Identification of free ceramide in human erythrocyte membrane

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Abstract Free ceramide was characterized in human erythrocytes and ghosts. Its concentration was found to be 5.6 μmol/100 ml of packed cells. It was isolated by thin-layer chromatography of its acetylated form and purified by thin-layer chromatography after deacetylation. It was constituted mainly of C16, C22, C24:0, and C24:1 nonhydroxy fatty acids and of C18:1 sphingosine. A small amount of 2-hydroxy fatty acids was also detected, containing mainly C24:0 hydroxy fatty acid. The structures of the ceramides and identification of the minor bases were confirmed by electron-impact and chemical ionization mass spectra of the trimethylsilylated ceramides.—**Bouhours, J-F., and D. Bouhours.** Identification of free ceramide in human erythrocyte membrane. *J. Lipid Res.* 1984. **25:** 613–619.

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Ceramide, the lipid moiety of sphingolipids, is the first substrate of the multiglycosyltransferase systems responsible for the synthesis of glycosphingolipids as well as the end product of their degradation by lysosomal glycosidases (1, 2).

Ceramide is also found in free form as a structural component of the cell membrane. It occurs in brain (2), kidney (3), liver (4), intestine (5), and skin (6). It has also been identified and quantified in human blood components such as plasma (7) and platelets (8). We report here on its occurrence in human red blood cells.

MATERIALS AND METHODS

Preparation of erythrocytes

Whole blood was drawn from normal humans on acid-citrate-dextrose anticoagulant (5 to 20 ml per subject, samples 1, 2, and 3). Blood was centrifuged at 1000 *g* for 10 min. The supernatant plasma and the buffy coat were removed. Erythrocytes were washed twice in saline.

Erythrocytes and ghosts were also prepared from outdated blood (1 month old) obtained at the local blood bank (samples 4 to 7). In this case, erythrocytes and ghosts were prepared according to the method of Dodge, Mitchell, and Hanahan (9).

Lipid extraction and purification

Washed erythrocytes or ghost pellets were mixed with 10 times their volume of cold methanol (−20°C). After warming of the mixture at room temperature under gentle stirring, 10 volumes of chloroform were added and the extraction was allowed to proceed overnight at room temperature. The mixture was then filtered and the residue was reextracted in 10 volumes of chloroform–methanol 2:1 (v/v) for 2 hr. The suspension was filtered and the lipid extract was concentrated to dryness in a rotary evaporator. After repeated drying with addition of 2-propanol, lipids were left in a desiccator under vacuum for 2 hr.

The dry lipids were acetylated with pyridine–acetic anhydride 3:2 (v/v) for 16 hr and chromatographed on a Florisil column according to the method of Saito and Hakomori (10). Acetylated glycosphingolipids and free ceramide were recovered in the dichloroethane–acetone 1:1 (v/v) fractions. They were analyzed by thin-layer chromatography (TLC) as their acetylated forms or after deacetylation.

Thin-layer chromatography

TLC was performed on analytical precoated silica gel 60 high-performance thin-layer plates (E. Merck, Darmstadt, West Germany) developed to a height of 10 cm. Native glycolipids were analyzed in solvent A: chloroform–methanol–water 60:35:8 (v/v). Native free ceramides were analyzed in solvent B: chloroform–methanol 90:10 (v/v). Acetylated ceramides were analyzed in solvent C: hexane–diethyl ether 3:7 (v/v). For preparative studies, lipids were detected under ultraviolet light after spraying the plates with a 0.01% solution of primuline (I.C.N. Pharmaceuticals, Inc., Plainview, NY) in acetone–water 90:10 (v/v). Otherwise, lipids were visualized by

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; EI, electron-impact mass spectrometry; CI, chemical ionization mass spectrometry.

charring at 200°C after spraying the plates with a 20% solution of ammonium sulfate in 4% sulfuric acid.

Gas–liquid chromatography

Free ceramide was hydrolyzed in methanol–concentrated hydrochloric acid–water 83:8.6:9.4 (v/v) at 80°C for 18 hr (11). Fatty acids and fatty acid methyl esters were extracted with hexane. The esterification of the fatty acids was completed by heating at 80°C for 2 hr in 0.8 N anhydrous methanolic hydrochloric acid. Fatty acid methyl esters were separated into hydroxy and nonhydroxy methyl esters by chromatography on a Florisil column (12). They were analyzed by gas–liquid chromatography (GLC) on a SE-30 wall-coated capillary column, 12 m long. The oven temperature was programed from 180° to 270°C at 5°/min. Hydroxy fatty acid methyl esters were trimethylsilylated before analysis.

Sphingoid bases were extracted from the methanolic phase remaining after hydrolysis of the sample and removal of the fatty acids. They were submitted to periodate oxidation as described by Sweeley and Moscatelli (13). Aldehydes were separated by GLC on a glass column (2 mm × 2 m) packed with Gas-Chrom P coated with 10% EGSS-X (Applied Science Laboratories, Inc., State College, PA) according to a published procedure (5).

GLC analyses were performed on a Hewlett-Packard 5710A gas chromatograph equipped with a double flame ionization detector. The detector signal was recorded on a Hewlett-Packard 3390A integrator.

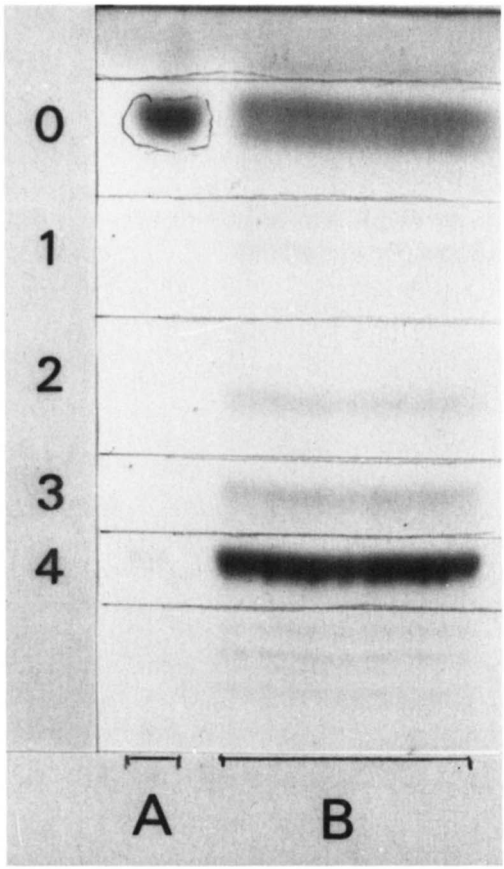

Fig. 1. TLC of red blood cell glycosphingolipids. TLC was performed in solvent A and lipids were visualized by charring. (A), Free ceramide standard; (B), red blood cell glycosphingolipids purified by Florisil column chromatography. Figures on the left margin refer to the number of sugar residues per glycolipid.

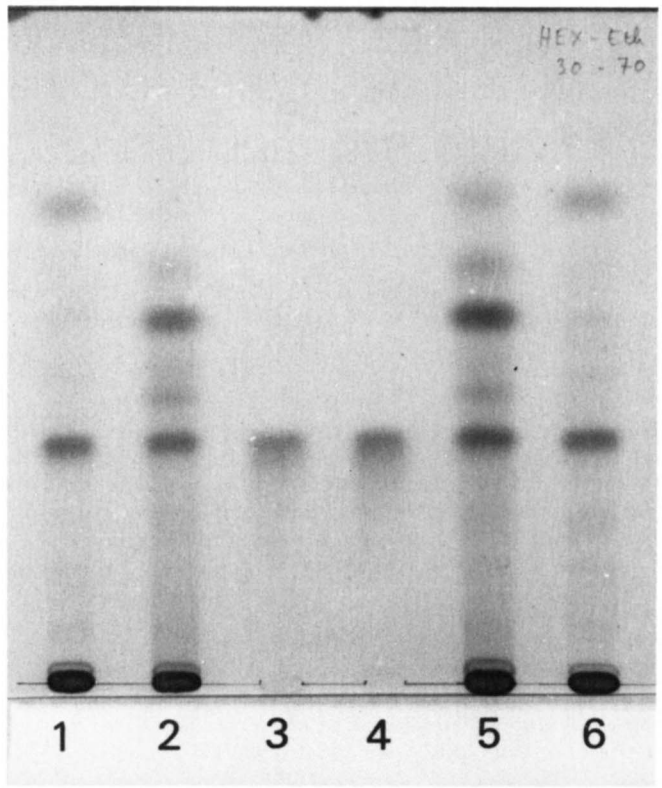

Fig. 2. TLC of acetylated glycolipids and ceramides. The acetylated glycolipids purified by Florisil column chromatography were analyzed by TLC in solvent C. (1) and (2), Ghosts and whole cells of sample 5, respectively; (3), ceramide standard containing nonhydroxy fatty acids; (4), ceramide standard containing 2-hydroxy fatty acids; (5), whole cells from sample 6; (6), whole cells from sample 4.

Mass spectrometry

Purified free ceramides were trimethylsilylated and introduced by the direct inlet probe in a VG Micromass 30F spectrometer. For analyses by the electron-impact technique (EI), the electron energy was 70 eV, the accelerating voltage was 8 KV, and the ionization current was 100 μA. The ion source temperature was set at 200°C and the probe was heated from ambient temperature to 250°C. Positive chemical ionization (CI) was produced by ammonia. For CI analyses, the source temperature

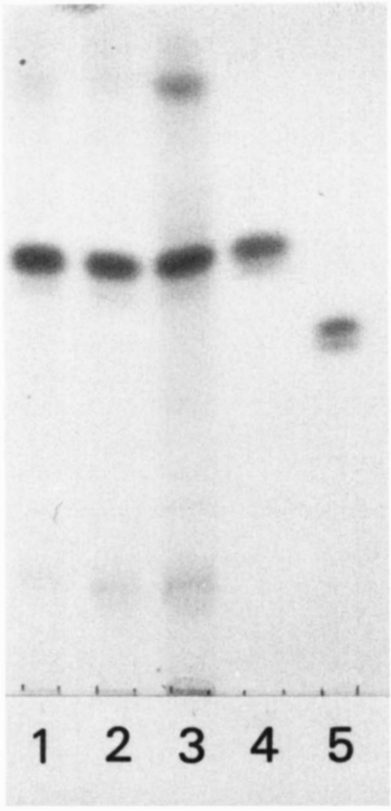

Fig. 3. TLC of deacetylated purified ceramides. Ceramides purified by preparative TLC of their acetylated form (Fig. 2) were deacetylated and analyzed in solvent B. (1), Ghosts of sample 7; (2), ghosts of sample 5; (3), whole cells of sample 6; (4), ceramide standard containing nonhydroxy fatty acids; (5), ceramide standard containing 2-hydroxy fatty acids.

was 170°C and the emission current was 500 μA. Mass spectra were recorded every 3 sec on a data acquisition system.

Colorimetric and fluorimetric assays

The cholesterol content of erythrocytes was assayed by the method of Zlatkis, Zak, and Boyle (14). Phospholipids were determined according to the method of Bartlett (15). The sphingosine content of free ceramide and glycosphingolipids was determined by fluorimetry after reaction with fluorescamine (Fluram, F. Hoffman-La Roche and Co., Basel, Switzerland) (16, 17). Proteins were determined by the method of Lowry et al. (18).

RESULTS

Free ceramide escapes detection when the sphingolipids are quantified either by their phosphorus content as sphingomyelin or by their sugar content as glycolipids. Previous investigations of the glycosphingolipid content of human erythrocytes relied upon sugar quantitation by GLC (19) or on high-performance liquid chromatography of derivatized glycolipids (20, 21). In either case, free ceramide was not detected, although it can be analyzed by high-performance liquid chromatography under appropriate conditions (4). When the glycolipid fraction of human erythrocytes purified by chromatography on a Florisil column was analyzed by TLC, it appeared that a noticeable amount of lipid material migrated at a position similar to that of standard free ceramide (**Fig. 1**). In order to ascertain the nature of that lipid, its base and fatty acid compositions were established and mass spectra of its trimethylsilyl derivatives were recorded.

Purification of free ceramide

Ceramide containing hydroxy fatty acids was not separated from ceramide containing nonhydroxy fatty acids

TABLE 1. Fatty acid composition of free ceramide from human erythrocytes from different blood samples

Fatty Acids	Nonhydroxy Fatty Acids				2-Hydroxy Fatty Acids
	Sample 3	Sample 5	Sample 6	Sample 7	Sample 7
			%		
16:0	12.7	12.1	13.2	12.5	18.0
16:1	1.2	0.3	0.4	0.7	
18:0	9.8	10.5	12.7	11.8	2.0
18:1	8.7	9.5	11.7	10.5	
20:0	1.7	1.7	1.4	2.1	
21:0	0.8	0.3	0.4	1.5	
22:0	12.4	9.6	8.1	9.1	10.5
22:1	0.3	0.7	0.8	0.6	
23:0	1.8	1.7	1.8	1.6	5.7
23:1	1.0	0.3	0.3	0.3	
24:0	19.8	23.5	21.1	20.8	58.7
24:1	23.4	25.5	24.1	24.3	5.1
25:0	1.0	0.6	0.7	0.6	
25:1	1.2	0.3	0.3	0.3	
26:0	2.5	2.0	2.3	2.0	
26:1	1.7	1.4	0.7	1.3	

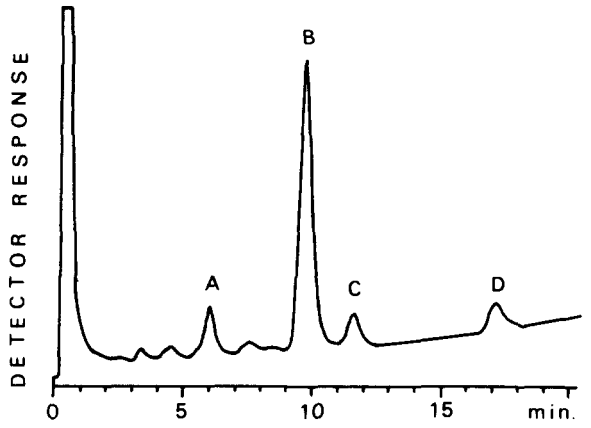

Fig. 4. GLC profile of aldehydes derived from the sphingoid bases of free ceramide. (A), C16 sphingosine; (B) and (D), C18 sphingosine.

when analyzed as their acetylated derivatives by TLC in solvent C (**Fig. 2**). We took advantage of this property to isolate the free ceramide of human erythrocytes by preparative TLC without discriminating between the two possible species of ceramide. The purified acetylated free ceramide was then deacetylated and analyzed by TLC in solvent B (**Fig. 3**). An intense spot migrating as reference ceramide containing nonhydroxy fatty acids was seen. The free ceramide of red blood cells was further purified by preparative TLC in solvent B in order to eliminate residual contaminants present in some samples. In one experiment, a large amount of free ceramide (corresponding to 300 mg of ghost protein of sample 7) was chromatographed in solvent B. Areas corresponding to the positions of nonhydroxy and hydroxy ceramides were scraped separately. After extraction, both were assayed for their sphingosine content. Under these conditions, the presence of ceramide containing hydroxy fatty acids was obvious, although it accounted for 0.9% of the free ceramide. Its constitutive hydroxy fatty acids were characterized (see below).

Fatty acid and sphingoid base composition

Free ceramides isolated from different samples of blood had a similar fatty acid composition with 43–49% of C24 fatty acids (**Table 1**). Nervonic acid was always more abundant than lignoceric acid. The small amount of hydroxy fatty acids was mainly contributed by cerebronic acid.

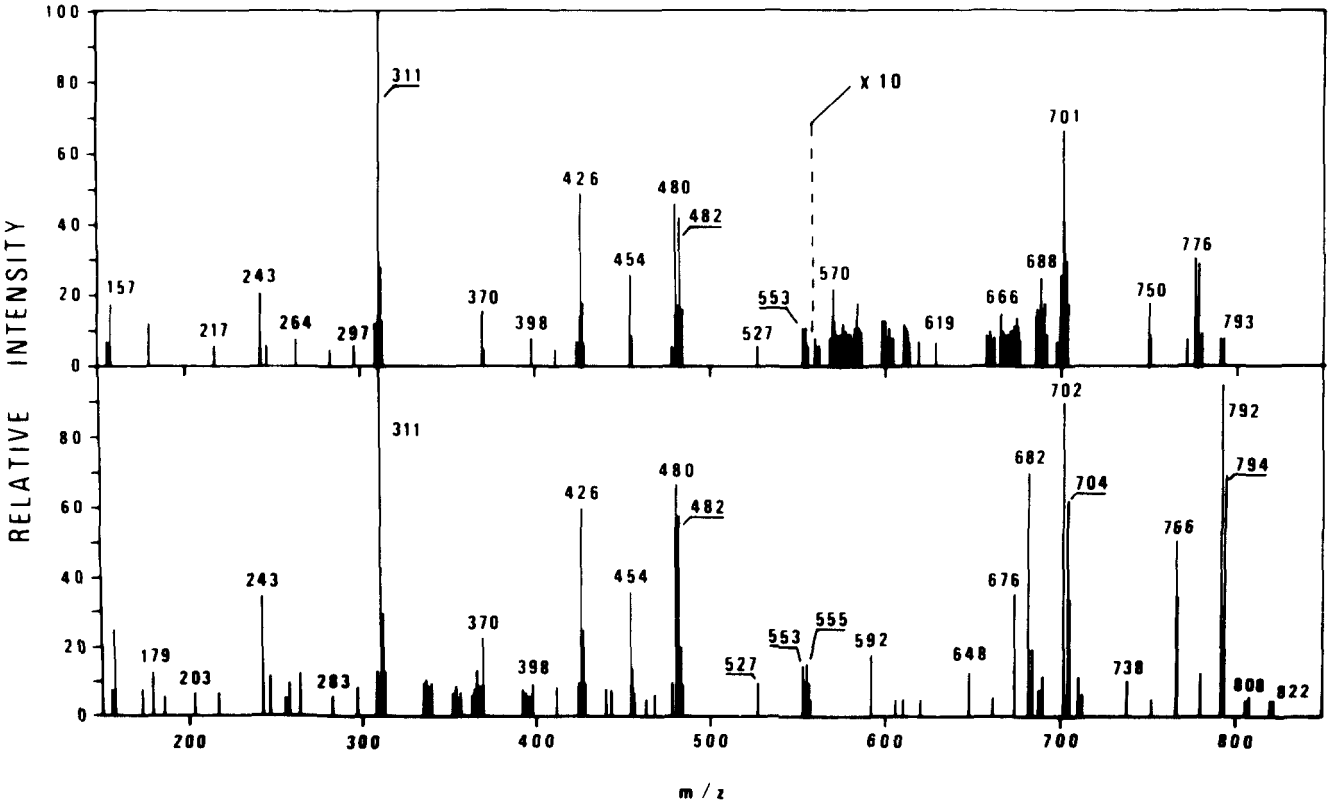

Fig. 5. Mass spectra of the trimethylsilylated ceramides purified from human erythrocytes. The upper box contains the EI spectrum. The lower box contains the CI spectrum of the same sample. The fragmentation is seen in Fig. 6. Spectra were recorded near the maximum intensity of the total ion current. Only peaks of intensity equal to 5% of the scale (or of the expanded scale of EI spectrum) or higher were reproduced.

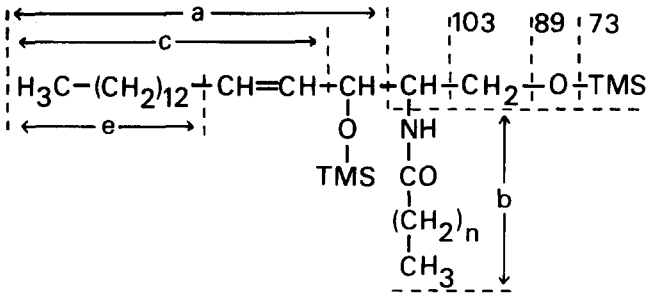

Fig. 6. Fragmentation of trimethylsilylated ceramide according to Samuelsson and Samuelsson (22).

The GLC profiles of aldehydes derived from sphingoid bases were almost identical from one blood sample to another with peaks of even and odd numbers of carbon from tetradecenal to nonadecenal (**Fig. 4**). C18 sphingosine was the most abundant base appearing as a large peak of hexadecenal (peak B) and a small peak of its 3-O-methyl derivative (peak D). It accounted for 75–80% of the bases. A small peak appearing at 11.5 min (peak C) might be interpreted as originating from C19 sphingosine. However, the corresponding fragment a at m/z 325 was not detected on the EI mass spectrum (**Fig. 5**). Peak C was then interpreted as coming from a C18:2 base. This was substantiated by the presence of a fragment a at m/z 309 on the EI mass spectrum (Fig. 5).

Mass spectrometry analyses were performed on free ceramides isolated from four different blood samples. Typical spectra are shown in Fig. 5. The major fragmentation occurred between C2 and C3 of the sphingoid base (**Fig. 6**). It gave rise to ions characteristic of the bases at m/z 311, 297, and 283 (fragment a) for the C18, C17, and C16 sphingosine, respectively. The other part of the molecule, containing the acylamide, gave intense ions (M-a) at m/z 480–482 and weaker ions (M-(a-73)) at m/z 553–555 for 24:1 and 24:0 fatty acids, respectively. The whole molecule was also detected with only peripheral fragmentation and elimination of a methyl group at m/z 776–778 (M-15), of a trimethylsilanol at m/z 701–703 (M-90), and a terminal $CH_2OSi(CH_3)_3$ at m/z 688–690 (M-103). The ceramides constituted of C18 sphingosine and C24 fatty acids also gave molecular ions at m/z 791–793. The ceramide containing C24:0 hydroxy fatty acid was detected by a small peak clearly emerging from the baseline, at m/z 570 corresponding to the fragment M-a (23). The trimethylsilylated molecule was also analyzed with CI. The CI spectrum displayed some fragmentations identical to the EI spectrum: fragment M-b + 1 at m/z 426, a, M-a and M-(a-73). There were no peaks arising from fragments M-15 and those corresponding to M-103 were small. The major interest of this technique was the appearance of very intense peaks corresponding to molecular ions M + 1 and M-90 + 1 for all ceramides whatever their fatty acids (Table 1).

Occurrence of free ceramide in human erythrocytes

Free ceramide occurred in equal concentrations in fresh blood or in outdated blood (**Table 2**). Therefore, it was not a degradation product of other sphingolipids which could have appeared upon aging of blood. Free ceramide was located in the membrane of erythrocytes (Fig. 2,

TABLE 2. Free ceramide content of human erythrocytes and ghosts

		Cholesterol	Phospholipids	Sphingolipids	Ceramide	Ceramide / Sphingolipids
		μmol/100 ml packed cells				*mol %*
Fresh blood						
Total erythrocytes	1	283	317	19.8	5.7	29.0
	2	276	312	21.2	4.6	21.7
	3	285	328	23.0	5.8	25.3
Outdated blood						
Total erythrocytes	4	288	350	18.3	4.9	26.7
	5	272	384	24.5	6.0	24.3
	6	286	378	22.2	6.7	30.3
		μmol/g total lipid				
Outdated blood						
Ghosts	5	568	669	48.0	10.9	22.8
	6	600	883	47.6	15.4	32.4
	7	552	635	42.0	12.0	28.5

Sphingolipids refer to total sphingolipids except sphingomyelin.

Table 2) and it accounted for 26.5 ± 3.4% of the sphingolipids, excluding sphingomyelin. The free ceramide concentration was 5.6 ± 0.7 μmol/100 ml of packed erythrocytes.

DISCUSSION

In the present study, free ceramide was demonstrated to be a component of human erythrocyte membrane. It was the second sphingolipid in concentration after globoside and it constituted 0.61 ± 0.13% of the erythrocyte lipids. In whole blood, the concentration of free ceramide contributed by erythrocytes was much higher (ca. 2.4 μmol/100 ml of blood) than that occurring in plasma (0.5 μmol) (7) or in platelets (0.08 μmol) (8).

A significant contribution of plasma to the free ceramide content of erythrocytes may be ruled out as the red cells were washed twice before lipid extraction. However, under our experimental conditions, other cells were spun down with red cells: 50–60% of the platelets and all the leucocytes. The free ceramide of platelets may be estimated to contribute 2% of the free ceramide of the erythrocyte pellet. The presence of free ceramide in human leucocytes is not documented at the present time. The leucocyte number is small, less than 0.2% of the erythrocyte number, and a white cell has a mean surface three times larger than a red cell. Thus, one can estimate that the contribution of the free ceramide of leucocytes is about 0.6% of the free ceramide found in the erythrocyte pellet, assuming a similar concentration of free ceramide in the plasma membrane of both cells. Therefore, the presence of other blood cells in the red cell pellets is responsible for only a few percent of the free ceramide content found in erythrocytes. Furthermore, the differences in concentration between individuals (Table 2) cannot be explained by differences in the level of contamination of red cells by other cells.

It was found that free ceramide of human erythrocytes contained mainly nonhydroxy fatty acids with 16, 22, and 24 carbon atoms. This fatty acid composition differed from that of platelet membrane where C22 fatty acid is the most abundant (8). It was noted that the monounsaturated C24 fatty acid was slightly more abundant than its saturated homologue. Hydroxy fatty acids were present in free ceramide, although not exceeding 1%. Such a hydroxylated ceramide was not found in human platelets (8) or in plasma (7). However, the presence of a small amount of hydroxylated galactosylceramide has been reported in human plasma (24).

The role of free ceramide in the plasma membrane remains unknown. It is likely to contribute to the structural organization of the lipid phase. It may also play a functional role. Clayton et al. (25) have shown that free ceramide is an active factor stimulating the maturation of rabbit erythroblasts in vitro. This active ceramide, isolated from rabbit leucocytes, is structurally very similar to that of human erythrocytes reported here. Therefore, free ceramide might also be an important factor in human erythropoiesis.

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