Degradation of glycerophosphatides during storage of saline-washed, saline-suspended red cells at −20°C

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SUMMARY When fresh intact red cells were washed and suspended in 0.153 M NaCl and then frozen-stored, the glycerophosphatide levels decreased significantly. Degradation began within 2 wk. Loss of phospholipid was not observed with hemoglobin-free red cell ghosts or plasma stored as long as 2 and 6 months, respectively.

KEY WORDS phospholipid · degradation · frozen-storage · red cells · red cell ghosts · plasma

THE LIPID COMPOSITION of biological tissues stored at temperatures below 0°C is often assumed to be the same as that of identical tissues extracted in the fresh state. This study documents striking changes in the lipid composition of human red cells suspended in 0.153 M NaCl and then frozen at −20°C for periods of 1 to 26 wk.

METHODS

Blood from hematologically normal human donors who had fasted 12 hr or more was collected in plastic syringes and discharged into glass vessels containing Na_2EDTA (1 mg/ml of blood) or ACD (NIH solution A, 1 ml/7 ml of blood). The erythrocytes were washed three times and then suspended to a hematocrit value of 40–60% in 0.153 M NaCl or 0.138 M NaCl containing 0.017 M EDTA (the latter solution henceforth is designated "EDTA–saline"). All solutions were maintained at 4°C. Aliquots of the fresh suspensions were removed for determination of hemoglobin and hematocrit value and for immediate extraction of lipids by procedure III of Ways and Hanahan (1). The remaining suspension was divided into appropriate-sized samples (10–20 ml) and stored at −20° C in glass or polypropylene containers.

At intervals ranging from 1 to 16 wk the samples were thawed, the hemoglobin concentration was determined, and the lipids were extracted from duplicate aliquots. Hemoglobin-free erythrocyte ghosts were prepared by the method of Dodge, Mitchell, and Hanahan (2). Lipid phosphorus determination (1), cholesterol determination (3), phospholipid distribution on silicic acid-impregnated paper (4), and neutral lipid thin-layer chromatography (1) were all done by previously described methods. Thin-layer chromatography of phospholipids was carried out on plates commercially coated with Silica Gel F 254,[1] in a solvent system composed of chloroform–methanol–water 95:35:4.

RESULTS

Table 1 illustrates the effect of storage at −20°C on the lipid composition of several red cell suspensions. The lipid phosphorus and cholesterol values were stable for 1 wk in all experiments. The lipid phosphorus fell after 2 wk in one (EDTA D) of two experiments and after 4 wk in two (EDTA D and D†) of three experiments. All suspensions stored for 6 wk or longer showed low levels of

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[1] Brinkmann Instruments, Great Neck, N. Y.

TABLE 1 Loss of Lipid Phosphorus during Frozen-Storage of Red Blood Cells*

Anticoagulant and Sample	Assay	Days Frozen-Stored 0	1	2	4	Weeks Frozen-Stored 1	2	4	6	8	16
ACD											
A	Lipid phosphorus	4.05	3.73	3.82	3.92	3.75					
	Cholesterol	3.62	3.40	3.49	3.49	3.38					
B	Lipid phosphorus	3.90	4.08	4.05	3.94	4.03					
	Cholesterol	3.67	3.96	3.82	3.75	3.71					
C	Lipid phosphorus	3.68								3.13	1.31
	Cholesterol	3.75								3.84	3.92
D	Lipid phosphorus	3.52				3.78	3.72	3.59	3.19	2.54	
	Cholesterol	3.83				3.85	3.57	3.59	3.67	3.44	
E	Lipid phosphorus	4.02								2.41	2.17
	Cholesterol	3.98								3.60	3.42
F	Lipid phosphorus	4.26								2.48	
	Cholesterol	4.04								3.42	
EDTA											
A	Lipid phosphorus	4.15	4.18	4.10	4.07	4.07					
	Cholesterol	3.39	3.59	3.65	3.48	3.47					
B	Lipid phosphorus	3.70	3.60	3.71	3.62	3.70					
	Cholesterol	3.54	3.54	3.48	3.34	3.46					
C	Lipid phosphorus	3.71								3.38	1.58
	Cholesterol	3.88								4.00	3.85
D	Lipid phosphorus	3.72				3.73	3.30	2.61	2.24	2.20	
	Cholesterol	3.99				3.74	3.71	3.71	3.95	3.65	
D†	Lipid phosphorus	3.72						2.55		2.16	
	Cholesterol	3.99						3.60		3.27	
E	Lipid phosphorus	3.85								2.59	
	Cholesterol	3.71								3.74	

* Lipid phosphorus expressed as mg/g of hemoglobin $\times 10^{-1}$; cholesterol expressed as mg/g of hemoglobin.

† This sample was suspended and frozen in "EDTA–saline" (see Methods) and stored in glass. All other samples were frozen in 310 milliosmolar NaCl and stored in glass.

TABLE 2 Changes in Phospholipid Distribution during Frozen-Storage of Red Cells

	Normal Fresh Cells (n = 7), Ref. 3	Experiment 1: 6 wk	Experiment 2: 0 time	Experiment 2: 8 wk
	% of total phospholipid present at zero time			
Lecithin	29.9	14.4	27.8	15.2
Sphingomyelin plus inositol phosphatides	25.3	28	24.7	25.8
Ethanolamine glycerophosphatides	24.3	4.6	27.7	4.4
Serine glycerophosphatides	14.8	4.5	14.3	4.6
Other	5.4	6.8	4.8	9.2
Recovery from chromatograms	99.0	106	93	90

lipid phosphorus; in some of these samples a slight decrease in cholesterol was also found. The data did not show clear differences between cells initially collected in EDTA and those collected in ACD. Washing, suspension, and storage in EDTA–saline did not appreciably diminish the phospholipid loss. In two experiments (not shown), portions of the same red cell suspension were stored in polypropylene as well as in glass tubes. There was no difference in the decrement of phospholipid.

In two experiments phospholipid distribution was determined. In both, significant reductions occurred in all of the diacyl phospholipids,[2] but no significant breakdown of sphingomyelin was evident (Table 2). Thin-layer chromatograms of the neutral lipid extracted from certain frozen-stored suspensions in which appreciable phosphoglyceride degradation had occurred showed higher than normal quantities of free fatty acid but no diglyceride or monoglyceride. In experiment 2 (Table 2) the neutral lipids were separated from the phospholipids after the sample had been stored for 8 wk, and the free fatty acids were quantified by titration. 1 μeq of free fatty acid was extracted per ml of packed cells—10 times more than the amount found in normal fresh cells (1). It can be calculated, however, that the amount of phospholipid "lost" during storage per ml of packed cells would require the appearance of 2.8 μeq of fatty acid if all of the loss had been due to deacylation.

The 8-wk and 16-wk extracts of experiment ACD-E were chromatographed on thin-layer plates which were then charred or stained with ninhydrin (Figs. 1 and 2).

[2] Since phosphatidyl inositol was eluted with sphingomyelin from the paper chromatograms, it is not certain that this phospholipid also decreased in amount.

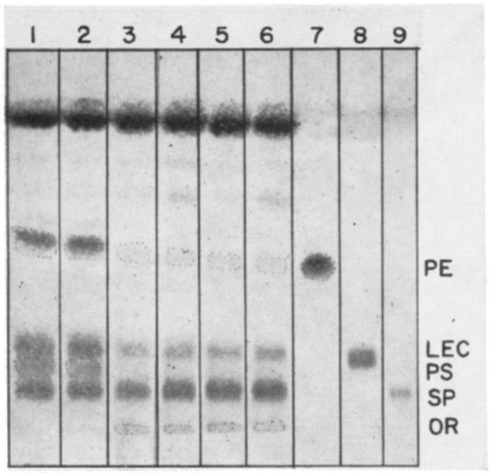

FIG. 1. Thin-layer chromatogram of red cell lipids extracted from fresh unstored cells and of several extracts of cells stored at −20°C. The plate was coated commercially (see Methods) and developed in chloroform–methanol–water 95:35:4. It was initially sprayed with ninhydrin; the positive areas have been encircled with dots. It was then charred after spraying with H_2SO_4. Lanes *1* and *2*: extract of fresh red blood cells originally anticoagulated with ACD and EDTA respectively (12 μg of lipid phosphorus applied). Lanes *3* and *4*: extracts of cells stored at −20°C in polypropylene for 2 and 4 months, respectively (7.5 μg of lipid phosphorus applied). Lanes *5* and *6*: extracts of cells stored at −20°C in glass for 2 and 4 months, respectively (9.5 μg of lipid phosphorus applied). Lane *7*, phosphatidyl ethanolamine (PE); lane *8*, lecithin (LEC); lane *9*, sphingomyelin (SP). In this solvent system phosphatidyl serine (PS) chromatographed to a position between lecithin and sphingomyelin. OR, origin.

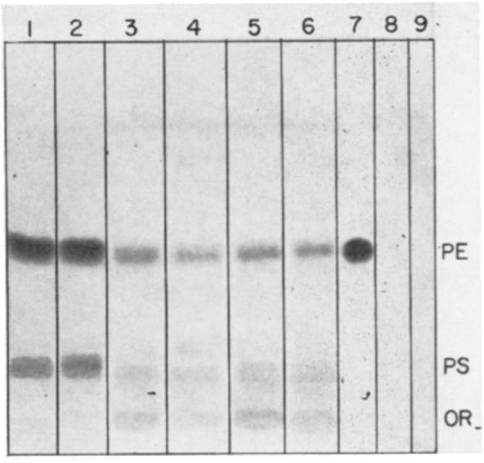

FIG. 2. A thin-layer chromatogram run under circumstances identical with those shown for the chromatogram in Fig. 1 but photographed after spraying with ninhydrin only. The standards in lanes *8* and *9* were omitted. Note the low ninhydrin reactivity in the phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) regions, and the relatively large amounts of ninhydrin-positive material at the origin (OR).

Ninhydrin-positive material was not only present in the area normally occupied by phosphatidyl ethanolamine and phosphatidyl serine, but was strongly concentrated at the origin. Lesser amounts were seen as a streak between lecithin and phosphatidyl ethanolamine.

In two experiments, "hemoglobin-free" red cell ghosts were prepared in 20 milliosmolar phosphate buffer, pH 7.4. They were then suspended in 0.153 M NaCl and frozen at −20°C for 4 and 8 wk, when they were found to contain the same amounts of phospholipid as at zero time (Table 3). Also by contrast, two samples of plasma frozen at −20°C for 13 and 26 wk and one frozen for 52 wk contained phospholipid in amounts comparable to those found in the same plasma extracted fresh (Table 3).

DISCUSSION

These studies demonstrate that the quantity of glycerophosphatides extractable from human red cells decreases significantly after more than 2 wk at −20°C. While the accumulation of free fatty acid in the phospholipid-deficient extracts suggests that deacylation is occurring, the amount of fatty acid is insufficient to account for all of the phospholipid loss. Dodge, Cohen, Kayden, and Phillips (5) have demonstrated decreased extractability of phosphatidyl ethanolamine and phosphatidyl serine after exposure to H_2O_2 of red cells from a patient with abetalipoproteinemia deficient in vitamin E. Similar results were obtained on red cells from rats deficient in vitamin E. A priori, the storage conditions used in the present studies would not be expected to encourage peroxidation. However, the distribution of ninhydrin-positive material on the thin-layer chromatograms of the stored extracts is similar to that demonstrated by Dodge and Phillips after peroxidation of lipid extracts (6). In addition, the ordered structure of ice is more conducive to the transfer of electrons than that of water (7), and certain enzyme reactions have been shown

TABLE 3 PRESERVATION OF PHOSPHOLIPID AND CHOLESTEROL DURING FROZEN-STORAGE OF HEMOGLOBIN-FREE RED CELL GHOSTS AND OF PLASMA

		Weeks Stored	Lipid Phosphorus	Cholesterol	Cholesterol / Lipid Phosphorus
			mg/2 ml of suspension		
Ghosts	A	0	0.339	3.38	10.0
		4	0.344	3.26	9.5
		8	0.332	3.15	9.6
	B	0	0.144	1.36	9.4
		4	0.147	1.33	9.1
		8	0.146	1.42	9.7
			mg/100 ml		
Plasma	A	0	8.9	211	
		13	9.0	213	
	B	0	6.3	176	
		26	7.0	169	
	C	0	4.8	112	
		52	4.7	96	

to occur readily at −20°C (8). That the storage temperature could, in some way, have been responsible for the inextractability of the glycerophosphatides is refuted by the normal quantities extracted from frozen-stored hemoglobin-free ghosts and from three frozen-stored samples of plasma.

Thus, the loss of extractable glycerophosphatides in frozen-stored red cells appears partly due to deacylation but is also compatible with the formation of lipid peroxides. Whether the reaction is physicochemical or enzymatic cannot be inferred from the data. It seems to be catalyzed by hemoglobin, or some fraction of hemoglobin, or some other soluble constituent of the intact cell.

The implications of these experiments for studies of red cell lipids are obvious. In addition, they suggest that one should determine the effect of freezing upon the lipid content of any tissue before attempting to freeze that tissue and then, later, perform lipid analyses on it.

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References

1. Ways, P., and D. J. Hanahan. 1964. *J. Lipid Res.* **5:** 318.
2. Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. *Arch. Biochem. Biophys.* **100:** 119.
3. Jones, J., and P. Ways. *J. Clin. Invest.* **46:** 1151.
4. Crowley, J., P. Ways, and J. W. Jones. 1965. *J. Clin. Invest.* **44:** 989.
5. Dodge, J. T., G. Cohen, H. J. Kayden, and G. B. Phillips. 1967. *J. Clin. Invest.* **46:** 357.
6. Dodge, J. T., and G. B. Phillips. 1966. *J. Lipid Res.* **7:** 387.
7. Klotz, I. M. 1962. *In* Horizons in Biochemistry. M. Kasha and B. Pullman, editors. Academic Press Inc., New York. 523–530.
8. Grant, N. H., and H. E. Alburn. 1966. *Nature.* **212:** 194.