PRESERVATION OF THE LIPIDS OF THE HUMAN ERYTHROCYTE STROMA DURING FIXATION AND DEHYDRATION FOR ELECTRON MICROSCOPY

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Recent reports of lipid and/or protein losses from biological material during fixation processes (Luft and Wood, 1963; Ashworth et al., 1966; Korn and Weisman, 1966; Morgan and Huber, 1967; Stein and Stein, 1967 *a*, *b*, 1968; Buschmann and Taylor, 1968; Ongun et al., 1968) emphasize the necessity for basing the interpretation of electron micrographs upon knowledge of the final composition of the fixed and dehydrated material being studied. In the experiments reported here, the amount of cholesterol and phospholipid lost from human erythrocyte stroma during fixation with glutaraldehyde and osmium tetroxide was measured. Even after dehydration with ethanol and propylene oxide, this loss was minimal provided the stroma had been freshly prepared.

MATERIALS AND METHODS

Erythrocyte stroma was prepared as described by Dodge et al. (1963) and was stored at 4°C in dilute phosphate buffer for 2, 7, or 10 days before fixation. Fixation was begun by mixing the stroma with glutaraldehyde for 3½ hr at 4°C (1.3% glutaraldehyde in 0.08 M cacodylate buffer, pH 7.4, [Ca^{++}] either 0.007 or 0.015 M). Following centrifugation the stroma was washed twice with 0.15 M cacodylate buffer, 0.014 M in Ca^{++}, then fixed in 2.5% OsO_4 for 1 hr at 4°C. The fixed stroma was then washed for 10-min periods with each of the following solvents in the order given: 30% ethanol, 70% ethanol, 95% ethanol, 100% ethanol, then twice with propylene oxide.

The supernatant solutions from each step of the above processes were individually extracted with three volumes of chloroform:methanol (2:1), and this extraction was repeated once on any remaining aqueous layer. The chloroform layers were evaporated, and the residues were taken up with 10 ml of chloroform followed by 5 ml of methanol. This chloroform-methanol extract was washed with 3 ml of 0.1 N KCl, the chloroform layer was evaporated, and the residue was then dissolved in a final volume of 10 ml of chloroform:methanol (2:1). Duplicate aliquots of

TABLE I

Per Cent Loss of Lipid from Erythrocyte Stroma during Fixation

Exp.	Type of stroma analyzed	Lipid fraction*	Buffer supernatant	Glutaraldehyde + cacodylate	Cacodylate wash	Cacodylate wash	OsO_4 fixative	30% Ethanol	70% Ethanol	95% Ethanol	100% Ethanol	Propylene oxide	Propylene oxide	Total extracted
			%	%	%	%	%	%	%	%	%	%	%	%
1	2 day old, Ca^{++} = 0.007 M	Chol	—	0.1	0.1	0.8	0.2	0.1	1.1	2.0	1.2	0.2	0.2	6.0
		PLS	—	0	0	0	0	0.1	0.5	0.5	0.9	0.1	0.1	2.2
2	10 day old, Ca^{++} = 0.007 M	Chol	—	0.2	0.1	<0.1	0	0.1	1.1	4.9	4.4	2.4	1.0	14.2
		PLS	—	1.4	<0.1	<0.1	0.6	0.3	0.8	1.7	2.1	0.4	0.3	7.7
3	2 day old, Ca^{++} = 0.015 M	Chol	—	0.1	0.1	0.1	<0.1	0.1	0.7	1.1	1.2	0.2	0.1	3.7
		PLS	—	0.4	0	0.5	0	0.5	0.4	0.5	0.9	0.1	0.1	3.4
4	7 day old, Ca^{++} = 0.015 M	Chol	0.6	0	0	0.2	0.6	0.2	0.9	1.7	2.0	1.6	0.8	8.6
		PLS	0.5	0.7	0.3	0.2	0.6	0.3	0.9	1.2	2.8	0.1	0.1	7.7
5	7 day old, Ca^{++} = 0.015 M	Chol	0.4	0.1	0	0.2	0.9	0.4	0.6	1.8	3.4	1.6	0.5	9.9
		PLS	0.3	1.1	0.4	0.3	0.8	0.1	1.2	1.3	1.7	0.6	0.2	8.0
6	10 day old, Ca^{++} = 0.015 M	Chol	—	0.2	0.1	<0.1	0	0.1	0.9	5.9	4.1	1.7	0.7	13.7
		PLS	—	0.6	<0.1	0	<0.1	0.4	0.6	2.2	1.7	0.4	0.3	6.3

* Chol = cholesterol; PLS = total phospholipid.

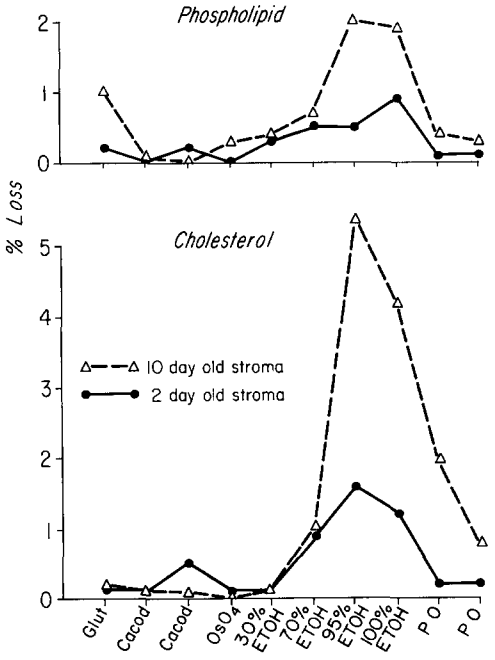

FIGURE 1 Per cent loss of lipid components from stroma during fixation and dehydration. See text for detailed composition of fixatives. Glut, glutaraldehyde fixative; Cacod, cacodylate buffer; PO, propylene oxide.

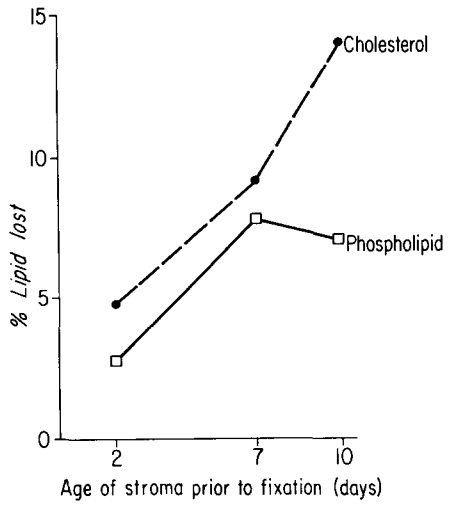

FIGURE 2 Correlation between total lipid loss and age of stroma.

fresh, unfixed stroma were extracted by the procedure of Mitchell and Hanahan (1966) to provide base-line values for the stromal cholesterol and lipid phosphorus.

Cholesterol was measured in aliquots of each lipid extract by the procedure of Courchaine et al. (1959); samples known to contain glutaraldehyde were first purified by thin-layer chromatography (Parker et al., 1968). Recovery of incremental amounts of pure cholesterol added to stromal samples prior to OsO_4 fixation was essentially complete (92–110%), verifying the efficiency of the extraction and assay techniques. Lipid phosphorus was measured by the procedure described by Parker and Peterson (1965), although to avoid the presence of arsenate in the final test for phosphate it was necessary to further purify the extracts from all cacodylate-containing samples. Aliquots from these extracts were applied to Silica Gel H thin-layer plates which were then developed with distilled water; the lipids remained at the origin, while the cacodylate contaminant was moved up the plate. Recovery of lipid phosphorus in this procedure ranged from 99 to 101%.

RESULTS AND CONCLUSIONS

The results obtained in these experiments are summarized in Table I and Fig. 1. The effect of ageing the hemoglobin-free stroma prior to fixation is summarized in Fig. 2. Fresh erythrocyte stroma fixed by this particular procedure retains most of the original stromal components, 95.2% of the cholesterol present in the unfixed stroma and 97.2% of the total phospholipid. The major loss of both cholesterol and phospholipid occurs during the ethanol dehydration procedure. The stroma becomes increasingly fragile if it is allowed to age prior to fixation, but the pattern of lipid loss remains the same.

It must be emphasized that this study describes the retention of lipids in human erythrocyte stroma only. Because each tissue is unique in its chemical composition and structural organization, specific measurements of preparative lipid and protein losses should be made for each individual tissue, as well as for each fixation or dehydration procedure.

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