free cholesterol were corrected in each determination for the recovery of the standard, which averaged 95%. The TLC was necessary to remove the BHT, which otherwise interfered with the ferric chloride method by producing a brown product that absorbed at 560 mµ. In the solvent system employed for development, BHT migrated just behind cholesteryl ester, but with considerable overlap.

Total red cell lipid phosphorus was determined in triplicate by an adaptation of Marinetti's modification of Bartlett's method (38, 39). An aliquot of lipid extract containing 0.01–0.15 µmole of phosphorus was transferred to a graduated Pyrex digestion tube (Kramer-Gittleman), and the organic solvents were evaporated off completely.[1] After addition of 0.75 ml of 70% perchloric acid and a Pyrex bead, the tube was placed in an electric heater at 190°C for 60 min. The tube was removed and allowed to cool, the following reagents were added, and the mixture was shaken after each addition: 2.4 ml of glass-distilled water; 2.0 ml of 1% ammonium molybdate; 0.2 ml of Fiske-Subbarow reagent (38); and water to the 6.0 ml mark. The tubes were then heated in boiling water for 10 min and allowed to cool, and the absorbancy of the solution was measured at 830 mµ.

[1] The digestion may proceed explosively unless organic solvents are evaporated off completely before addition of perchloric acid.

Downloaded from www.jlr.org by guest, on June 20, 2012

Phospholipid Fractionation

The phospholipid distribution was determined in triplicate by TLC of aliquots of the total red cell lipid extract at 4°C on Silica Gel HR, 0.5 mm thick, in chloroform–methanol–glacial acetic acid–water 25:15:4:2 (27) to which we added BHT at a concentration of 50 mg/100 ml to prevent autoxidation during chromatography (37); the TLC plates were prepared with water ("neutral" plates). Use of a "wedged-tip technique" for applying the lipid sample at the origin of the plate (40) resulted in excellent separations of the individual phospholipids. In particular, the method appeared to provide complete separation between phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), lecithin, and sphingomyelin; a discrete spot migrated between PS and lecithin that was tentatively identified as phosphatidyl inositol (27). The spots were made visible in UV light by spraying with a solution of 2,7-dichlorofluorescein (33.3 mg/100 ml of aqueous 2 mM NaOH) and then scraped directly into Kramer-Gittleman tubes, where the phospholipids were digested at 190°C for 60 min with 1.0 ml of 70% perchloric acid.[2] The remainder of the procedure was performed as described above, except that after color development, the silica gel was removed by centrifugation at 3000 *g* for 5 min and the absorbancy was determined on the clear supernatant solution. Corrections were made for the absorbancy of corresponding areas of blank lanes. The very low levels of phosphorus contaminating the Silica Gel HR as supplied by the manufacturer were comparable to those obtained with Silica Gel H after the latter had been washed with chloroform–methanol–formic acid (41).

For preparation of methyl ester and dimethyl acetal derivatives of red cell lipid fractions, we isolated the total phospholipids by TLC of the lipid extract at 4°C on Silica Gel HR, 0.5 mm thick, in hexane–diethyl ether–glacial acetic acid 70:30:1 containing BHT (50 mg/100 ml). In this system, the phospholipids remained at the origin, while the less polar lipids, the large amount of BHT added during the extraction procedure, and BHT oxidation products migrated up the plate and were not included in the methylation reaction. Individual phospholipids were then separated and stained as described above; the corresponding spots in six lanes were pooled. The appropriate areas of silica gel were rapidly scraped into ampoules and 2.0 ml of boron trifluoride–methanol reagent (Applied Science Laboratories Inc., State College Pa.) was added without delay; the ampoules were flushed with nitrogen, sealed, and heated in boiling water for the time periods specified by Morrison and Smith (30). The methyl esters and dimethyl acetals were extracted into pentane by addition of 4.0 ml of pentane containing BHT (5 mg/100 ml) followed by 2 ml of 5 N NaOH, which was added dropwise with stirring at 0°C, and collection of the upper phase. A second extraction carried out with 2.0 ml of pentane was pooled with the first. The base was added to prevent reversion of the dimethyl acetals (30). The dichlorofluorescein stain remained in the aqueous phase.

Methyl esters and dimethyl acetals from total red cell phospholipid were separated by TLC at 4°C on Silica Gel HR, 0.5 mm thick, with toluene containing BHT (50 mg/100 ml) as the developing solvent. Toluene gave a separation comparable to that obtained with benzene as described by Morrison and Smith (30). After the appropriate regions of silica gel had been scraped into centrifuge tubes, the separated derivatives were extracted from the silica gel once with a 2.0 ml and twice with a 1.0 ml aliquot of chloroform containing BHT (5 mg/100 ml). The methyl ester and dimethyl acetal fractions were then analyzed separately by GLC.

As an aid in identification and quantification of fatty acids in overlapping peaks on GLC, we further fractionated the fatty acid methyl esters of total red cell phospholipid according to their degree of unsaturation by TLC of the acetoxymercurimethoxy derivatives as described by Mangold (31).

Gas–Liquid Chromatography

GLC was performed on hexane-dissolved samples with a Barber-Colman instrument, model 5000, equipped with paired 8-ft columns of EGSS-X (an ethylene glycol succinate polyester combined with a silicone) 8% on Gas-Chrom P, 100–120 mesh (Applied Science Laboratories Inc.) and dual flame ionization detectors. The nitrogen flow rate was 50 ml/min at the inlet. The column temperature was maintained at 165°C for 10 min after injection of the sample, then increased at 2°C/min to 200°C. Fig. 1 shows that such a programmed run took approximately one-half the time of an isothermal run at 180°C without significantly sacrificing peak separation; the programming, furthermore, did not appear to change the order of peak elution observed isothermally. The peaks were identified on isothermal runs at 185°C by use of a plot of the log retention time versus the carbon number, and by calculation of Types I and II separation factors according to Ackman and Burgher (42). Known fatty acid methyl ester mixtures corresponding to NHI mixtures KB, KD, and KF and mixtures K107, L203, L205, and L207 (Applied Science Laboratories Inc.) were used as primary and cod liver oil fatty acid methyl

[2] Bartlett noted that color development is influenced by the acidity of the reaction (38). In the present system, with a final total volume of 6.0 ml, a constant absorbancy was obtained when 0.5–1.1 ml of 70% perchloric acid was used. Since we found that Silica Gel HR neutralized perchloric acid, a large excess of acid (1.0 ml) was used with the silica gel to assure sufficient acidity. Insufficient acidity resulted in artifactually increased absorption.

Downloaded from www.jlr.org by guest, on June 20, 2012

ASBMB JOURNAL OF LIPID RESEARCH

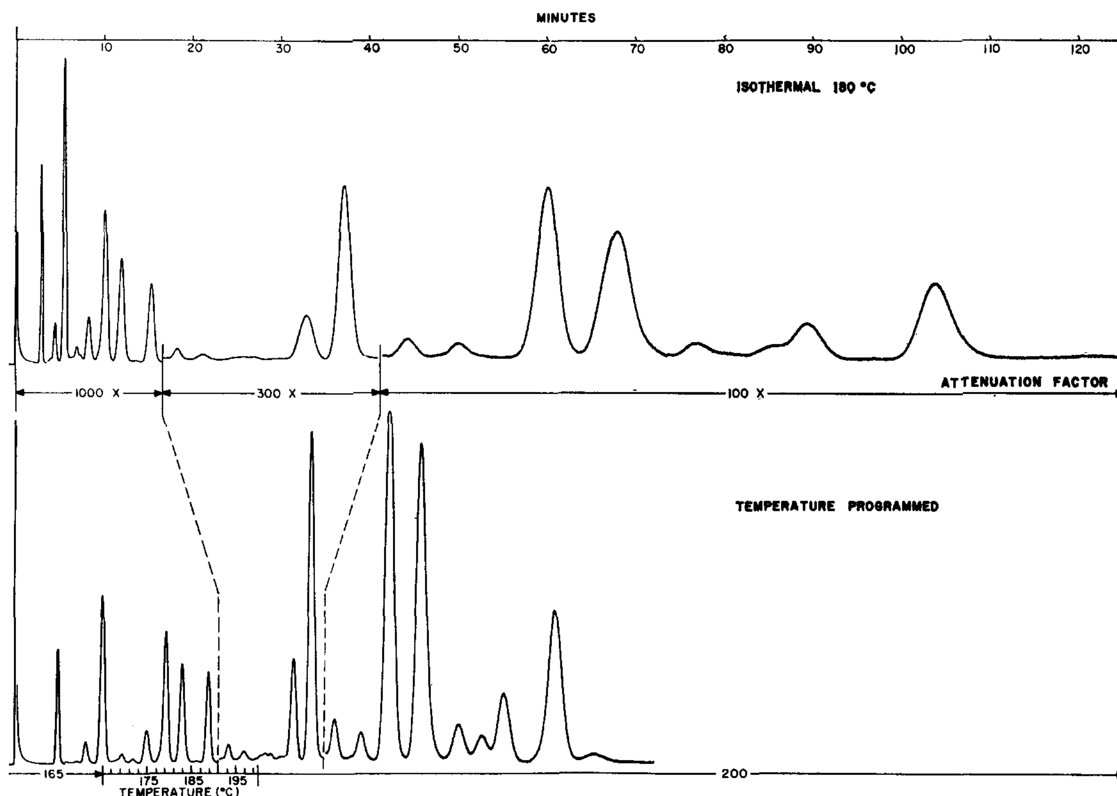

FIG. 1. Comparison of isothermal and temperature-programmed gas–liquid chromatographic pattern of fatty acid methyl esters and dimethyl acetals of total phospholipid of normal human red cells.

esters as secondary reference standards (29, 43). Identification was confirmed by GLC of the red cell total phospholipid methyl esters on a less polar column, EGSS-Y 12% (Applied Science Laboratories Inc.) at 190°C, and by GLC of methyl ester fractions separated according to their degree of unsaturation (31). Samples of red cell total phospholipid fatty acid methyl esters were run by the programmed method for as long as 7 hr to rule out the presence of later peaks. Fig. 2 shows the GLC patterns of red cell total phospholipid fatty acid methyl ester fractions separated according to degree of unsaturation by TLC of the acetoxymercurimethoxy derivatives followed by removal of the mercuric acetate (31). The TLC separation of these derivatives was incomplete and somewhat variable; this accounts for the presence of certain fatty acids in two fractions. Nevertheless, this method provided a useful means of identification and quantification of individual components, especially when components merged in the same peak during GLC of the total methyl ester sample. The decrease in the polarity of the GLC column with time, which presumably was a consequence of a decrease in concentration of the stationary phase (44) as a result of "bleeding," affected the elution pattern to some extent, e.g., initially, 26:0 emerged before 22:6ω3, whereas later (Fig. 2) the elution sequence was reversed. The GLC pattern of the isolated dimethyl acetal fraction is also shown in Fig. 2. The procedures used were periodically carried out on blank samples to exclude the presence of artifactual peaks.

Peak areas were calculated by means of a disc integrator and were considered to be directly proportional to the weight of the component fatty acids or aldehydes without application of correction factors. Quantitative results with NHI fatty acid methyl ester standard mixtures KB, KD, and KF (Applied Science Laboratories Inc.) agreed with stated composition data with a relative error of less than 2% for major components (>10% of total mixture) and less than 3.5% for minor components (<10% of total mixture). Data in moles/100 moles were calculated by division of each peak area by a detector response factor that was derived from the number of "effective carbon atoms" per molecule of the peak's component (45). The detector response factor used for fatty acids was the number of carbon atoms in the methyl ester minus one, and for fatty aldehydes, the number of carbon atoms in the dimethyl acetal minus one (45); e.g., the response factors for stearic (18:0), arachidic (20:0), and arachidonic (20:4ω6) acids were 18, 20, and 20, respectively, and for the fatty aldehydes 18:0 and 18:1 the factor was 19.

BHT emerged with a retention time similar to that of methyl myristate, and when taken through the methyla-

Downloaded from www.jlr.org by guest, on June 20, 2012

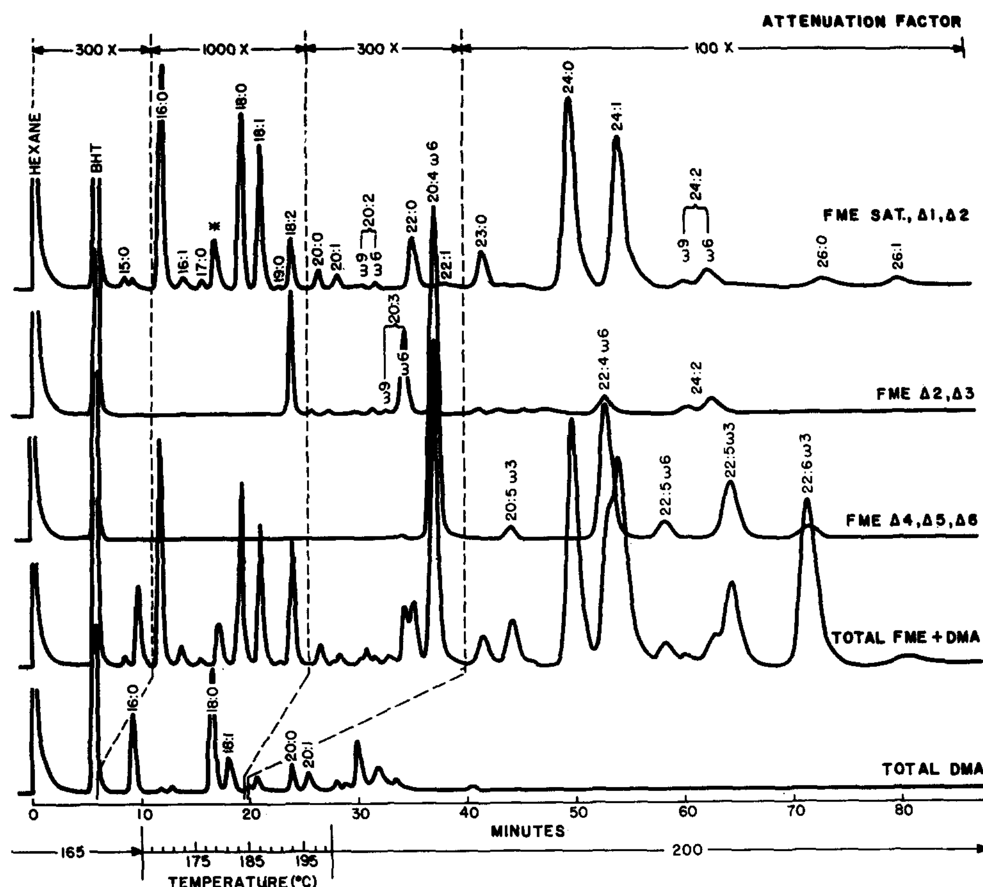

Fig. 2. Gas–liquid chromatographic patterns of normal human red cell total phospholipid fatty acid methyl ester (FME) fractions separated according to degree of unsaturation by TLC of the acetoxymercurimethoxy derivatives followed by removal of the mercuric acetate. The pattern for GLC of the isolated dimethyl acetal (DMA) fraction is also shown.

tion procedure in the presence of boron trifluoride–methanol reagent gave rise to an additional peak with a retention time similar to that of methyl palmitoleate (25). Therefore, myristic acid was not quantified in this investigation; palmitoleic acid was quantified by the mercuric acetate adduct method.

Solvents

Chloroform was washed with water, distilled in an all-glass system, and methanol was added (1:50) as a preservative. Pentane was distilled over $KMnO_4$. Hexane was "spectroanalyzed" grade and all other chemicals were certified reagent grade materials from Fisher Scientific Company, Fair Lawn, N.J., unless specified otherwise.

RESULTS

Table 1 shows the mean values and standard deviations for the lipid phosphorus and free cholesterol content and for the phospholipid distribution of the red cells from the 10 normal subjects studied. Esterified cholesterol and triglyceride were present only in trace amounts and were not quantified.

With the same lipid extracts from these 10 subjects, we determined the distribution of the fatty acids and aldehydes in the isolated total red cell phospholipid and recorded the values both in moles/100 moles and weight (g/100 g) in Table 2. The mercuric acetate adduct method was used in all of the samples to facilitate identification and quantification in mixed peaks (Fig. 2). Additional minor components were present that were too small in amount for reliable quantification. No spot for hydroxy acid methyl esters was detected on TLC of the red cell total phospholipid following the boron trifluoride procedure (30).

We fractionated the lipid extracted from five of the subjects into the individual phospholipids by TLC and analyzed the mixed fatty acid methyl esters and dimethyl acetals of each phospholipid fraction by GLC. The mean values and ranges for the fatty acid components of PE, PS, lecithin, and sphingomyelin are given in Table 3. Since the mercuric acetate adduct method was not used and the individual components of mixed peaks were not estimated, the value for each peak is expressed only as weight (g/100 g). Because of interference by a BHT artifact, 16:1 was not estimated. The results for the minor

Downloaded from www.jlr.org by guest, on June 20, 2012

ASBMB JOURNAL OF LIPID RESEARCH

TABLE 1 PHOSPHOLIPID AND CHOLESTEROL COMPOSITION* OF RED CELLS FROM 10 NORMAL SUBJECTS

Determination	Results	
	µmoles/ml red cells	*µmoles × 10⁻¹⁰/red cell*
Free cholesterol	3.15 ± 0.29	3.23 ± 0.14
Lipid phosphorus	3.90 ± 0.23	4.00 ± 0.17
Phospholipid distribution	*moles/100 moles of phosphorus recovered*	
Polyglycerol phosphatide†	1.1 ± 0.5	
Phosphatidyl ethanolamine	27.5 ± 1.5	
Phosphatidyl serine	14.8 ± 1.7	
Phosphatidyl inositol†	0.6 ± 0.5	
Lecithin	29.2 ± 1.5	
Sphingomyelin	25.4 ± 1.4	
Lysolecithin	1.0 ± 0.8	
Point of origin on TLC plate	0.4 ± 0.6	
Recovery of phosphorus after TLC (%)	94.5 ± 4.0	

* Values are shown as mean ± SD.
† Not positively identified.

phospholipids are not shown because of the considerable error involved in their estimation as a result of base line instability and interference by artifacts during GLC of the small amounts of material at high sensitivity. Furthermore, evidence that some lysolecithin was lost in the lipid extract wash indicated that the fraction recovered on the TLC plate might not have been representative.

DISCUSSION

Although the fatty acid distribution of human red cell total lipid or phospholipid has been analyzed previously (2–4, 6, 9, 12, 14, 17, 18), the improved methods used in the present study allowed a more comprehensive analysis than has hitherto been achieved. Thirty-three fatty acids and five aldehydes were separated, quantified, and tentatively identified. Moreover, scrutiny of the chromatographic record indicated that additional minor components were present. The greater number of fatty acids quantified and the inclusion of the aldehydes in the calculation of distribution tended to make the relative amount of the major fatty acids less than in previous reports. For example, 24:0 and 24:1, which were not reported in previous studies of red cell total phospholipids except by Ways and Hanahan (18), together comprised about 10% of the total fatty acid and aldehyde by weight in the present study. Where the relative amounts of the major peaks alone were calculated, the values found were quite similar to those reported by Ways and Hanahan (18). Several previous estimations of the relative amounts of 20:4ω6 (3, 4, 9, 12, 14) and of 22:6ω3 (3, 4, 9, 12) were considerably lower than those found in the present study. The lower values in previous studies could have been due to autoxidation of these highly susceptible fatty acids (25, 46, 47). To protect against autoxidation in the present study, we used the antioxidant BHT during the extraction, storage, and chromatography of the lipids (25, 32). The smaller degree of variation in the individual fatty acids between subjects observed in this study compared to that in previous studies (2–4, 17, 18) suggested an improved reliability in methodology. Some of the variation between subjects could have been related to diet, since the distribution of red cell fatty acids, especially 18:2, has been shown to respond to dietary lipid alteration (3, 4, 14, 20). It is of interest, therefore, that the largest standard deviation of the major fatty acids observed in

TABLE 2 FATTY ACID AND ALDEHYDE COMPOSITION* OF TOTAL PHOSPHOLIPID FROM RED CELLS OF 10 NORMAL SUBJECTS

Components†	Distribution (% of total fatty acid + aldehyde)	
	moles/100 moles	*g/100 g*
15:0	0.16 ± 0.05	0.13 ± 0.04
16:0 (DMA)‡	2.2 ± 0.2	1.9 ± 0.2
16:0	21.4 ± 0.8	18.2 ± 0.7
16:1ω9	0.88 ± 0.29	0.74 ± 0.25
17:0	0.44 ± 0.09	0.40 ± 0.08
18:0 (DMA)‡	3.5 ± 0.3	3.6 ± 0.3
18:1 (DMA)‡	1.3 ± 0.3	1.3 ± 0.3
18:0	14.0 ± 0.7	13.4 ± 0.6
18:1ω9	12.6 ± 0.7	12.0 ± 0.6
19:0	—§	—§
18:2ω6 ‖	9.0 ± 1.1	8.6 ± 1.1
20:0 (DMA)‡	0.06 ± 0.01	0.07 ± 0.01
20:1 (DMA)‡	0.06 ± 0.02	0.06 ± 0.02
20:0	0.40 ± 0.12	0.42 ± 0.13
20:1ω9 + 18:3ω3¶	0.37 ± 0.08	0.39 ± 0.08
20:2ω9** + 20:2ω6** + 21:0¶	0.55 ± 0.19	0.58 ± 0.20
20:3ω9	0.28 ± 0.04	0.29 ± 0.04
20:3ω6	1.2 ± 0.4	1.3 ± 0.4
22:0	1.7 ± 0.2	1.9 ± 0.2
20:4ω6	11.9 ± 0.8	12.6 ± 0.8
22:1ω9	0.25 ± 0.08	0.29 ± 0.09
23:0 + 20:4ω3¶	0.34 ± 0.13	0.41 ± 0.15
20:5ω3 + 22:3ω9¶ + 22:2ω9¶ + 22:2ω6¶	0.46 ± 0.13	0.51 ± 0.12
24:0	3.7 ± 0.4	4.7 ± 0.5
22:4ω6	2.6 ± 0.7	3.1 ± 0.8
24:1ω9	3.8 ± 0.4	4.8 ± 0.5
22:5ω6	0.67 ± 0.19	0.79 ± 0.22
24:2ω9** + 24:2ω6**	0.49 ± 0.15	0.62 ± 0.19
22:5ω3	1.7 ± 0.1	2.0 ± 0.2
26:0	0.20 ± 0.06	0.27 ± 0.09
22:6ω3	3.6 ± 0.6	4.2 ± 0.7
26:1ω9 + 24:4ω6¶	0.19 ± 0.04	0.29 ± 0.07

* Values are shown as mean ± SD.
† This shorthand designation is explained in the *Abbreviations* (p. 667).
‡ DMA, dimethyl acetal derivative of the aldehyde.
§ This peak was too small for quantification at the sensitivities used in this part of the analysis, but was estimated to be <0.5% of the total.
‖ May contain 18:2ω9.
¶ Trace component, ≦0.05% of the total.
** The ratio of the ω6 to the ω9 component was about 3 to 1.

Downloaded from www.jlr.org by guest, on June 20, 2012

TABLE 3 Fatty Acid Composition of the Four Major Phospholipids of Red Cells from 5 Normal Subjects

Components*	Fatty acid distribution (g/100 g of Fatty Acid and Aldehyde)							
	Phosphatidyl Ethanolamine		Phosphatidyl Serine		Lecithin		Sphingomyelin	
	Mean	*Range*	*Mean*	*Range*	*Mean*	*Range*	*Mean*	*Range*
16:0	12.9	11.6 –15.6	2.7	2.5 – 3.0	31.2	30.4 –31.7	23.6	20.9 –27.3
17:0	0.33	0.17– 0.51	0.38	0.31– 0.44	0.69	0.41– 1.10	0.64	0.38– 0.89
18:0 + 18:1 (DMA)	11.5	10.4 –11.9	37.5	37.2 –37.7	11.8	11.1 –12.3	5.7	4.8 – 6.4
18:1ω9	18.1	16.0 –19.6	8.1	7.8 – 8.6	18.9	17.2 –20.5	0.82	0.72– 0.92
18:2ω6†	7.1	5.4 – 8.2	3.1	1.8 – 6.4	22.8	20.6 –24.9	0.24	0.11– 0.38
20:0	0.25	0.05– 0.46	0.25	0.18– 0.32	0.19	0.12– 0.25	1.9	1.5 – 2.0
20:1ω9 + 18:3ω3	0.72	0.53– 0.99	0.36	0.23– 0.43	0.48	0.43– 0.52	0.18	tr. – 0.37
20:2‡ + 20:3ω9 + 21:0	1.3	0.21– 2.1	0.77	0.45– 0.94	1.1	0.67– 1.3	0.59	0.21– 1.06
20:3ω6 + 22:0	1.5	1.2 – 1.8	2.6	1.7 – 4.2	1.9	1.5 – 2.4	9.5§	8.5 –10.3
20:4ω6 + 22:1ω9	23.7	19.7 –25.9	24.2	22.0 –26.1	6.7	5.4 – 8.7	1.4	1.0 – 1.8
23:0 + 20:4ω3	0.06	tr. – 0.09	0.06	tr. – 0.11	0.06	tr. – 0.10	2.0‖	1.5 – 2.4
20:5ω3 + 22:3ω9 + 22:2‡	1.2	0.96– 1.4	0.31	0.24– 0.40	0.31	0.23– 0.38	0.51	tr. – 1.3
24:0	0.25	0.17– 0.33	0.61	0.40– 0.74	0.19	0.10– 0.32	22.8	19.5 –25.0
22:4ω6 }	7.5	6.7 – 9.1	4.0	3.3 – 4.8	0.43	0.31– 0.54		
24:1ω9 }							24.0	23.0 –25.8
22:5ω6	1.0	0.56– 1.4	1.5	1.0 – 1.7	0.23	0.14– 0.33	0.54	0.21– 0.80
24:2‡	tr.	0.00– tr.	0.12	tr. – 0.19	tr.	tr. – 0.13	3.2	2.1 – 4.8
22:5ω3	4.3	4.1 – 4.4	3.4	3.1 – 3.6	0.54	0.45– 0.61		
26:0							1.0	0.73– 1.2
22:6ω3	8.2	7.5 – 9.7	10.1	8.3 –13.7	2.1	1.7 – 2.5		
26:1ω9 + 24:4ω6	0.24	0.12– 0.40	0.36	0.33– 0.39	0.14	0.09– 0.22	1.4	1.0 – 2.2

* See †, Table 2. DMA, dimethyl acetal; tr., ≦ 0.05% of total. Blank spaces: component not detected.
† May contain 18:2ω9.
‡ Contains ω9 and ω6 components.
§ Appears to be largely 22:0.
‖ Appears to be largely 23:0.

the present study was for 18:2, followed by 20:4ω6 and 22:4ω6, and that the subject who had had the highest intake of polyunsaturated fatty acids also had the highest relative amounts of 18:2 and 22:6ω3.

As is evident from Fig. 2, a complex mixture of aldehydes is also present in human red cell phospholipid. Detailed quantitative analysis of this mixture has been described by Kates, Allison, and James (9) in red cell total lipid and by Farquhar (12, 48) in red cell phospholipid. The distribution of the major aldehydes 18:0, 16:0, and 18:1 found in the present study was similar to that found by these investigators. Only the major aldehydes, which constituted about 90% of the total aldehydes, were estimated in the present study and were included with the distribution of fatty acids shown in Table 2.

The fatty acid distribution of each of the major individual phospholipids, i.e., PE, PS, lecithin, and sphingomyelin, showed a characteristic pattern with striking differences in individual fatty acids (Table 3). The major unsaturated fatty acid in PE and PS was 20:4ω6, whereas in lecithin it was 18:2, both made up about one-fourth of the total fatty acid. Other polyunsaturated fatty acids, such as 22:4ω6, 22:5ω6, 22:5ω3, and 22:6ω3, were also at a much higher concentration in PE and PS than in lecithin. About 37% of the total fatty acid in PS was 18:0, while only about 3% was 16:0; in PE and lecithin, 16:0 was the major saturated fatty acid, with the level in lecithin being over twice that in PE. The relative amount of 18:1 was also much lower in PS than in PE and lecithin. Since Farquhar showed that 67% of the PE, 8% of the PS, and 10% of the lecithin of human red cells is in the plasmalogen form (12), and since the aldehyde appears to be largely if not entirely confined to the 1-position of the glycerophospholipid (49), inclusion of the aldehydes in the calculation of fatty acid distribution would reduce the percentages of the individual fatty acids in Table 3 by about 34% for PE, 4% for PS, and 5% for lecithin. If one uses Farquhar's figures for aldehyde distribution (12), the saturated fatty acids-plus-aldehydes, expressed as a percentage of total fatty acids-plus-aldehydes, can be calculated to be 50, 45, and 48% for PE, PS, and lecithin, respectively, the last two figures being lower than Farquhar's. These results are consistent with the concept that the 2-position of the glycerophospholipids is esterified to an unsaturated fatty acid while the 1-position is linked to a saturated fatty acid or aldehyde (49, 50).

The fatty acid distribution of sphingomyelin differed markedly from that of the glycerophospholipids, in particular in the greater degree of saturation. Only about 33% of the fatty acids were unsaturated; in addition, less than 6% of the fatty acids appeared to have more than one double bond and less than 3% more than two double bonds. The 16:0, 24:0, and 24:1 made up almost 70% of the total fatty acids. Essentially all of the 24:0 and most of the 24:1 of the human red cell phospholipid appeared to reside in sphingomyelin. These two fatty

ASBMB JOURNAL OF LIPID RESEARCH JLR

Downloaded from www.jlr.org by guest, on June 20, 2012

acids were also found by others in human red cell sphingomyelin, but in different relative amounts (18, 20, 23, 24).

The fatty acid distribution of human red cell PE (12, 14, 18, 20, 24), PS (12, 14, 18, 20, 24), lecithin (5, 12, 14, 16, 18, 20, 24), and sphingomyelin (5, 14, 18, 20, 23, 24) has been described previously. A major difficulty in these previous studies was that the individual phospholipids were separated by silicic acid column chromatography, a method that did not allow complete separation of the phospholipids, especially lecithin from sphingomyelin. Since the beginning and end of a phospholipid peak eluted from the column appear to have different fatty acid compositions (51, 52), a sample taken from the middle of the peak would not be representative of that particular phospholipid and a sample of the whole peak would include contamination by other phospholipids. In the present study, the major phospholipids were discretely separated by TLC and the whole, apparently uncontaminated, phospholipid spot analyzed. The fatty acid distributions found, nevertheless, were generally similar to those described by Farquhar (12), Ways and Hanahan (18), Hill, Kuksis, and Beveridge (20), and Williams, Kuchmak, and Witter (24) with occasional outstanding differences.

The distribution of the individual phospholipids determined by TLC was similar to that found previously in this laboratory by silicic acid column chromatography (1) and to the results of certain other investigations using silicic acid paper and column chromatography (5, 7, 10, 12, 13, 19, 20). TLC, which was used previously for human red cell phospholipid analysis (13, 19–21), afforded apparently complete separation of the major phospholipids and required considerably less time than the paper and column methods. The lower relative amount of "cephalin" reported in previous studies using silicic acid paper (8, 9, 11, 15, 22) or TLC (21, 22) may have been due to autoxidation of the polyunsaturated fatty acids (25). The free (unesterified) cholesterol and total lipid phosphorus content of the red cells found in this study was similar to that found by others (for review, see 53).

We gratefully acknowledge the technical assistance of Mrs. Carole Rockmore and Miss Cathy Berzin. The generous counsel of Dr. R. G. Ackman of the Technological Research Laboratory, Fisheries Research Board of Canada, Halifax, Nova Scotia, in establishing a method for fatty acid analysis by GLC is deeply appreciated.

This investigation was supported by PHS Research Grant HE 02907 from the National Heart Institute, U.S. Public Health Service.

Manuscript received 1 May 1967; accepted 14 August 1967.

References

1. Phillips, G. B., and N. S. Roome. 1959. *Proc. Soc. Exptl. Med.* **100:** 489.
2. James, A. T., J. E. Lovelock, and J. P. W. Webb. 1959. *Biochem. J.* **73:** 106.
3. Leibetseder, F., and E. H. Ahrens, Jr. 1959. *Brit. J. Haematol.* **5:** 356.
4. Horwitt, M. K., C. C. Harvey, and B. Century. 1959. *Science.* **130:** 917.
5. Hanahan, D. J., R. M. Watts, and D. Pappajohn. 1960. *J. Lipid Res.* **1:** 421.
6. Kögl, F., J. de Gier, I. Mulder, and L. L. M. van Deenen. 1960. *Biochim. Biophys. Acta.* **43:** 95.
7. Reed, C. F., S. N. Swisher, G. V. Marinetti, and E. G. Eden. 1960. *J. Lab. Clin. Med.* **56:** 281.
8. Gier, J. de, and L. L. M. van Deenen. 1961. *Biochim. Biophys. Acta.* **49:** 286.
9. Kates, M., A. C. Allison, and A. T. James. 1961. *Biochim. Biophys. Acta.* **48:** 571.
10. Klibansky, C., and Z. Osimi. 1961. *Bull. Res. Council Israel, Sect. E.* **9:** 143.
11. Blomstrand, R., F. Nakayama, and I. M. Nilsson. 1962. *J. Lab. Clin. Med.* **59:** 771.
12. Farquhar, J. W. 1962. *Biochim. Biophys. Acta.* **60:** 80.
13. Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. *Arch. Biochem. Biophys.* **180:** 119.
14. Farquhar, J. W., and E. H. Ahrens, Jr. 1963. *J. Clin. Invest.* **42:** 675.
15. Westerman, M. P., L. E. Pierce, and W. N. Jensen. 1963. *J. Lab. Clin. Med.* **62:** 394.
16. Ways, P., C. F. Reed, and D. J. Hanahan. 1963. *J. Clin. Invest.* **42:** 1248.
17. Gier, J. de, L. L. M. van Deenen, M. C. Verloop, and C. van Gastel. 1964. *Brit. J. Haematol.* **10:** 246.
18. Ways, P., and D. J. Hanahan. 1964. *J. Lipid Res.* **5:** 318.
19. Bradlow, B. A., R. Rubenstein, and J. Lee. 1964. *S. African J. Med. Sci.* **29:** 41.
20. Hill, J. G., A. Kuksis, and J. M. R. Beveridge. 1965. *J. Am. Oil Chemists' Soc.* **42:** 137.
21. Gastel, C. van, D. van den Berg, J. de Gier, and L. L. M. van Deenen. 1965. *Brit. J. Haematol.* **11:** 193.
22. Szeinberg, A., J. Zaidman, and L. Clejan. 1965. *Biochim. Biophys. Acta.* **98:** 598.
23. Svennerholm, E., S. Ställberg-Stenhagen, and L. Svennerholm. 1966. *Biochim. Biophys. Acta.* **125:** 60.
24. Williams, J. H., M. Kuchmak, and R. F. Witter. 1966. *Lipids.* **1:** 391.
25. Dodge, J. T., and G. B. Phillips, 1966. *J. Lipid Res.* **7:** 387.
26. Skipski, V. P., R. F. Peterson, J. Saunders, and M. Barclay. 1963. *J. Lipid Res.* **4:** 227.
27. Skipski, V. P., R. F. Peterson, and M. Barclay. 1964. *Biochem. J.* **90:** 374.
28. Ackman, R. G., and P. M. Jangaard. 1963. *J. Am. Oil Chemists' Soc.* **40:** 744.
29. Ackman, R. G., and R. D. Burgher. 1964. *J. Fisheries Res. Board Can.* **21:** 319.
30. Morrison, W. R., and L. M. Smith. 1964. *J. Lipid Res.* **5:** 600.
31. Mangold, H. K. 1965. *In* Thin-layer Chromatography. E. Stahl, editor. Academic Press Inc., New York. 174.
32. Wren, J. J., and A. D. Szczepanowska. 1964. *J. Chromatog.* **14:** 405.

Downloaded from www.jlr.org by guest, on June 20, 2012

33. Wintrobe, M. M. 1956. *In* Clinical Hematology. Lea and Febiger, Philadelphia. 4th edition. 367.
34. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. *J. Biol. Chem.* **226:** 497.
35. Mann, G. V. 1961. *Clin. Chem.* **7:** 275.
36. Fieser, L. F. 1953. *J. Amer. Chem. Soc.* **75:** 5421.
37. Williams, J. H., M. Kuchmak, and R. F. Witter. 1965. *J. Lipid Res.* **6:** 461.
38. Bartlett, G. R. 1959. *J. Biol. Chem.* **234:** 466.
39. Marinetti, G. V. 1962. *J. Lipid Res.* **3:** 1.
40. Stahl, E. 1965. *In* Thin-layer Chromatography. E. Stahl, editor. Academic Press Inc., New York. 35.
41. Parker, F., and N. F. Peterson. 1965. *J. Lipid Res.* **6:** 455.
42. Ackman, R. G., and R. D. Burgher. 1963. *J. Chromatog.* **11:** 185.
43. Ackman, R. G., and R. D. Burgher. 1965. *J. Am. Oil Chemists' Soc.* **42:** 38.
44. Supina, W. R., 1964. *In* Biomedical Applications of Gas Chromatography. H. A. Szymanski, editor. Plenum Press, New York. 284–286.
45. Perkins, G., Jr., G. M. Rouayheb, L. D. Lively, and W. C. Hamilton. 1962. *In* Gas Chromatography. N. Brenner, J. E. Callen, and M. D. Weiss, editors. Academic Press Inc., New York. 269.
46. Swern, D. 1961. *In* Autoxidation and Antioxidants. W. O. Lundberg, editor. Interscience Publishers, Inc., New York. **1:** 1.
47. Dodge, J. T., G. Cohen, H. J. Kayden, and G. B. Phillips. 1967. *J. Clin. Invest.* **46:** 357.
48. Farquhar, J. W. 1962. *J. Lipid Res.* **3:** 21.
49. Marinetti, G. V., J. Erbland, and E. Stotz. 1959. *J. Am. Chem. Soc.* **81:** 861.
50. Tattrie, N. H. 1959. *J. Lipid Res.* **1:** 60.
51. Collins, F. D. 1960. *Nature.* **186:** 366.
52. Harris, P. M., D. S. Robinson, and G. Getz. 1960. *Nature.* **188:** 742.
53. Deenen, L. L. M. van, and J. de Gier. 1964. *In* The Red Blood Cell. C. Bishop and D. M. Surgenor, editors. Academic Press Inc., New York. 243.

Downloaded from www.jlr.org by guest, on June 20, 2012