Abnormal phospholipid molecular species of erythrocytes in sickle cell anemia[1]

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Abstract As the lipid composition of cell membranes has significant effects upon cellular function, we hypothesized that the membranes of sickle cells might have a distorted lipid composition. Accordingly, we analyzed the molecular species of the choline and ethanolamine glycerophospholipids, the fatty acid composition of the total phospholipids and of the five major individual phospholipids of erythrocytes from 8 patients with sickle cell anemia and from 14 normal subjects. Of the 31 molecular species identified for each subclass of the glycerophospholipids, 12 were found to be distinctly abnormal. Sickle cells contained more molecular species with saturated and monounsaturated fatty acid at the sn-2 position and fewer molecular species with polyunsaturated fatty acids at the sn-2 position. The values ranged from 20 to 60% above or below normal values. In diacyl choline glycerophospholipids (outer membrane leaflet), sickle erythrocytes contained lower amounts of the 16:0–18:2 species and higher 16:0–18:1 and 16:0–16:0 species. In diacyl ethanolamine glycerophospholipid (inner membrane leaflet), sickle erythrocytes had lower amounts of 18:0–22:6; 16:0–22:4; 18:0–18:2; 18:1–18:2; and 18:1–20:3. In phosphatidylcholine and phosphatidylethanolamine, sickle erythrocytes contained less linoleic acid, less docosahexaenoic acid (30–40%) and more oleic and palmitic acids (20–30%) compared to normal erythrocytes. These same differences were seen also in the total phospholipids. Our data demonstrated distinct abnormalities of the phospholipid molecular species composition in the membrane lipids of sickle erythrocytes. These defects might have a role in one or more known metabolic abnormalities of sickle cell disease including cation imbalance, dehydration, disturbed membrane phospholipid asymmetry, and hypercoagulability. Furthermore, detailed information of the phospholipid molecular species composition of normal erythrocytes was provided.—**Connor, W. E., D. S. Lin, G. Thomas, F. Ey, T. DeLoughery, and N. Zhu.** Abnormal phospholipid molecular species of erythrocytes in sickle cell anemia. *J. Lipid Res.* 1997. **38:** 2516–2528.

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Hemoglobin S is the most common abnormal hemoglobin in human populations and the resultant sickle cell anemia is the cause of serious morbidity and greatly enhanced mortality in individuals of African descent (2, 3). Pioneering work in the 1950s by Pauling et al. (4) demonstrated that hemoglobin S resulted from a single amino acid substitution of valine for glutamic acid at position 6 of the beta globulin chain. Sickle cell anemia became the first inherited disease to be understood at the molecular level (4, 5). Despite the fact that the molecular nature of the disease has been known for more than 30 years, treatment remains largely symptomatic and supportive, and definitive treatment has remained elusive (2). Recent progress has been made in attempting to increase hemoglobin F production using pharmacological agents to ameliorate the effects of hemoglobin S. Many questions remain about factors that could modify the clinical course of this lethal disease.

Although the primary defect of sickle cell disease occurs from the abnormal hemoglobin molecule, other abnormalities in the erythrocyte membrane have been

Abbreviations: DHA, docosahexaenoic acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; HPLC, high pressure liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. Phospholipid molecular species nomenclature: the first symbol is the fatty acid (i.e., 16:0) at the *sn*-1 position of the phospholipid molecule. The first number of the fatty acid represents the number of carbons (i.e., 16). The second number represents the number of double bonds (i.e., 0). The second symbol after the dashed line (—) is the fatty acid (i.e., 18:1) at the *sn*-2 position of the phospholipid molecule. Diacyl choline glycerophospholipid has a choline base and two fatty acids esterified at the *sn*-1 and *sn*-2 positions. In contrast, alkenylacyl and alkylacyl choline glycerophospholipid have ether linkages rather than ester linkages at the *sn*-1 position. Fatty acid nomenclature: the first number indicates the length of the carbon chain; the second number, after the colon, specifies the number of double bonds; the third number, after n, gives the number of carbon atoms before the first double bond, counting from the methyl end of the chain.

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reported. These include altered cation homeostasis with cellular dehydration (2, 6), changes in membrane proteins (7, 8), and abnormalities in the phospholipid bilayer (9–11). Kuypers et al. (12) found an improvement in cellular hydration and deformability by changing the molecular species of phosphatidyl choline in the sickle cell erythrocytes. These results suggested that abnormalities in the molecular species composition of phospholipid might be present in sickle erythrocytes and that these could be corrected by facilitating the exchange of molecular species of phosphatidylcholine. However, there has been no definite information about the phospholipid molecular species composition of sickle erythrocytes. Even the information about the phospholipid molecular species composition of normal erythrocytes is scanty. In biologic membranes, many phospholipid molecular species exist, as has now been demonstrated, in the brain, retina, and sperm (13–15). Each molecular species is defined by the chemical nature of the polar head group, the type of linkage to glycerol and to the aliphatic chains at both the *sn*-1 and *sn*-2 positions. Different molecular species have different metabolic and physical properties. The molecular species composition of membrane phospholipids is associated with membrane fluidity and the function and activity of membrane bound enzymes (16–18).

In the present study, we analyzed the membrane phospholipid molecular species and fatty acid composition of sickle erythrocytes from eight patients with sickle cell anemia and compared them to normal erythrocytes. Distinct abnormalities were uncovered with low levels of several essential polyunsaturated fatty acids and a considerable difference in the phospholipid molecular species composition.

METHODS

The normal subjects included both white and black individuals of both sexes. The patients with sickle cell anemia had a homozygous SS genotype based on hemoglobin electrophoresis and the classic clinical diagnostic criteria for this disease. All of the sickle cell patients had low (<2%) or undetectable levels of fetal hemoglobin. The sickle cell patients were asymptomatic at the time of study. They had not been recently transfused. We did not study them during periods of crisis but only in the steady state condition. The blood of eight individuals with the sickle cell trait was also examined. We considered it highly desirable to study relatives of patients living in the same family environment (i.e., siblings and parents). We thought it desirable to establish whether individuals with sickle cell trait had minor fatty acid abnormalities as compared with sickle cell patients. Sickle cell trait implies the heterozygote state or the carrier state. In addition, blood from patients with other hemolytic diseases and from patients with Hb SC, Hb SF, and Hb SβoTh. These patients were classified according to standard definition. For example, patients with sickle-fetal hemoglobin, HbSF, met the standard definition of those patients with HbS who had a fetal hemoglobin over 10% and mild clinical disease. These patients were distinct from those with "SS" disease. No genetic study was done to determine whether any of these patients had hereditary persistence of fetal hemoglobin.

Blood samples were collected in tubes containing EDTA from 14 normal subjects and 8 sickle cell anemia patients. Plasma and erythrocytes were separated immediately by centrifugation. Reticulocyte counts were measured by standard methods. The erythrocytes were washed three times with saline. The lipids of the erythrocytes were extracted by the procedure of Rose and Oklander (19) using chloroform and isopropanol, because the use of isopropanol in place of methanol avoids attracting heme pigment. Butylated hydroxytoluene (5 mg/100 ml) was added as an antioxidant (20).

The erythrocytes of 6 patients and 2 subjects with sickle trait were fractionated by discontinuous stractan (arabine gulatan [sigma]) density gradient centrifugation (21) into two fractions: younger cells and older cells (light and dense fractions respectively). The lipids of these two fractions were extracted and their fatty acids were measured and compared with the fatty acids of the original erythrocytes.

The phospholipid molecular species were analyzed by the methods reported previously (13). Phospholipid classes were separated by the thin-layer chromatography (TLC) system described previously. Choline and ethanolamine glycerophospholipids were extracted from gel scrapings with two washes of 5 ml of chloroform–methanol 1:1 (vol/vol), followed by one wash with 5 ml of chloroform–methanol–water 65:45:12 (by volume) and one more with 5 ml of chloroform–methanol 1:1 (vol/vol). The molecular species of choline and ethanolamine glycerophospholipids were analyzed based on the method described by Blank et al. (22). Briefly, ethanolamine glycerophospholipids were hydrolyzed with phospholipase C for 4 h at room temperature. Diradylglycerols were extracted from the hydrolysate by the method of Bligh and Dyer (23) and benzoate derivatives were prepared by reaction with benzoic anhydride and 4-dimethylaminopyridine for 1 h at room temperature. The reaction was stopped with concentrated ammonium hydroxide and the resulting diradylglycerobenzoates were extracted with hexane. Diradylglycerobenzoates were separated into the diacyl,

alkenylacyl, and alkylacyl subclasses by TLC on silica gel G with benzene–hexane–ethyl ether 50:45:4 (by volume). Bands were scraped into a 1:1 ethanol–water mixture and the diradylglycerobenzoates were extracted with hexane. The samples were then filtered (Millex-HV 0.43 μm filter unit; Millipore Corp., Bedford, MA), dried under nitrogen, and redissolved in acetonitrile–isopropanol 70:30 (vol/vol) for HPLC injection.

Separation of the molecular species was accomplished by HPLC. It was performed at ambient temperature with a Perkin-Elmer Model 41-LC BioPump system fitted with a μBondapak C18 precolumn insert and a 3.9 mm × 30 cm analytical column packed with Novapak C18 (Waters Associates, Milford, MA). Peaks were monitored at 230 nm with a Perkin-Elmer LC-235 diode array detector and quantitated on a Perkin-Elmer LcI-100 integrator. Molecular species within the diacylalkenylacyl- and alkylacylglycerobenzoates were separated by isocratic elution with acetonitrile–isopropanol in the ratios of 70:30, 63:35, and 63:37 (vol/vol), respectively. Column flow rate was 1 ml/min.

Identification of molecular species was accomplished by comparison with retention times in samples of bovine brain ethanolamine glycerophospholipid, as established by Blank et al. (22) and by gas chromatographic analysis of the collected peaks. The elution profile was similar to that obtained by Blank et al. except that four additional species were identified. Two new peaks, containing 16:0–22:5n–3 and 18:0–22:5n–3, were seen between peaks 2 and 3 and between peaks 7 and 8, respectively, whereas 18:1–22:5n–6 and 18:0–20:5n–3 eluted within peaks 4 and 5, respectively. As a control for possible losses due to oxidation, an aliquot of bovine brain ethanolamine glycerophospholipid was included on every TLC plate and was worked up in parallel to experimental samples. Molecular species analysis of the three subclasses of the control bovine sample gave consistent results similar to those reported. Furthermore, to avoid the possible variations, normal and sickle erythrocytes were paired for the analysis of the phospholipid molecular species. Ethanolamine glycerophospholipid from beef brain, phospholipase C from *Bacillus cereus*, benzoic anhydride, and 4-dimethylaminopyridine were purchased from Sigma (St. Louis, MO). Chloroform, acetonitrile, 2-propanol, methanol, hexane, and benzene were HPLC grade from Burdick and Jackson (Muskegon, MI) and anhydrous ethyl ether was from Mallinckrodt (Paris, KY).

The relative distribution of the diacyl, alkenylacyl, and alkylacyl subclasses within each phospholipid was determined by comparison of chromatogram areas. Diradylglycerobenzoates from each phospholipid group were separated into the three subclasses on the same TLC plate and recovered by exhaustive extraction as described above. Aliquots of extract were then injected into the HPLC and total peak areas from each chromatogram were calculated. The relative percentage of each of the three subclasses was then calculated by dividing the total peak area for the respective subclass by the sum of the total peak areas for all three subclasses.

The fatty acid composition of total phospholipids and individual phospholipids was analyzed by the same methods reported in our previous publications (24). The lipid extracts of erythrocytes were separated into four major classes (phospholipids, free fatty acids, triglycerides, and cholesteryl esters) by thin-layer chromatography (25) on silica gel G plates (500 μm, Analtech, Newark, DE). The solvent system was hexane–chloroform–ethyl ether–acetic acid 80:10:10:1. The individual phospholipids in the lipid extracts were separated by a different thin-layer chromatography system (26), using pre-coated K6 silica gel 60A plates (Whatman Inc., Clifton, NJ) and a solvent system of chloroform–methanol–petroleum ether (bp 35°)–acetic acid–boric acid 40:20:30:10:1.8 (v/v/v/v/w). The fatty acids in each lipid class or phospholipid class were transmethylated with boron trifluoride–methanol (27).

Plasma lipids were extracted by the procedure of Bligh and Dyer (23). The lipid extract was dried under nitrogen and saponified with alcoholic KOH. Fatty acids were recovered by an acidifying aqueous phase and extracted with hexane and then methylated (27).

Methyl esters of fatty acids were analyzed by gas–liquid chromatography using an instrument equipped with a hydrogen flame ionization detector (Perkin-Elmer Model 8500, Norwalk, CT) and a 30-meter SP-2330 fused silica capillary column (Supelco, Bellefonte, PA). The temperatures of the column, detector and injection port were 195°, 250° and 250°C, respectively. Helium was used as the carrier gas and the inlet pressure was 80 psi. The split ratio was 1:170. Individual fatty acids were identified and quantified by computer. A mixture of fatty acid standards was run daily.

Statistical analyses were performed by standard methods (28).

RESULTS

The composition of the membrane phospholipid molecular species was abnormal in sickle cell disease. Twelve molecular species were found to be different between sickle cells and normal erythrocytes (**Table 1**). Differences of molecular species existed in both choline and ethanolamine glycerophospholipids with variations between 20–60% below or above normal. In general, sickle

TABLE 1. Summary of molecular species differences between sickle cells and normal erythrocytes

Phospho-lipids	Subclasses	Molecular Species	Differences from Normal	*P* Value
CGP	Diacyl	16:0–18:2	−19%	<0.027
CGP	Alkylacyl	16:0–22:6	−57%	<0.010
CGP	Diacyl	16:0–18:1	+17%	<0.041
CGP	Diacyl	16:0–16:0	+36%	<0.005
EGP	Diacyl	16:0–22:4	−23%	<0.006
EGP	Diacyl	18:0–22:6	−20%	<0.042
EGP	Diacyl	18:0–18:2	−38%	<0.001
EGP	Diacyl	18:1–18:2	−23%	<0.015
EGP	Diacyl	18:1–20:3	−20%	<0.027
EGP	Alkylacyl	16:0–20:4	−35%	<0.001
EGP	Alkylacyl	16:0–22:6	−41%	<0.005
EGP	Alkylacyl	16:0–18:1	+57%	<0.019

CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids.

cells contained fewer molecular species having polyunsaturated fatty acids at the *sn*-2 position and more molecular species having monounsaturated and saturated fatty acids at the *sn*-2 position than normal erythrocytes.

The molecular species differences between sickle cells and normal erythrocytes are most clearly depicted in **Fig. 1.** While the species 16:0–18:1 and 16:0–16:0 in diacyl choline glycerophospholipids were increased in sickle cells, there were striking reductions in the species containing polyunsaturated fatty acids at the *sn*-2 position: 16:0–18:2 in diacyl choline glycerophospholipids and 16:0–22:6 in alkylacyl choline glycerophospholipids. The ratio of saturated plus the n–9 species to the n–3 plus n–6 species in the diacyl choline glycerophospholipids of sickle erythrocytes was much higher than in normal erythrocytes (0.88 vs. 0.72, $P < 0.05$) (**Table 2**).

In the diacyl ethanolamine glycerophospholipids (Table 1 and **Table 3**), sickle erythrocytes contained lower 18:0–22:6 (−20%, $P < 0.042$); 18:1–18:2 (−23%, $P < 0.015$); 18:0–18:2 (−38%, $P < 0.001$), 16:0–22:4 (−23%, $P < 0.006$) and 18:1–20:3 (−20%, $P < 0.027$) than normal erythrocytes. In alkylacyl ethanolamine glycerophospholipids, sickle erythrocytes also had several species abnormalities: lower 16:0–22:6 (−41%, $P < 0.005$) and 16:0–20:4 (−35%, $P < 0.001$). The total 18:1 species in this subclass was higher in sickle erythrocytes (+25%, $P < 0.000$).

The detailed molecular species of choline and ethanolamine glycerophospholipids in normal and sickle erythrocytes are documented in Tables 2 and 3. Thirty-one molecular species were identified and individually measured. While the *sn*-1 position of these molecular species contained exclusively 16:0, 18:0, or 18:1, the unsaturated n–3, n–6, and n–9 fatty acids as well as saturated fatty acids were found in the *sn*-2 position. In the erythrocyte membranes, there were seven species having n–3 fatty acids at the *sn*-2 position, fifteen with the

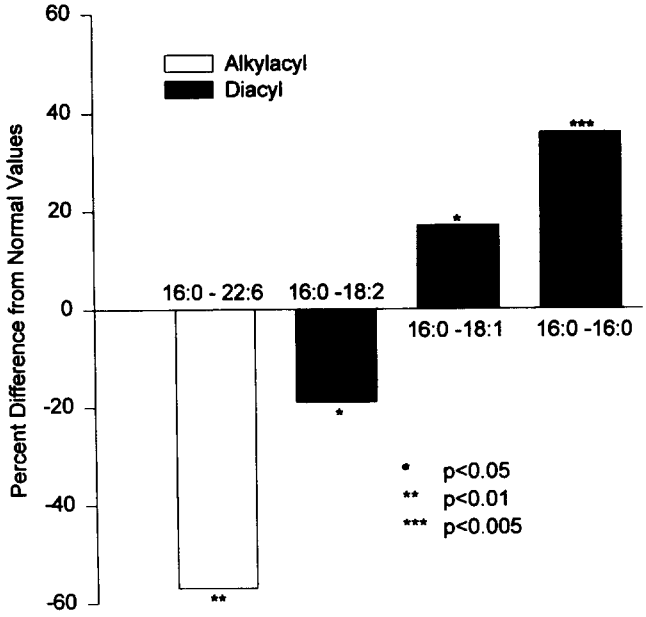

Fig. 1. Major abnormal molecular species in diacyl and alkylacyl choline glycerophospholipids (PC) of sickle cell membranes.

n–6 species, seven with the n–9 species, and two with saturated species. The major n–3, n–6, n–9, and saturated species were 16:0–22:6, 16:0–18:2, 16:0–18:1, and 16:0–16:0, respectively, in the diacyl choline and ethanolamine glycerophospholipids. Interestingly, in alkenylacyl and alkylacyl subclasses, 16:0–20:4 and 16:0–22:4 were the major n–6 species instead of the 16:0–18:2 as seen in the diacyl subclass.

We calculated the relative concentrations of the three subclasses (diacyl, alkenylacyl, and alkylacyl) of the individual phospholipids (14, 22). In normal erythrocytes, the relative concentrations of diacyl, alkenylacyl, and alkylacyl subclasses of choline glycerophospholipids were 93.0, 3.8, and 3.2%, respectively. These same distributions were found in the sickle erythrocytes as well. The relative concentrations of the three subclasses of ethanolamine glycerophospholipids in normal erythrocytes were 61.3, 35.5, and 3.2%, respectively, again the distribution being similar in normal and sickle erythrocytes. Thus, the abnormalities of sickle erythrocytes were found only in individual phospholipid molecular species.

The fatty acid composition of the total phospholipids of sickle erythrocytes was distinctly different from normal erythrocytes (**Table 4**). Sickle erythrocytes contained fewer polyunsaturated fatty acids and more saturated and monounsaturated fatty acids than normal erythrocytes. In sickle cell patients, the linoleic acid 18:2n–6 was 26% lower than normal ($P < 0.000$) and docosahexaenoic acid 22:6n–3 was 27% lower than nor-

TABLE 2. Major molecular species of choline glycophospholipids in normal and sickle erythrocytes (mol percent)

Molecular Species	Diacyl		Alkenylacyl		Alkylacyl	
	Normal (n = 6)	Sickle (n = 6)	Normal (n = 6)	Sickle (n = 6)	Normal (n = 6)	Sickle (n = 6)
n–3						
16:0–22:6	3.3 ± 1.6	3.2 ± 0.9	34.5 ± 14.1	32.9 ± 11.9	12.6 ± 5.0	5.4 ± 2.5[b]
18:0–22:6	0.5 ± 0.7	0.5 ± 0.1	2.1 ± 0.8	1.9 ± 1.0	5.8 ± 5.5	2.6 ± 0.4
18:1–22:6	0.5 ± 0.3	0.6 ± 0.8	1.2 ± 2.0	2.2 ± 2.9	3.9 ± 2.4	2.1 ± 1.2
Total 22:6	4.2 ± 1.5	4.3 ± 1.7	37.8 ± 12.5	37.0 ± 10.4	20.8 ± 11.1	10.1 ± 2.1
16:0–22:5	0.8 ± 0.2	0.9 ± 0.2	0.7 ± 0.5	1.2 ± 1.2	0.8 ± 0.2	2.0 ± 0.8
18:0–22:5	0.4 ± 0.7	0.0 ± 0.0	0.1 ± 0.2	0.5 ± 1.2	0.1 ± 0.3	0.0 ± 0.0
16:0–20:5	0.2 ± 0.1	0.3 ± 0.3	0.3 ± 0.5	0.6 ± 0.8	1.0 ± 0.7	0.6 ± 0.3
18:0–20:5	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2
n–6						
16:0–20:4	5.8 ± 1.0	6.7 ± 0.9	7.5 ± 2.5	7.8 ± 1.3	6.8 ± 1.2	9.2 ± 2.9
18:0–20:4	3.5 ± 1.7	4.6 ± 0.4	7.7 ± 6.3	6.7 ± 4.5	5.8 ± 2.4	6.6 ± 2.1
18:1–20:4	3.4 ± 0.9	2.9 ± 0.8	4.0 ± 2.8	3.6 ± 3.7	4.6 ± 1.2	5.9 ± 2.5
Total 20:4	12.7 ± 2.2	14.2 ± 1.4	19.2 ± 9.0	18.1 ± 5.4	17.7 ± 3.3	21.7 ± 7.2
16:0–18:2	26.5 ± 3.9	21.5 ± 2.7[a]	2.3 ± 1.3	1.8 ± 0.6	2.9 ± 0.8	2.2 ± 0.5
18:0–18:2	6.2 ± 0.6	6.1 ± 0.8	0.4 ± 0.2	0.4 ± 0.2	0.9 ± 0.3	1.2 ± 0.3
18:1–18:2	2.1 ± 0.6	2.1 ± 0.1	0.6 ± 0.2	0.6 ± 0.3	1.8 ± 1.7	0.8 ± 0.1
Total 18:2	34.8 ± 4.1	29.7 ± 3.2[a]	3.3 ± 1.6	2.8 ± 0.6	5.4 ± 1.4	4.1 ± 0.3[a]
16:0–22:4	0.9 ± 0.1	0.6 ± 0.1	5.8 ± 3.2	5.5 ± 1.9	7.2 ± 2.1	6.8 ± 1.4
18:0–22:4	0.4 ± 0.0	1.1 ± 0.1	2.3 ± 1.0	2.7 ± 1.5	5.2 ± 1.6	8.1 ± 1.9
18:1–22:4	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.4	1.0 ± 0.5	3.0 ± 2.8	1.3 ± 0.2
16:0–20:3	1.6 ± 0.2	0.2 ± 0.0	0.5 ± 0.3	0.8 ± 0.3	0.6 ± 0.2	1.0 ± 0.2
18:0–20:3	0.6 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.7 ± 0.2	0.6 ± 0.1
18:1–20:3	0.2 ± 0.1	0.8 ± 8.1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.2	0.1 ± 0.0
16:0–22:5	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.6
18:0–22:5	0.9 ± 0.9	0.1 ± 0.2	1.9 ± 1.9	2.0 ± 2.5	0.1 ± 0.3	0.8 ± 1.2
18:1–22:5	0.4 ± 0.1	0.9 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.6 ± 0.2
n–9						
16:0–18:1	19.0 ± 1.7	22.3 ± 3.0[b]	4.3 ± 1.8	4.4 ± 2.4	9.6 ± 3.0	13.0 ± 3.1
18:0–18:1	6.7 ± 1.6	5.5 ± 1.5	2.7 ± 1.3	4.7 ± 2.6	4.7 ± 3.8	5.2 ± 1.3
18:1–18:1	2.3 ± 1.5	3.5 ± 0.6	2.7 ± 0.9	2.7 ± 1.4	6.4 ± 2.3	6.0 ± 1.7
Total 18:1	28.0 ± 1.8	31.3 ± 2.9[a]	9.7 ± 2.3	11.7 ± 3.5	20.7 ± 4.4	24.2 ± 4.5
16:0–20:1	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.05	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0
18:0–20:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.8	0.0 ± 0.0	0.0 ± 0.0
18:1–20:1	1.1 ± 1.2	0.9 ± 0.8	1.7 ± 1.6	2.4 ± 1.7	1.3 ± 2.0	1.1 ± 1.1
17:0–18:1	2.0 ± 1.1	1.7 ± 0.9	8.5 ± 3.9	7.2 ± 3.2	1.7 ± 2.4	1.1 ± 0.8
Saturated						
16:0–16:0	6.1 ± 1.0	8.3 ± 1.1[b]	3.4 ± 0.6	3.2 ± 1.4	6.6 ± 4.6	9.8 ± 3.6
16:0–18:0	4.1 ± 2.0	4.1 ± 1.7	2.5 ± 1.6	1.9 ± 2.1	4.3 ± 4.7	5.6 ± 3.2
Total saturated	10.2 ± 2.6	12.4 ± 2.3	5.9 ± 1.4	5.1 ± 3.0	10.9 ± 8.7	15.4 ± 5.7
Sat. + mono. / Polyunsaturated	0.72 ± 0.12	0.88 ± 0.11[a]	0.35 ± 0.05	0.38 ± 0.10	0.56 ± 0.22	0.74 ± 0.20

Compared to normal: [a]$P < 0.05$, [b]$P < 0.01$.

mal ($P < 0.016$). On the other hand, the percentage of the total fatty acids as saturated and monounsaturated fatty acid was increased, especially palmitic (16:0) and oleic (18:1) acids. The ratio of saturated plus monounsaturated fatty acid to polyunsaturated fatty acids was thus increased to 1.63 for sickle erythrocytes over the 1.22 for normal erythrocytes ($P < 0.008$). For sickle cell trait patients, the fatty acid composition of erythrocytes was normal. The percentages of fetal hemoglobin in the blood of the patients with SS disease was 0% or less than 2%, so that we could not make a correlation between membrane lipid abnormalities and fetal hemoglobin levels. None of these patients were being treated with hydroxyurea. This would be an interesting study to do in patients treated with hydroxyurea, which would produce more fetal hemoglobin.

We fractioned the erythrocytes of six sickle cell patients into light and dense fractions and also the erythrocytes of two sickle trait subjects as controls. The fatty acid composition of the light and dense fractions of erythrocytes was not different within either group (sickle and sickle trait). However, expected differences

TABLE 3. Major molecular species of ethanolamine glycerophospholipids in normal and sickle erythrocytes (mol percent)

Molecular Species	Diacyl		Alkenylacyl		Alkylacyl	
	Normal (n = 6)	Sickle (n = 6)	Normal (n = 6)	Sickle (n = 6)	Normal (n = 6)	Sickle (n = 6)
n–3						
16:0–22:6	5.3 ± 0.5	5.1 ± 0.8	3.9 ± 2.2	4.8 ± 2.9	8.3 ± 2.2	4.9 ± 0.7[c]
18:0–22:6	1.5 ± 0.3	1.2 ± 0.1[a]	3.2 ± 0.5	3.5 ± 0.6	4.3 ± 2.1	5.0 ± 1.7
18:1–22:6	1.6 ± 0.4	1.4 ± 0.2	4.0 ± 2.2	2.1 ± 1.2	4.9 ± 1.7	4.3 ± 3.7
Total 22:6	8.3 ± 0.8	7.7 ± 0.7	11.1 ± 1.6	10.4 ± 1.5	17.5 ± 3.5	14.3 ± 3.3
16:0–22:5	1.9 ± 0.3	2.2 ± 0.4	2.1 ± 0.4	3.1 ± 0.5	1.9 ± 0.9	4.0 ± 0.5
18:0–22:5	0.3 ± 0.5	0.4 ± 0.7	4.1 ± 0.7	4.3 ± 1.3	1.0 ± 2.5	0.7 ± 1.6
16:0–20:5	0.5 ± 0.1	0.4 ± 0.1	1.1 ± 0.6	0.6 ± 0.3	1.3 ± 0.5	1.2 ± 1.0
18:0–20:5	0.2 ± 0.3	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 1.2	0.0 ± 0.0
n–6						
16:0–20:4	13.1 ± 1.1	15.0 ± 2.1	10.5 ± 1.5	10.0 ± 2.1	14.3 ± 2.0	9.3 ± 2.0[d]
18:0–20:4	10.6 ± 2.2	13.7 ± 2.9	19.0 ± 1.7	18.3 ± 1.7	14.6 ± 1.7	13.0 ± 2.7
18:1–20:4	9.0 ± 1.4	8.6 ± 1.6	11.6 ± 2.0	9.5 ± 1.6	10.3 ± 5.1	12.0 ± 1.5
Total 20:4	32.7 ± 3.4	37.3 ± 3.4[a]	41.0 ± 3.9	37.8 ± 2.6	39.2 ± 5.0	34.3 ± 3.3
16:0–18:2	4.8 ± 0.6	5.7 ± 0.9	2.0 ± 0.7	2.4 ± 0.5	2.5 ± 0.8	2.6 ± 0.8
18:0–18:2	2.6 ± 0.3	1.6 ± 0.3[d]	0.7 ± 0.0	0.8 ± 0.1	0.6 ± 0.3	0.9 ± 0.1
18:1–18:2	6.2 ± 1.1	4.8 ± 0.4[a]	1.0 ± 0.2	1.0 ± 0.2	1.3 ± 0.6	1.5 ± 0.5
Total 18:2	13.6 ± 1.4	12.2 ± 1.2	4.5 ± 0.7	4.3 ± 0.4	4.3 ± 0.9	4.9 ± 0.9
16:0–22:4	6.6 ± 0.9	4.9 ± 0.8[b]	7.2 ± 1.7	7.4 ± 1.5	6.3 ± 1.9	7.8 ± 2.4
18:0–22:4	0.2 ± 0.0	0.4 ± 0.1	4.2 ± 0.1	5.7 ± 0.4	3.3 ± 1.6	6.0 ± 0.5
18:1–22:4	1.0 ± 0.1	0.8 ± 0.05	1.6 ± 0.3	1.8 ± 0.3	2.1 ± 1.1	2.6 ± 0.9
16:0–20:3	1.3 ± 0.2	1.6 ± 0.3	0.6 ± 0.1	1.1 ± 0.2	0.5 ± 0.2	1.1 ± 0.3
18:0–20:3	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.5 ± 0.2	0.4 ± 0.0
18:1–20:3	0.5 ± 0.08	0.4 ± 0.05[a]	0.1 ± 0.05	0.1 ± 0.05	0.2 ± 0.1	0.2 ± 0.06
16:0–22:5	0.4 ± 0.6	0.5 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 2.9	0.0 ± 0.0
18:0–22:5	0.7 ± 1.0	0.4 ± 0.7	0.0 ± 0.0	1.0 ± 1.2	0.3 ± 0.8	0.9 ± 1.5
18:1–22:5	0.5 ± 0.0	0.2 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
n–9						
16:0–18:1	14.1 ± 1.4	15.9 ± 3.0	7.8 ± 0.2	9.3 ± 0.6	6.1 ± 3.0	9.6 ± 0.7[a]
18:0–18:1	5.5 ± 1.1	5.4 ± 1.5	5.4 ± 2.5	5.0 ± 1.3	3.9 ± 1.0	5.0 ± 1.2
18:1–18:1	5.8 ± 1.4	4.2 ± 0.7	2.4 ± 0.7	2.9 ± 0.6	3.5 ± 3.2	3.2 ± 0.4
Total 18:1	25.5 ± 1.1	25.4 ± 1.9	15.6 ± 2.7	17.2 ± 1.9	13.5 ± 1.6	17.9 ± 0.8[d]
16:0–20:1	0.2 ± 0.04	0.5 ± 0.1	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.08	0.0 ± 0.0
18:0–20:1	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 1.8	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.6
18:1–20:1	1.3 ± 0.9	1.2 ± 0.8	1.0 ± 1.2	1.0 ± 0.6	0.3 ± 0.5	0.7 ± 1.4
17:0–18:1	1.1 ± 1.2	1.2 ± 0.8	0.8 ± 0.7	1.0 ± 0.9	1.4 ± 1.7	0.2 ± 0.3
Saturated						
16:0–16:0	1.6 ± 0.9	1.1 ± 1.3	1.2 ± 0.6	1.3 ± 0.7	0.7 ± 0.9	0.9 ± 0.7
16:0–18:0	1.4 ± 2.8	0.9 ± 1.4	0.2 ± 0.6	0.3 ± 0.5	0.7 ± 0.5	0.6 ± 0.9
Total saturated	3.0 ± 3.6	2.1 ± 2.7	1.4 ± 1.0	1.6 ± 0.9	1.4 ± 1.2	1.5 ± 0.9
Sat. + mono. / Polyunsaturated	0.46 ± 0.13	0.44 ± 0.09	0.27 ± 0.11	0.27 ± 0.03	0.21 ± 0.05	0.27 ± 0.04

Compared to normal: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.005$, [d]$P < 0.001$.

in fatty acid composition between sickle cells and sickle trait erythrocytes occurred in both the light and dense fractions as well. For example, the linoleic acid was 9.5 ± 1.1, 9.2 ± 1.2, and 9.1 ± 1.1% in the sickle erythrocytes before fractionation and the same compositions were in the light and dense fractions after fractionation. In comparison, the linoleic acid content was 11.1 ± 0.5, 11.6 ± 0.4, and 12.0 ± 1.3% in the whole and respective fractions of sickle trait erythrocytes.

The differences in fatty acid composition between sickle and normal erythrocytes were even more accentuated in the five individual phospholipids, especially in phosphatidyl choline and ethanolamine (**Table 5**). In phosphatidyl choline, a principal outer membrane leaflet constituent of the erythrocyte, the ratio of saturated plus monounsaturated fatty acids to polyunsaturated fatty acids was 2.58 for sickle erythrocytes and 1.72 for normal erythrocytes ($P < 0.000$). Sickle erythrocytes had reduced total polyunsaturated fatty acids (−23%, $P < 0.000$) as well as lower n–6 fatty acids (−23%, $P < 0.001$) and n–3 fatty acids (−30%, $P < 0.019$) than normal. Both the saturated fatty acids and monounsat-

TABLE 4. Fatty acid composition of the phospholipids of erythrocytes of normal subjects, sickle cell patients, and sickle cell trait subjects

Fatty Acids	Normal (n = 14)	Sickle Cell (n = 8)	Sickle Trait (n = 8)
16:0	19.0 ± 1.3	21.0 ± 2.3[a]	19.0 ± 1.4
18:0	15.1 ± 1.5	15.2 ± 1.9	14.4 ± 0.6
Total saturated	36.5 ± 1.9	39.9 ± 3.3[b]	35.7 ± 1.4
18:1	15.2 ± 2.3	18.9 ± 1.6[c]	13.8 ± 1.1
Total monounsaturated	16.2 ± 2.3	20.1 ± 1.4[c]	15.7 ± 1.8
18:2 n–6	11.5 ± 1.0	8.5 ± 1.6[c]	12.3 ± 0.9
20:3 n–6	1.6 ± 0.2	1.5 ± 0.1	1.6 ± 0.2
20:4 n–6	17.0 ± 1.4	16.2 ± 1.1	16.2 ± 1.6
22:4 n–6	4.0 ± 0.5	5.1 ± 0.5[c]	4.3 ± 0.8
22:5 n–6	1.0 ± 0.3	1.3 ± 0.1[a]	1.3 ± 0.4
Total n–6	35.6 ± 1.3	33.0 ± 2.9[b]	36.3 ± 2.3
18:3 n–3	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.05
20:5 n–3	0.5 ± 0.2	0.7 ± 1.0	0.6 ± 0.2
22:5 n–3	2.2 ± 0.4	1.9 ± 0.4	2.3 ± 0.2
22:6 n–3	3.7 ± 1.0	2.7 ± 0.5[a]	4.8 ± 1.4[a]
Total n–3	6.6 ± 1.3	5.4 ± 0.9[a]	7.9 ± 1.6[a]
Polyunsaturated	42.4 ± 1.1	39.0 ± 3.9[b]	44.7 ± 3.0
n–6/n–3	5.5 ± 1.1	6.3 ± 0.8	4.7 ± 0.9
Sat. + mono. / Polyunsaturated	1.22 ± 0.09	1.63 ± 0.3[b]	1.16 ± 0.08

Results given as percent of total fatty acids, mean ± SD.
Compared to normal: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$.

urated fatty acids were higher in sickle cells, 9% ($P < 0.028$) and 27% ($P < 0.011$) higher than normal. Individually, linoleic acid (18:2n–6) and DHA (22:6n–3) were 33% ($P < 0.000$) and 39% ($P < 0.019$) lower than normal while palmitic acid (16:0) and oleic acid (18:1 n–9) were increased 19% ($P < 0.003$) and 27% ($P < 0.013$) higher than normal (**Fig. 2**).

In phosphatidylethanolamine, a chief inner leaflet constituent of erythrocytes, sickle cells especially contained less n–3 polyunsaturated fatty acids (−19%, $P < 0.002$), mainly from a lower content of docosahexaenoic acid 22:6n–3 (−22%, $P < 0.009$). In phosphatidyl serine (Table 5), another inner leaflet constituent, sickle erythrocytes had more long chain n–6 fatty acids: 22:4–6 (+53%, $P < 0.005$); 22:5n–6 (+63%, $P < 0.005$). There were no changes in the 18:2n–6 and 22:6n–3 fatty acids.

The plasma fatty acid composition of sickle cell patients was also abnormal (**Table 6**). Sickle cell patients had lower polyunsaturated fatty acids and higher monounsaturated and saturated fatty acids. The linoleic acid level in sickle cell plasma was 15% lower than normal plasma ($P < 0.011$). Both palmitic acid and oleic acid were higher in the plasma of sickle cell patients. Palmitic acid was 11% ($P < 0.050$) and oleic

TABLE 5. Fatty acid composition of the major phospholipids in normal and sickle erythrocytes

	Phosphatidyl Choline		Phosphatidyl Ethanolamine		Phosphatidyl Serine		Phosphatidyl Inositol		Sphingomyelin	
Fatty Acids	Normal (n = 9)	Sickle (n = 8)	Normal (n = 9)	Sickle (n = 8)	Normal (n = 9)	Sickle (n = 8)	Normal (n = 9)	Sickle (n = 8)	Normal (n = 9)	Sickle (n = 8)
16:0	32.1 ± 4.6	38.2 ± 1.9[c]	14.7 ± 2.6	12.8 ± 4.9	2.4 ± 2.6	2.1 ± 0.9	8.5 ± 2.9	8.4 ± 1.0	34.6 ± 12.0	36.0 ± 7.4
18:0	9.8 ± 1.6	7.2 ± 1.3[c]	9.9 ± 2.1	10.2 ± 2.6	33.1 ± 4.6	35.3 ± 7.4	26.7 ± 7.2	27.4 ± 5.9	6.3 ± 1.2	5.4 ± 1.4
Saturated	42.8 ± 3.9	46.6 ± 2.2[a]	26.8 ± 2.3	26.8 ± 4.2	36.3 ± 4.8	38.2 ± 6.7	37.3 ± 8.2	37.7 ± 3.9	69.6 ± 3.9	67.3 ± 5.0
18:1	17.7 ± 2.9	22.5 ± 4.1[a]	18.6 ± 2.8	16.7 ± 2.6	9.6 ± 5.7	9.0 ± 2.7	11.7 ± 3.1	13.5 ± 2.0	1.3 ± 0.3	1.4 ± 0.4
Mono-unsaturated	18.6 ± 2.8	23.6 ± 4.2[a]	19.3 ± 2.7	17.7 ± 2.5	10.2 ± 5.9	9.6 ± 2.8	13.3 ± 2.9	14.7 ± 1.5	19.3 ± 5.5	22.8 ± 4.0
18:2 n–6	24.4 ± 2.6	16.6 ± 3.6[a]	6.1 ± 0.8	4.5 ± 1.1[c]	1.9 ± 1.2	2.1 ± 1.2	6.4 ± 2.2	5.7 ± 1.6	0.4 ± 0.2	0.5 ± 0.6
20:3 n–6	1.9 ± 0.6	1.2 ± 0.2[b]	1.2 ± 0.1	1.1 ± 0.1	0.8 ± 0.4	2.5 ± 0.6	2.5 ± 0.7	2.6 ± 0.5	0.2 ± 0.2	0.2 ± 0.1
20:4 n–6	6.3 ± 1.5	7.1 ± 1.8	25.1 ± 2.2	25.5 ± 2.7	28.8 ± 6.4	26.2 ± 5.0	26.4 ± 7.7	26.6 ± 2.0	0.5 ± 0.2	0.6 ± 0.5
22:4 n–6	0.2 ± 0.1	0.6 ± 0.2[d]	6.8 ± 1.1	8.4 ± 2.3	4.0 ± 1.0	6.1 ± 1.3[c]	2.0 ± 0.8	3.0 ± 0.9	0.7 ± 0.4	0.8 ± 0.5
22:5 n–6	0.3 ± 0.3	0.3 ± 0.1	0.2 ± 0.4	1.5 ± 0.4	1.9 ± 0.5	3.1 ± 0.9[c]	1.2 ± 0.7	1.7 ± 1.6	0.4 ± 0.4	0.4 ± 0.3
Total n–6	33.7 ± 2.9	26.4 ± 3.3[d]	40.7 ± 3.3	41.2 ± 2.5	38.7 ± 6.1	40.2 ± 6.2	39.2 ± 8.8	39.9 ± 3.0	1.7 ± 0.9	2.1 ± 1.1
18:3 n–3	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.0	0.1 ± 0.1	0.2 ± 0.2	0.3 ± 0.3	0.1 ± 0.0	0.2 ± 0.1
20:5 n–3	0.5 ± 0.2	0.2 ± 0.0	0.8 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.2
22:5 n–3	0.4 ± 0.1	0.4 ± 0.1	3.3 ± 0.8	3.0 ± 0.7	3.2 ± 1.0	3.2 ± 1.0	1.8 ± 0.5	1.5 ± 0.3	3.2 ± 1.2	3.1 ± 0.7
22:6 n–3	1.3 ± 0.5	0.8 ± 0.2[a]	4.9 ± 0.8	3.8 ± 0.3[c]	7.3 ± 3.6	6.0 ± 1.2	3.4 ± 1.6	2.7 ± 0.7	0.1 ± 0.1	0.2 ± 0.1
Total n–3	2.3 ± 0.7	1.6 ± 0.3[a]	9.3 ± 1.5	7.5 ± 0.8[b]	10.8 ± 4.6	9.5 ± 2.0	5.8 ± 2.3	4.6 ± 0.5	4.4 ± 1.2	4.0 ± 0.9
Polyun-saturated	36.1 ± 3.3	27.8 ± 3.4[d]	50.2 ± 2.8	49.9 ± 3.5	49.6 ± 8.0	49.7 ± 7.7	45.1 ± 9.0	44.6 ± 3.1	6.1 ± 1.0	6.0 ± 1.8
n–6/n–3	16.8 ± 4.4	17.2 ± 4.2	4.5 ± 0.9	5.5 ± 0.4	3.9 ± 1.1	4.3 ± 0.7	7.8 ± 3.5	8.7 ± 1.2	0.5 ± 0.5	0.5 ± 0.3
Sat. + mono. / Polyunsaturated	1.7 ± 0.2	2.6 ± 0.5[d]	0.9 ± 0.1	0.9 ± 0.2	1.0 ± 0.4	1.0 ± 0.3	1.2 ± 0.7	1.2 ± 0.2	15.0 ± 2.8	16.3 ± 5.3

Results given as percent of total fatty acids, mean ± SD.
Compared to normal: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.005$, [d]$P < 0.001$.

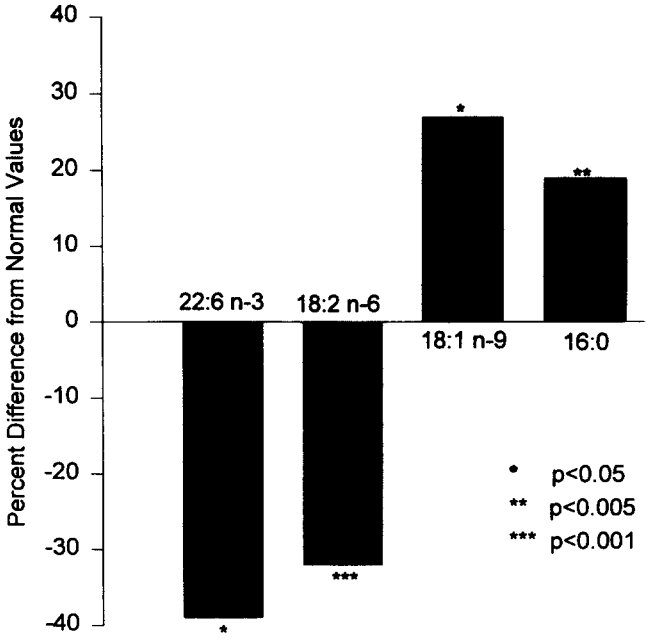

Fig. 2. Major abnormal fatty acids in phosphatidyl choline of sickle cell membranes.

TABLE 6. Plasma fatty acid composition of normal subjects and sickle cell patients

Fatty Acids	Normal (n = 13)	Sickle Cell (n = 7)
16:0	19.9 ± 2.1	22.0 ± 2.2[a]
18:0	6.8 ± 0.8	7.0 ± 1.2
Total saturated	26.0 ± 7.9	30.6 ± 2.1
18:1	19.5 ± 2.9	25.7 ± 4.9[c]
Total monounsaturated	10.4 ± 6.8	28.3 ± 4.8[a]
18:2 n–6	32.1 ± 2.8	27.3 ± 4.9[a]
20:3 n–6	1.5 ± 0.2	0.8 ± 1.5
20:4 n–6	8.3 ± 2.7	7.3 ± 1.5
22:4 n–6	0.3 ± 0.1	0.3 ± 0.2
22:5 n–6	0.3 ± 0.1	0.3 ± 0.1
Total n–6	43.3 ± 3.7	36.5 ± 6.0[c]
18:3 n–3	0.6 ± 0.2	0.5 ± 0.2
20:5 n–3	0.5 ± 0.2	0.2 ± 0.1[c]
22:5 n–3	0.5 ± 0.1	0.5 ± 0.1
22:6 n–3	1.6 ± 0.6	1.1 ± 0.3
Total n–3	3.1 ± 1.1	2.3 ± 0.4
Total polyunsaturated	46.9 ± 4.1	39.2 ± 6.0[c]
n–6/n–3	13.3 ± 2.1	16.1 ± 3.6[b]
Sat. + mono. / Polyunsaturated	1.1 ± 0.2	1.6 ± 0.4[c]

Results given as percent of total fatty acid, mean ± SD.
Compared to normal: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.005$.

acid was 32% ($P < 0.002$) higher than normal. Consequently, the ratio of saturated plus monounsaturated fatty acids to polyunsaturated fatty acids was 1.6 for sickle cell patients and 1.1 for normal subjects ($P < 0.001$). Unlike erythrocytes, the plasma DHA levels of sickle cell patients were not significantly lower than that of normal plasma. However, the n–6/n–3 ratios were significantly higher in the plasma of sickle cell patients (16.1 vs. 13.3 in normal plasma, $P < 0.010$).

The mean reticulocyte count for the sickle cell patients was invariably elevated at 11.1 ± 5.5%.

DISCUSSION

Our data clearly demonstrated a second molecular abnormality in sickle erythrocytes, in addition to the abnormal protein hemoglobin S. The second molecular abnormality lies in the disturbed structure of the phospholipids of sickle erythrocytes. An abnormal molecular species composition was found in both choline glycerophospholipids (the major lipid component in the outer membrane leaflet) and ethanolamine glycerophospholipids (the major lipid component in the inner membrane leaflet). We examined the membrane phospholipids of erythrocytes in three different ways: the composition of the phospholipid molecular species, the fatty acid composition of the individual phospholipids, and the fatty acids of the total phospholipids. Each analysis provided information about different aspects of the abnormal biochemistry. In the total phospholipids, the lower amount of polyunsaturated fatty acids in sickle erythrocytes could mainly be ascribed to the lower quantities of linoleic (18:2n–6) and docosahexaenoic acid (22:6n–3). Muskiet et al. (29, 30) found that the total linoleic acid was reduced in sickle cells but docosahexaenoic acid, the most highly polyunsaturated fatty acid, was not found to be abnormal. No analyses of individual phospholipids or molecular species were reported. Currently, improved methodology now makes these analyses possible as in the present paper. Analysis of the fatty acid composition of individual phospholipids demonstrated that the reduced level of linoleic acid was confined mainly to phosphatidyl choline (present in the outer leaflet of the erythrocyte), whereas the reduced level of docosahexaenoic acid in the sickle cells was mainly present in phosphatidyl ethanolamine, a constituent of the inner leaflet.

The data about the phospholipid molecular species indicate that the lower 18:2n–6 in phosphatidyl choline of sickle erythrocytes occurred mainly from the lower amount of the molecular species 16:0–18:2 in the diacyl choline glycerophospholipids. This lower 16:0–18:2 species was also seen in the alkenylacyl and alkylacyl subclasses. The decreased 16:0–18:2 species was compensated for by an increase of a species with a monounsaturated fatty acid at the *sn*-2 position, 16:0–18:1 spe-

cies and a species with saturated fatty acid at the *sn*-2 position, 16:0–16:0. The species 16:0–22:6 was greatly decreased in the alkylacyl subclass (−57%).

In the ethanolamine glycerophospholipids, there was a trend to lower docosahexaenoic acid 22:6 species in all three subclasses of phospholipids in the sickle erythrocyte. Significantly lower 18:0–22:6 was seen in the diacyl subclass and lower 16:0–22:6 in the alkylacyl subclass. Interestingly, there was a higher total 20:4 species in the diacyl subclass and a lower 16:0–20:4 in the alkylacyl subclass in sickle cells. Therefore, the consistent results from three different techniques of analysis strengthen the conclusion that the membrane phospholipids of sickle erythrocytes are abnormal, having lower levels of polyunsaturated fatty acids and higher levels of saturated and monounsaturated fatty acids. The analysis of phospholipid molecular species pinpoints the defect in specific molecular species that cannot be detected by fatty acid analysis alone.

The question may be asked whether the observed abnormal membrane lipid composition of sickle cells has any relevance to the pathophysiology of the disease. The existing information points to several possibilities. In 1987, using PC specific transfer proteins in an in vitro study, Kuypers et al. (12) changed the phospholipid molecular species composition of sickle cells without affecting its protein and other components. They found that there was an improvement in cellular dehydration and deformability. Therefore, they concluded that abnormalities in phospholipid molecular species composition might be present in sickle erythrocytes and that this could be corrected by facilitating exchange of molecular species of phosphatidyl choline. Studying erythrocytes from normal and hyperlipidemic subjects with the same technique and with detailed membrane lipid analysis, scientists from Netherlands and Germany demonstrated the association between membrane lipid composition and ion transport (31–34). Analyzing phospholipid molecular species of erythrocytes from 7 normal and 16 hyperlipidemic subjects, Englemann et al. (33) found that there was a negative correlation between the concentration of the 16:0–18:2 PC species in diacyl PC and the rate of Na/Li counter transport. To confirm this relationship established by correlation association, these authors selectively introduced 16:0–18:2 PC into the erythrocyte membrane by means of a PC specific transfer protein. As a result, the modified erythrocyte had a 24% and a 14% increase, respectively, in the 18:2 and 16:0 concentration in its phosphatidyl choline and a 25% decrease in Na/Li counter transfer (31–33). In another experiment, when 20% native PC of the erythrocytes from healthy volunteers was replaced by 16:0–16:0 PC with PC specific transfer protein, there was 34% increase in the concentration of 16:0 in PC and 40% increase in Na/Li counter transport and 25% decrease in Na/K pump in the modified erythrocytes. Introducing 16:0–18:1 PC to normal erythrocytes also caused decrease in the Na/K pump rate (31).

In summary, these data demonstrated that an increase in molecular species of 16:0–18:1 PC and 16:0–16:0 PC or a decrease in 16:0–18:2 PC in erythrocytes will decrease the Na/K pump rate and increase Na/Li counter transport. The magnitude required to have this effect was a 14–34% change of these fatty acids in phosphatidyl choline.

Moreover, in the present study we found that the concentrations of molecular species of 16:0–16:0 PC and 16:0 and 18:1 PC in sickle cells were 36% and 17% higher than normal (Fig. 1) and 16:0–18:2 PC was 19% lower than normal. Similarly, the concentrations of 16:0 and 18:1 in PC of sickle erythrocytes were 19% and 27% higher than normal and 18:2 was 32% lower than normal (Fig. 2). Based on the in vitro data mentioned above, these deviations of molecular species and fatty acid composition in sickle cells from normal erythrocytes might be expected to increase their Na/Li counter transport and decrease their Na/K pump rate. This deduction seems to fit the known problem of cation imbalance (increase in Na^+ and Ca^{2+}, decrease in K^+) of sickle cell disease. Comparison of our data with those in vitro data may, therefore, indicate that abnormal membrane lipid composition in sickle cells may have a role in their abnormal cation transport.

A defect in cation transport may have many consequences. Cation transport was found to have significant effects on cell dehydration (35–38). Dehydration in sickle cells was suggested to increase the chance of polymerization of Hb SS and in turn, more pain crises (39). Cation transport also plays a role in the scrambling of lipids over the membrane bilayer. Intracellular influx of Ca^{2+} represents the key event of cellular activation and initiates a variety of cellular responses. Migration of amniophospholipids to the outer leaflet of the membrane is one of the consequences (40–45).

The exposure of phosphatidyl serine in the outer leaflet membrane of the red blood cells was found to be responsible for the hypercoagulable state in sickle cells (46–48). Membrane lipid composition was also found to have significant effect on cell deformability (49–52). Cation imbalance, cell dehydration, reduced deformability, disrupted membrane phospholipid asymmetry, and hypercoagulability are the known metabolic abnormalities of sickle cell disease (6, 53). From the evidence described above, it seems reasonable to suggest that abnormal membrane lipid composition in sickle cells may have a role in one or more of these events directly or indirectly.

While the causes for these lipid abnormalities are unknown at this time, there are several possibilities. First, as mature red cells cannot synthesize fatty acids, the fatty acids of red blood cells can be influenced by plasma fatty acid composition. We analyzed the plasma fatty acid composition of both sickle cell patients and normal controls (Table 6). The plasma of sickle cell patients contained lower polyunsaturated fatty acid and higher monounsaturated and saturated fatty acids, similar to sickle cells. Most of the differences in polyunsaturated fatty acids was from a low linoleic acid concentration. Increased palmitic and oleic acids were the main contributors to the increased monounsaturated and saturated fatty acids, respectively. In view of the similar differences between normal subjects and sickle cell patients in erythrocyte and plasma fatty acid composition, one of the possible causes of abnormal fatty acid in sickle erythrocytes, therefore, could be the abnormal plasma fatty acid composition of these patients. Abnormal plasma fatty acid composition may be derived from different dietary fatty acid intake or altered lipid metabolism. For the former possibility, we doubt that the low content of linoleic acid in the phospholipid molecular species is the result of dietary deficiency of this n–6 fatty acid. In n–6 essential fatty acid deficiency, the n–6 arachidonic acid (20:4) is also reduced in the plasma and this was not the case. The monounsaturated fatty acid eicosatrianoic acid (20:3n–9) that commonly occurs in essential acid deficiency was not elevated at all. Indeed, some highly polyunsaturated n–6 fatty acids were actually elevated in the erythrocytes instead of being depressed, as might be the case if their precursor synthetic matrix, linoleic acid, was deficient in the diet. Furthermore, the dietary histories of our patients with sickle cell disease indicated a pattern of food consumption similar to the usual American pattern with no essential fatty acid deficiencies. The intake of docosahexaenoic acid was also not abnormal.

We also studied the fatty acid composition of the erythrocytes of the parents and siblings from four families who had one child each with sickle cell disease. A low linoleic and docosahexaenoic acid content was found only in the erythrocytes of the sickle cell patients. The family members consuming the same diet had normal linoleic and docosahexaenoic acid concentrations in their erythrocyte membranes. These data, therefore, indicated that the low polyunsaturated fatty acids observed in plasma and erythrocytes of sickle cell patients were not from a dietary deficiency of these fatty acids. However, the abnormal plasma fatty acid composition in sickle cell patients could occur from altered lipid metabolism. The increased erythropoiesis, increased cardiac output, and alterations of hepatic function seen in sickle cell anemia could change lipid metabolism.

The increased peroxidation of polyunsaturated fatty acids known to occur in sickle cell disease could be another possible cause of the deficiency of linoleic and docosahexaenoic acids (54–56). However, this seems unlikely because, while the level of linoleic acid with two double bonds was depressed, other polyunsaturated fatty acids with four and five double bonds were not depressed, indeed even increased. It has been reported that sickling affected membrane phospholipid organization (10). There were increases in phosphatidyl ethanolamine and serine in the outer leaflet in the deoxygenated sickle cell. Franck et al. (11) found increased transbilayer movement of phosphatidyl choline in deoxygenated (sickled) erythrocytes. Chiu, Lubin, and Shohet (9) observed that fatty acid incorporation into membrane phospholipids was decreased in the sickled state. Mohandas et al. (57) reported that the structural organization of membrane skeletal proteins plays an important role in regulating the rate of transbilayer movement of lipids across the erythrocyte membrane. The possibility that the process of sickling itself could change the configuration of the membrane and consequently the composition of phospholipid molecular species remains to be studied.

Red blood cells in patients with sickle cell anemia are heterogenous including reticulocytes and reversibly and irreversibly sickled cells. As our patients had high reticulocyte counts, the abnormal fatty acid composition of sickle cells could have resulted from very young cells which may have had a different fatty acid composition than older cells. As young and aged cells have different densities (58), we fractionated the erythrocytes from six sickle cell patients and two subjects with sickle trait and analyzed the fatty acid composition of the light (young cells) and dense (old cells) fractions. We found that the fatty acid compositions of these two fractions were similar. Fatty acid differences between sickle cell and erythrocytes from sickle trait existed in light and dense fraction as well. Therefore, the high number of reticulocytes in sickle cell anemia is likely not the cause of the abnormal fatty acid composition observed.

As sickle cell patients have rapid hemolysis, we analyzed the fatty acid composition of the erythrocytes from patients with other hemolytic diseases. In one patient with the hemolytic disease glucose-6-phosphate dehydrogenase deficiency and one patient with peroxysomal maternal hemoglobinuria, the erythrocytes had a completely normal fatty acid composition. However, in two patients with unstable hemoglobins, the fatty acid composition of their erythrocytes was similar to that of sickle cells. The unstable hemoglobins in some patients are thought to interact abnormally with the red cell cytoskeleton. Thus this may be another example of hemoglobin defects altering cytoskeletal organi-

zation associated with changes in membrane lipid composition.

The possible causes for the abnormal lipid molecular species in sickle cell disease certainly include the genetic makeup of the erythrocyte. This abnormal lipid composition may well result from the Hb SS protein which alters the lipid configuration per se. The membrane formed in erythrocytes with the abnormal hemoglobin S may require a different pattern of phospholipid molecular species. For the sickle cell variants such as Hb SC, Hb SF, or Hb SβoTh, the molecular species abnormalities were less pronounced than in sickle cell anemia, HB SS. There was only a lowering of linoleic acid (9.0–9.6 vs. 8.5 and 11.5% in Hb SS and normal, respectively) and no abnormality of docosahexaenoic acid (3.6–4.4 vs. 2.7 and 3.7% in Hb SS and normal, respectively). In individuals with the sickle cell trait, there were no fatty acid abnormalities even though some 30–40% of the hemoglobin was sickle hemoglobin. The lipid abnormality seems related to the severity of the disease.

In the early 1990s, Muskiet et al. (30) studied the effects of zinc, α-tocopheral, vitamin C, soybean oil, and fish oil on sickle cell disease. When soybean oil rich in linoleic acid was fed to sickle cell patients, no change of fatty acid composition of erythrocytes was found. The dose administered may have been too small to influence the very large body pool of linoleic acid. There was no parallel control group studied. When a fish oil supplement was also given to these patients, increased n–3 fatty acids in the erythrocytes were observed. (Multiple parameters were measured, yet no clear conclusion was made.) Interestingly, the number of irreversible sickle cells was reduced by both supplementations. This was attributed to zinc which may have influenced heme synthesis. The authors commented that longer studies of such supplementation in which frequency, intensity, and duration of pain and parameters of platelet activation are monitored, may be required.

In the present study, we have established the phospholipid molecular species composition of normal erythrocytes. This information is scanty in the literature. In 1989, using elaborate analytical procedures, Myher, Kuksis, and Pind (59) reported the phospholipid molecular species composition of the erythrocytes of one normal male. In their study of the aminophospholipid molecular species asymmetry in human erythrocytes, Hullin, Bossant, and Salem (60) analyzed the phospholipid molecular species of normal erythrocytes. However, their data were limited to aminophospholipids, mainly ethanolamine glycerophospholipids. Furthermore, the complete data of three subclasses (diacyl, alkenylacyl, and alkylacyl) were not reported. In this study, we have analyzed the phospholipid molecular species composition of all three subclasses in both choline and ethanolamine glycerophospholipids of the erythrocytes from six normal subjects. Interestingly, despite the different analytical procedures used, the results were similar.

In conclusion, our data have demonstrated distinct abnormalities of the phospholipid molecular species of sickle erythrocytes. The deficiencies of molecular species containing the polyunsaturated linoleic and docosahexaenoic acids and their replacement by species containing saturated and monounsaturated fatty acids may have a direct bearing upon the dehydration and abnormal sodium and potassium metabolism known to occur in sickle erythrocytes. In combination with the protein abnormality of hemoglobin, these molecular changes in membrane lipids may accentuate the sickling phenomena and perhaps, in part, the crises of these patients. These fatty acid abnormalities in sickle cell anemia offer further understanding of the pathophysiology, the possibility of correction by exogenous fatty acids, and perhaps also another diagnostic criteria for the disease.

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