NMR lipid profiles of cells, tissues, and body fluids: proton NMR analysis of human erythrocyte lipids

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Abstract One- and two-dimensional high resolution NMR spectroscopy was applied to determine quantitatively and qualitatively the lipids extracted from human erythrocyte membranes. The relative amounts of the major lipids were determined from the spectra of unfractionated lipid extracts. After HPLC fractionation of the lipid extracts and NMR analysis of the fractions, it was possible to determine the features of the component lipids of each lipid class and to compare, especially, the fatty acid types and composition of the individual major glycerophospholipids. The results of this proton NMR analysis were compared to those obtained elsewhere using classical lipid analytical techniques and found to be in substantial agreement.—**Adosraku, R. K., G. T. Y. Choi, V. Constantinou-Kokotos, M. M. Anderson, and W. A. Gibbons.** NMR lipid profiles of cells, tissues, and body fluids: proton NMR analysis of human erythrocyte lipids. *J. Lipid Res.* 1994. **35:** 1925–1931.

Supplementary key words erythrocyte membrane • NMR • phospholipids • fatty acid

The integrity of erythrocyte membrane is vital for the shape and function of the red blood cell. Defects in the lipid organization of the erythrocyte membrane produce abnormal cells leading to diseases such as hereditary hemolytic anemia (1). Cholesterol and phospholipids are present in about equal proportions in the erythrocyte membrane (2). High phosphatidylcholine (PC) hemolytic anemia, a defect in the active incorporation pathway which results in increased cation permeability of the erythrocyte membrane, occurs when PC levels are raised significantly and phosphoethanolamine (PE) levels are reduced but the overall phospholipid level remains normal (1). Lipid analyses are, therefore, important not only in routine scientific research but also for clinical application.

Several methods are commonly used for the analysis of lipids including TLC thin-layer chromatography (3), ion exchange chromatography (4), high performance liquid chromatography HPLC (5, 6), gas chromatography GC (7) and mass spectrometry GC–MS (8). Comprehensive information on lipids usually calls for several chromatographic steps, normally accompanied by chemical reactions, e.g., in the determination of ether and diacyl phospholipids in a mixture, sphingomyelin and phosphatidylcholine, preparation of fatty acid methyl esters for fatty acid determination by GC (7). However, without any chemical modification, comprehensive information can be obtained when total lipid extracts and/or their intact components are analyzed using high resolution proton NMR spectroscopy. NMR proton chemical shift characteristics have been determined for individual standard lipids (9), fatty acids (10) and mixtures of extracted lipids (11). Using Alzheimer's brain tissue, Kwee, Nakada, and Ellis (12) determined the unsaturation indexes for membrane phospholipid extracts by proton NMR and compared them with age-matched controls and found elevation in unsaturated fatty acids. The proton NMR spectra of human lymphocyte cell lines were shown to differ from each other, the changes attributable to variation in membrane fluidity and composition (13). Using 1-D and 2-D proton NMR (500 MHz) techniques on total lipid extracts of rat liver (14) and human peripheral blood lymphocytes (15), all major phospholipids and neutral lipids as well as fatty acid profiles were determined from characteristic NMR resonances. The results were reported to agree with traditional methods.

In this analysis of erythrocyte lipids, high resolution proton NMR operating at 500 MHz was used in all experiments on total lipid extracts and lipids fractionated by HPLC in order to examine individual lipids in greater detail. No chemical reactions were carried out so that the determinations were carried out on intact lipid molecules.

Abbreviations: NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphoethanolamine; HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; UV, ultraviolet; FID, flame ionization detector; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

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Different types of phospholipids as well as neutral lipids were identified and quantified from their NMR spectra. Information on the fatty acid composition of, particularly, the major glycerophospholipids was also obtained from the proton NMR spectra as well as average levels of unsaturation.

EXPERIMENTAL PROCEDURES

Fractionation of whole blood to obtain erythrocytes

Whole blood was centrifuged at low speed (500 rpm) for 5 min, the platelet-rich supernatant was removed and kept, and the interfacial white fluff was removed and discarded. The packed red cells were washed three times with three volumes each of cold normal saline and centrifuged. The washings were discarded and the cells were kept.

Preparation of erythrocyte membranes from red blood cells

Erythrocyte membranes were obtained by hypotonic lysis of the red blood cells. Four volumes of ice-cold acetic acid solution (0.2%) was added to the washed red cells obtained above, vortexed, and kept cold for 5 min, with occasional shaking, to achieve osmotic lysis of the cells. The suspension was then centrifuged (2500 rpm) for 10 min and the supernatant hemoglobin solution was discarded. The membrane pellet was washed with fresh acetic acid solution and centrifuged. The washing was repeated until the membrane pellet was free of hemoglobin, which was indicated by the clear supernatant of the last few washings. The erythrocyte membranes thus obtained were immediately extracted with chloroform–methanol mixtures.

Extraction of total lipids from erythrocyte membranes

About ten volumes of chloroform–methanol 2:1 was added to the erythrocyte membrane pellet obtained above in a glass tube, vortexed occasionally for 5 min, and centrifuged. The supernatant aqueous layer was discarded and the lower chloroform layer was retained. The pellet was re-extracted twice with the chloroform–methanol mixture and the lower chloroform layers were pooled and washed twice with equal volumes of 0.5 M KCl in 50% methanol to remove non-lipid substances. After centrifugation, the lipid-rich chloroform layer was removed and dried in a stream of nitrogen, re-dissolved in the chloroform–methanol mixture, and kept at −20°C awaiting NMR analysis.

Separation of lipid extracts by HPLC

The lipids, about 4 mg/400 μl, were injected 4 times onto a Spherisorb SW10 silica gel column (4.6 × 250 mm, Altech) and run with a gradient of 5 mM KH_2PO_4 into acetonitrile (2.5–15% from 5–20 min), on a Gilson 714 HPLC instrument interfaced with an IBM PC1 computer. Ultraviolet (UV) detection at 205 nm was used to monitor lipid separation. Peaks were collected manually, dried in a stream of nitrogen, and redissolved in methanol-d_4–chloroform-d_1 2:1 for NMR determination.

Proton NMR of extracted lipids

All NMR spectra were determined using a Bruker AM500 NMR spectrometer. Chemical shifts were referenced to the residual methanol peak at 3.31 ppm. For the 1-dimensional (1-D) NMR determination, a 45° pulse was applied with solvent presaturation during relaxation to remove excess HOD signal at about 4.7 ppm. The FID was acquired with 16 K data points in the Fourier transform mode; the temperature was regulated at 298 K. In all cases, dried lipids were dissolved in 600 μl of methanol-d_4–chloroform-d_1 2:1 for the NMR determination. The 2-dimensional (2-D) COSY experiment was performed on the total lipid extracts in the non-phase sensitive mode, with presaturation during relaxation to remove excess HOD signal. The presaturation power was then switched to a minimum level during pulses and evolution to hold the saturation; 512 FIDs of 48 scans each were acquired with 2 K data points per increment.

Calculation of lipid proportions

Chemical shifts were identified as described elsewhere (9, 14, 16) and by obtaining spectra of authentic phospholipid samples and also from 2-D COSY spectra of the lipids. After baseline correction, characteristic peaks in the 1-D NMR spectra were integrated. The integrals directly related to the amounts of each lipid present, correcting for any signal overlap. The number of protons giving rise to the signal was considered in the calculations. In estimating the fatty acid composition of phospholipids, the integral at about 0.86 ppm was taken as a measure of total fatty chains. The integrals of characteristic fatty acid signals were compared to this value.

RESULTS AND DISCUSSION

Erythrocyte membrane total lipids

The 1-D proton NMR spectrum of the total lipid extract of the erythrocyte membrane lipids is shown in **Fig. 1** while the 2-D COSY (homonuclear correlated spectroscopy) spectrum is shown in **Fig 2.** Proton resonances between 0.68 ppm and 6.0 ppm can be divided into three broad regions (14): *1,* fatty chain and sterol methyl and methylene proton resonances between 0.65 ppm and 2.9 ppm (10); *2,* phospholipid head group and glycerol backbone proton resonances between 3.05 ppm and 5.25 ppm; and *3,* vinyl proton signals between 5.3 ppm and 5.95 ppm.

Choline phospholipids were identified by their characteristic-$N^+(CH_3)_3$ proton singlets at about 3.2 ppm that consisted of two partially overlapping singlets. These two sig-

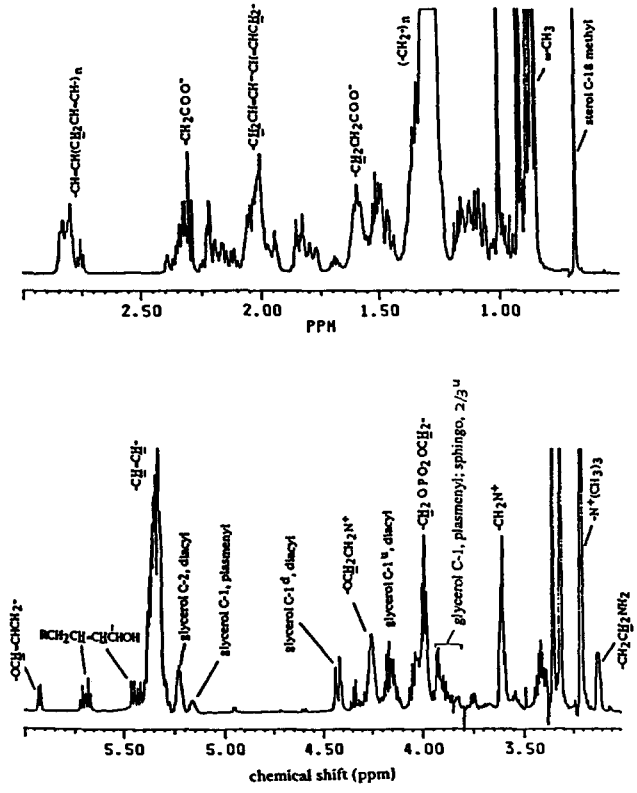

Fig. 1. Proton NMR spectrum of human erythrocyte membrane total lipids. The spectrum was obtained on 10 mg/ml of lipid extract in CD_3OD-$CDCl_3$ 2:1 at a temperature of 298 K; 256 scans were obtained for Fourier transformation and the residual methanol peak at 3.31 ppm was used as the reference chemical shift.

nals suggested the presence of two principal choline lipids that were later identified as phosphatidylcholine (3.2065 ppm) and sphingomyelin (3.196 ppm) after chromatographic separation and NMR analysis of the fractions. The proportions of the two types of choline lipids were estimated from the integrals and/or heights of the singlets. Any induced changes in these lipids may, therefore, be easily noted by running a proton NMR experiment on the total lipid extract. The two choline head group methylene protons-$OCH_2CH_2N^+$, resonated at 3.6 ppm(-CH_2N^+) and 4.25 ppm (-O CH_2-), were confirmed by their cross peaks in the 2-D COSY spectrum of the total lipids (Fig. 2) (14).

Ethanolamine phospholipids were also identified by their characteristic head group-CH_2NH_2 methylene proton resonance at about 3.12 ppm. The shape of this signal indicated the presence of a mixture of ethanolamine lipids, which were later identified as diacyl and plasmenyl glycerophosphoethanolamine after HPLC separation of the total lipids for further NMR determination. Examination of the 2-D COSY spectrum of the total lipids showed the cross peak (3.12 ppm, 3.98 ppm), which identified the -OCH_2 head group methylene protons at 3.98 ppm (14).

Diacylglycerophospholipids were represented by the backbone glycerol *sn*-2 proton multiplet at 5.21 ppm. The magnetically inequivalent glycerol *sn*-1 methylene protons resonated at 4.42 ppm (downfield) and 4.15 ppm (upfield), while both glycerol *sn*-3 methylene proton resonances overlapped at about 4.0 ppm. Coupling between these glycerol backbone protons was confirmed by cross peaks in the 2-D COSY spectrum of the total lipids and unequivocably gave their assignments.

Ether phospholipids gave characteristic resonances identified in the NMR spectrum of the total lipids. The multiplet at 5.15 ppm, arising from the glycerol *sn*-2 proton, represented all the ether lipids, both alkylacyl and alkenylacyl (plasmenyl). The ratio of diacyl to total ether lipids was determined from the integrals of their glycerol *sn*-2 proton signals. The glycerol *sn*-1 proton signals were, however, not readily recognized in the 1-D spectrum of the total lipids, because of overlapping signals from other phospholipid protons. Plasmenyl lipids gave their characteristic vinyl proton resonances at 5.92 ppm (doublet) and 4.33 ppm (quartet) (14). The difference between the integrals at 5.15 ppm and 5.92 ppm, therefore, represented the measure of 1-alkyl-2-acyl glycerophospholipids present (**Table 1**).

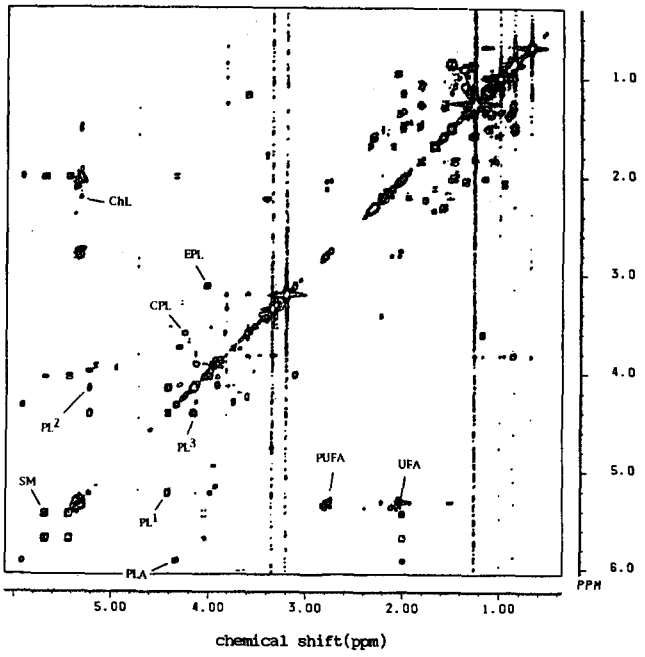

Fig. 2. 2-D COSY spectrum of total membrane lipids. SM, sphingosine vinyl protons; PLA, plasmalogen -OCH = $CHCH_2R$; PL^1, phospholipid glycerol C-1(downfield proton) and C-2 proton; PL^2, glycerol C-1 (upfield proton) and C-2 proton; PL^3, glycerol C-1 upfield and downfield protons; CPL, choline methylenes; EPL, ethanolamine methylenes; PUFA, -CH = $CHCH_2CH$ = CH-; UFA, -CH = $CHCH_2CH_2R$; ChL, cholesterol C-6 and C-7 protons.

TABLE 1. Erythrocyte membrane lipid compositions determined from NMR integrals of characteristic signals, and compared with values obtained from TLC/chemical analysis

	mol % of Total Lipids	mol % of Total Phospholipids	
Lipids	NMR	NMR	TLC/Phosphorous[a]
Phosphatidylcholine	14.0 ± 2.5	26.2 ± 2.5	31.7 ± 2.1
Total ethanolamine lipids	16.0 ± 2.3	30.0 ± 2.3	27.1 ± 2.3
Diacyl-PE	8.4	-	-
Alkenylacyl-PE	6.9	-	-
Alkylacyl-PE	0.7	-	-
Sphingomyelin	18.7 ± 3.0	35.0 ± 3.0	28.0 ± 1.2
Sphingenine analogue	16.6	-	-
Sphinganine analogue	2.1	-	-
Cholesterol	46.6 ± 2.8	-	-
(PS, PI)	4.7 ± 1.6	8.8 ± 1.6	12.5 ± 0.4
LPC	trace	trace	0.8 ± 0.4

[a]Ref. 19.

Values are the average of three determinations on the same blood sample. Lipid recovery from the HPLC column was not less than 89%. The values represent the proportions of individual integrals of characteristic signals to the sum of these integrals. PE, phosphoethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; LPC, lysophosphatidylcholine.

Sphingenine lipids were identified by the specific vinyl proton resonances of the sphingenine moeity at 5.7 ppm (multiplet) and 5.44 ppm (quartet of singlets). The coupling of these vinyl protons was confirmed by cross peaks in the 2-D COSY spectrum of the total lipids (Fig. 2) (14). They were shown to be mainly sphingomyelin after HPLC separation and NMR determination.

Some difficulties were encountered in the determination of phospholipids from the NMR spectra of the total lipid extracts which included the following: acidic phospholipids such as phosphatidylserine, phosphatidylinositol, and phosphatidylglycerol, gave head group resonances that largely overlapped with the corresponding glycerol and head group signals from non-acidic phospholipids. It was, therefore, possible but not simple to identify and quantify all the acidic lipids. Thus when non-acidic phospholipids are present mainly in the diacyl form, the head group region of the spectrum is less complicated and inositol lipids may be identified and quantified from one or more of the inositol ring triplets (14, 15) at about 3.8 ppm. However, when significant proportions of ether lipids are present, the head group region of the spectrum is much more complicated by additional glycerol *sn*-1 proton signals, so that even inositol lipids are not easily determined. Again, it was difficult at this stage to determine the proportions of the head groups (choline and ethanolamine) as diacyl, ether, or ceramide lipids. These problems were largely resolved by chromatographic separation of the lipids and NMR analysis of the chromatographic fractions.

Cholesterol was identified in the 1-D NMR spectrum of the total lipids by its characteristic C-18 methyl singlet at 0.68 ppm (9, 14) from which it was quantified. Other diagnostic cholesterol resonances easily identified in the spectrum included the C-19 methyl singlet at 1.0 ppm and the C-3 proton multiplet at 3.41 ppm. Examination of the spectrum suggested that cholesterol was the only major neutral lipid present in the erythrocyte membranes. Triglycerides were clearly absent from the lipid extract due to the absence of one of their characteristic glycerol C-1/C-3 proton resonances at 4.33 ppm (14, 16). This signal would have overlapped the plasmenyl vinyl proton quartet at the same resonance frequency.

Fatty chains

The fatty acid chains of the erythrocyte membrane phospholipids consisted of MUFAs and different PUFAs. The complex signal at 5.33 ppm arose collectively from vinyl ($-C\underline{H} = C\underline{H}-$) protons in the fatty acid chains and cholesterol. The contribution of the cholesterol proton was subtracted and the remaining integral of this resonance was a measure of the extent of unsaturation in the fatty acid chains. The presence of polyunsaturated fatty chains was indicated by the overlapping resonances at ca 2.8 ppm. These signals arose from the methylene protons within the series of double bonds in the chain $\{-CH = CH-C\underline{H}_2-(CH = CH-CH_2-)_n\}$. For linoleic acid, n = 1, the specific methylene gave rise to the triplet at 2.75 ppm which was resolved and, therefore, readily quantified. Cross peaks in the 2-D COSY spectrum confirmed coupling between these methylene and vinyl protons of the various unsaturated fatty acids (Fig. 2). Tetraenoic (i.e., arachidonic) and related fatty acid chains gave rise to the multiplet at 1.69 ppm as a result of the characteristic beta methylene groups, $= CHCH_2C\underline{H}_2CH_2C\,O\,O^-$. The ω-CH_3 protons resonated at ca. 0.86 ppm with interference from cholesterol methyl resonances. It was

possible to deduce, from the integral at 0.68 ppm, the appropriate cholesterol methyl resonance integral, and hence obtain a measure of the total fatty acid chains from the 0.68 ppm resonance. Again, it was not possible at this stage to determine the variation of fatty acid chains among the phospholipids. This was possible after chromatographic separation, which also removed overlapping cholesterol resonances and revealed characteristic fatty acid peaks more clearly. The total lipid extract was, therefore, subjected to HPLC to obtain individual lipids and lipid classes for further analysis by proton NMR. The usefulness of the HPLC prior to NMR determination is that it allows structural comparison between phospholipids from the same or different sources. Subspecies of a particular phospholipid (e.g., diacyl, alkylacyl, plasmenyl) are also readily quantified from their NMR spectra. In this way, specific variations in particular phospholipids caused by lipid-modifying agents can easily be determined and quantified. This is particularly significant as no chemical degradative processes are necessary prior to analysis. Only representative spectra of HPLC-separated lipids are shown in this report.

Lipids isolated by HPLC

Erythrocyte membrane PC eluted from the column at about 25 min after sample injection (**Fig. 3**) and its NMR spectrum is shown as **Fig. 4.** The fatty acid region (0.8 ppm–2.9 ppm) was much simpler than in the total lipids. The average unsaturation, i.e., the ratio of the integral of the vinyl peak at 5.34 ppm to that of the ω-CH_3 peak at 0.86 ppm, was calculated at 0.91. This value, less than unity, suggested low levels of PUFAs. Total unsaturated chains were calculated at about 48% of total fatty chains, i.e., ratio of integral of peak at 2.04 ppm to that of the

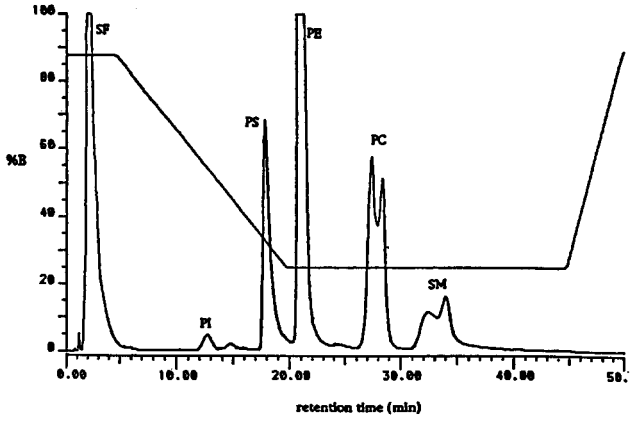

Fig. 3. HPLC profile of erythrocyte membrane lipids. One mg of lipid extract was injected each time with UV detection at 205 nm; solvent A, acetonotrile–5 mM KH_2PO_4 4:1; solvent B, acetonitrile; SF, solvent front; PI, inositol phospholipids; PS, phosphatidylserine; PE, ethanolamine phospholipids; PC, phosphatidylcholine; SM, sphingomyelin.

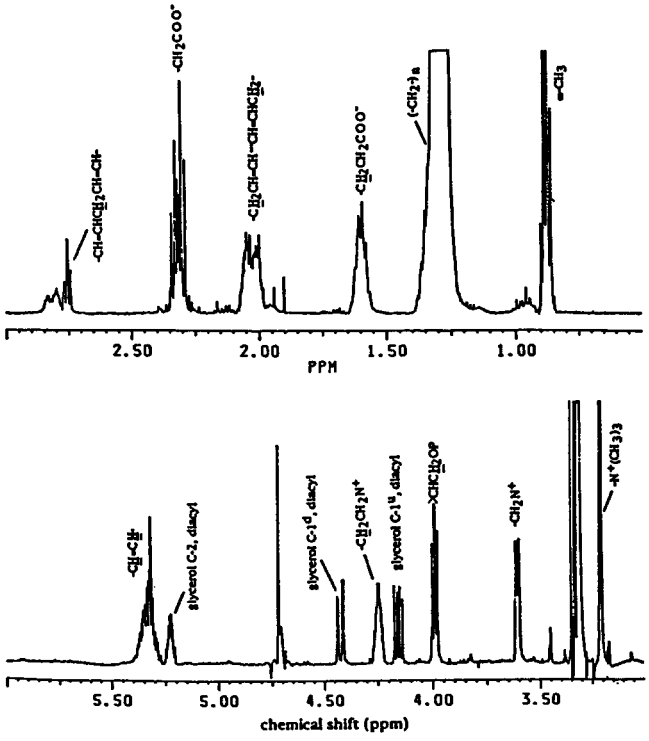

Fig. 4. Proton NMR spectrum of erythrocyte membrane phosphatidylcholine. The erythrocyte membrane phosphatidylcholine was obtained from 4 mg of lipid extract and the spectrum was recorded as described under Experimental Procedures.

ω-CH_3 signal. This confirmed the presence of roughly equal proportions of saturated and unsaturated fatty chains in erythrocyte membrane PC (**Table 2**). Examination of the fatty acid region confirmed that linoleic acid (2.75 ppm) was the only significant polyunsaturated fatty chain present (17), i.e., 23% of total chains which compares with the 22.63% obtained by Hsiao et al. (18) using GC. Other values obtained by Hsiao et al. (18) e.g., monounsaturated (19.53%), tetraenoic (6.39%), hexaenoic (1.32%), compared reasonably with the values in Table 2.

Erythrocyte membrane ethanolamine lipids eluted at about 21 min from the HPLC column (Fig. 3) and gave the proton NMR spectrum shown in **Fig. 5.** The shape of the $-CH_2C\underline{H}_2NH_2$ signal at 3.1 ppm depicted a heterogeneous mixture of ethanolamine lipids. Further spectral examination revealed the presence of two principal types of glycerophosphoethanolamine lipids, the diacyl and ether lipids, distinguishable by their glycerol *sn*-2 proton resonances at 5.23 ppm (diacyl) and 5.15 ppm (ether). The integral of these resonances confirmed that the diacyl lipids made up about 52% while the ether lipids (alkylacyl and plasmenyl) made up about 48% of the ethanolamine phospholipids (Table 1).

Hexaenoic acid (2.4 ppm), arachidonic and related PUFAs (1.69 ppm, 2.12 ppm), and n-3 fatty chains (0.95

TABLE 2. Fatty acid composition of erythrocyte membrane choline and ethanolamine glycerolipids as determined from NMR integrals

Fatty Chain	Chemical Shift (ppm)	Percentage of Total Fatty Chains	
		Erythrocyte PC	Erythrocyte PE
Total unsaturated	2.02	48.4 ± 2.6	63.9 ± 3.2
Total saturated	-	51.6 ± 2.6	36.1 ± 3.2
Linoleic (C:2)	2.75	23.0 ± 2.3	7.9 ± 1.3
Arachidonic (C:4)	1.69	4.3 ± 0.5	18.0 ± 2.2
Hexaenoic (C:6)	2.4/0.96	0.8 ± 0.2	4.6 ± 0.5
Trienoic (C:3)	0.96	2.0 ± 0.3	4.3 ± 0.5
Monounsaturated	-	18.3 ± 2.2	29.1 ± 2.3

C:n denotes fatty acid chains with n double bonds. The values were obtained as an average of three determinations. Integrals of characteristic resonances were compared to that of the ω-CH_3 resonance at about 0.86 ppm which represented total fatty chains.

ppm), which were likely to consist predominantly of trienoic linolenic acid chains, were almost entirely confined to the ethanolamine phospholipids. Linoleic acid (2.75 ppm) was, however, less abundant in ethanolamine than choline phospholipids. Again, the GC analysis of Hsiao et al. (18) e.g., monounsaturated (25.49), dienoic (12.45), tetraenoic (21.34), hexaenoic (5.49), reflect the NMR values in Table 2.

The integrals of the sphingosine moeity vinyl resonances compared to that of the choline N^+-methyl singlet (**Fig. 6**) showed that 89% of the sphingolipids of the erythrocyte membrane occurred as the sphingenine analogue of sphingomyelin. This, therefore, suggested that the sphinganine analogue was present at ca. 11% of the sphingolipids. It was noted that no HPLC peak, when analyzed by NMR, showed evidence of sphingosine moeities with ethanolamine head groups, confirming the absence of ceramide phosphoethanolamine lipids. Using TLC/phosphorus analysis, Hsiao et al. (18) reported the

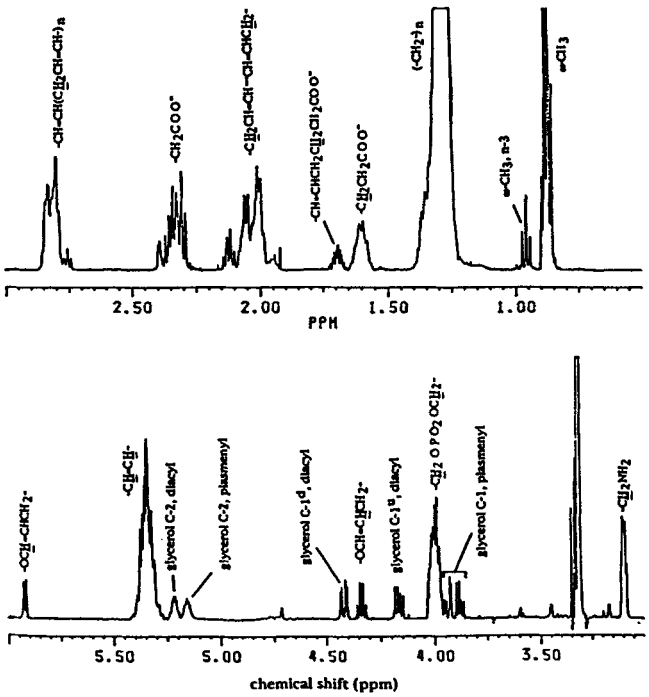

Fig. 5. Proton NMR spectrum of membrane glycerophosphoethanolamine lipids. The erythrocyte membrane ethanolamine lipids were obtained from 4 mg of lipid extract and the spectrum was recorded as described under Experimental Procedures.

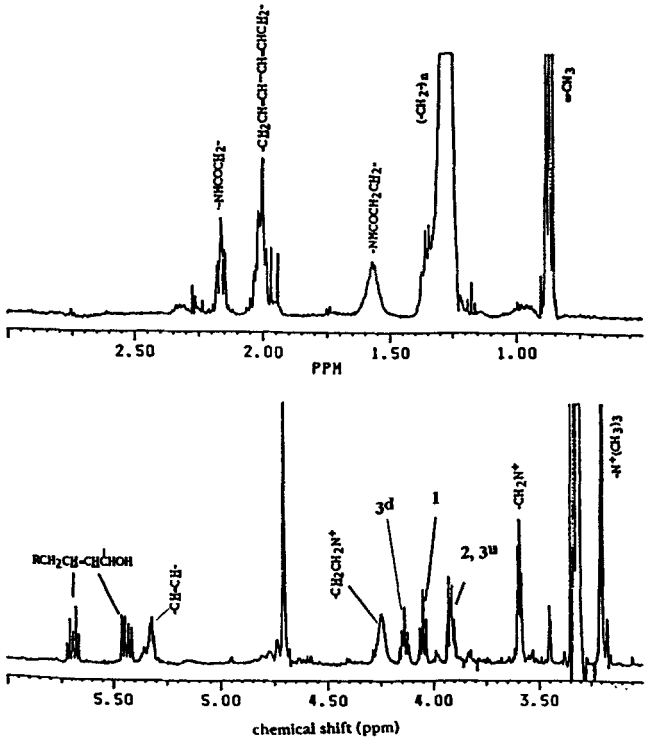

Fig. 6. Proton NMR spectrum of erythrocyte membrane sphingomyelin. One, 2, and 3, sphingosine backbone carbons 1, 2, and 3; u and d, upfield and downfield protons. The sphingomyelin was obtained from 4 mg of lipid extract and the spectrum was recorded as described under Experimental Procedures.

following values for the major phospholipids of the erythrocyte membrane: PC 31.7%; PE 27.1%; sphingomyelin 28.0% of phospholipids. These compare closely with the NMR values of 26.2%, 30.0%, and 35.0%, respectively (Table 1). The small variations are likely due to differences in the samples of blood used (19).

Minor lipids

These were the acidic phospholipids that eluted between 12.5 and 18 min of the HPLC separation (Fig. 3). At least three broad peaks were located in this region of the chromatogram at 13, 15, and 18 min, and were indicative of the presence of acidic head groups containing glycerol, inositol, and serine (comparison with authentic samples). The low levels of these lipids resulted in poorly defined NMR signals that, however, indicated the presence of high proportions of PUFAs in these acidic lipids, a factor that contributed to the enhanced UV absorption peaks observed in the chromatogram. The small proportions of these lipids have been documented (2).

CONCLUSION

The predominant lipids identified in the erythrocyte membrane by the proton NMR analysis included choline and ethanolamine glycerophospholipids, sphingomyelin, and cholesterol. About half the ethanolamine lipids were present as the plasmalogen lipid. The glycerophosphocholine lipid was, however, present entirely in the diacyl form. Unlike phosphatidylcholine, the ethanolamine phospholipids carried high proportions of PUFAs. The sphingolipids consisted of sphingenine and sphinganine bases with choline head groups, the sphingenine analogue being quantitatively more significant.

The proportions of all the major lipids and several minor lipids in both the erythrocyte membrane and plasma were readily obtained by acquiring and integrating a one-dimensional proton NMR spectrum of the total lipid extracts. Without using any chromatographic or chemical procedures, the NMR approach provides a quick and effective diagnostic means of determining many phospholipid and neutral lipid levels in clinical blood samples. The NMR method should also provide a useful alternative means of assessing the effectiveness of lipid-lowering agents during drug treatment and clinical trials, and any lipid inbalances due to genetic or other pathological or toxicological conditions.

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