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IS LIPID LOST DURING PREPARATION OF ERYTHROCYTE MEMBRANES?

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Summary

In contrast to the findings of Turner and Rouser (Turner, J.D. and Rouser, G. (1974) Lipids 9, 49—54), it is shown that lipid is not lost from erythrocyte membranes carefully prepared by hypotonic hemolysis. The present study confirms earlier reports showing excellent recovery of lipid in hemoglobin-free membranes.

In a recent publication, Turner and Rouser [1] reported that, in their laboratory, preparation of membranes (ghosts) from human erythrocytes by the method of Dodge et al. [2] resulted in a substantial removal of lipid. Furthermore, Turner and Rouser [1] argued that their results agreed with those of Burger et al. [3] who observed lipid loss during membrane preparation from bovine erythrocytes. In the latter case, however, Turner and Rouser [1] have misinterpreted or misread the results presented by Burger et al. [3] since membranes of the bovine erythrocyte undergo fragmentation during preparation by the low osmolar buffer procedure of Dodge et al. [2] with concomitant loss of lipid and protein (as monitored by acetylcholine esterase release) only if calcium or magnesium are not present in the hemolysing buffer Mazia and Ruby [4] also observed fragmentation of bovine erythrocyte (membranes) during hemolysis in ion-free water. In any event, the data of Turner and Rouser [1] were provocative since earlier reports by Weed et al. [5] and Dodge et al. [2] had shown excellent recovery of lipid in hemoglobin-free membranes. In the ensuing years, our laboratory has prepared membranes many times with no evidence of loss of lipid or of a typical membrane en-

TABLE I

LIPID CONTENT IN HUMAN ERYTHROCYTES AND HEMOGLOBIN-FREE MEMBRANES USING PROCEDURE A

Specific details are presented in the text. Subject, S.S.

Sample	Anti-coagulant	Wash buffer	Hemolyzing buffer	Lipid in μmoles per ml packed cells	
				Cholesterol	Phosphorus
Intact cells	Heparin	Tris	—	3.13	4.39
	Heparin	Phosphate	—	3.11	4.30
Intact cells	EDTA	Tris	—	3.25	4.36
	EDTA	Phosphate	—	3.12	4.36
Membranes	Heparin	Tris	Tris	2.89	4.03
	Heparin	Phosphate	Phosphate	3.07	4.39
Membranes	EDTA	Tris	Tris	3.05	4.36
	EDTA	Phosphate	Phosphate	3.08	4.36

TABLE II

LIPID CONTENT AND ACETYLCHOLINE ESTERASE LEVELS IN HUMAN ERYTHROCYTES AND HEMOGLOBIN-FREE MEMBRANES USING PROCEDURE B

Specific details are presented in the text. Subject, M.H.

Sample	Cholesterol in μmoles/ml packed cells	Lipid phosphorus in μmoles/ml packed cells		Acetylcholine esterase levels	
		Total	Chloroform-rich phase of biphasic system	Units/ml packed cells	% recovery
Intact cells*	2.76	5.40	5.66	50.6	100
Membranes*	3.20	5.48	5.52	47.6	94
Intact cells**	3.23	5.61	5.25	(same as above)	
Membranes**	3.22	5.30	5.30		

*Lipids were extracted, purified and analyzed as described in Procedure A.
**Lipids were extracted and purified as described in Procedure B and analyzed as described in Procedure A.

zyme, acetylcholine esterase. Nevertheless, realizing the frailties of humans and scientific experimentation, we undertook to compare the methods employed in our laboratory with those outlined by Turner and Rouser [1] for the preparation of membranes from human erythrocytes. The details of these procedures and the results are presented below.

Essentially the two following procedures were employed for collection of cells and preparation of membranes:

(A) Blood was withdrawn from the antecubital vein of healthy volunteers and collected in an anticoagulant, either (Na^+) heparin (14.3 units/ml blood) or EDTA (1 mg per ml blood). Subsequent to the removal of the buffy coat by centrifugation at 1000 x *g* for 20 min at 4 °C, aliquots of the cell suspension in plasma were washed 3 times with 0.172 M Tris—HCl buffer*, pH 7.6 or with 0.155 M phosphate buffer*, pH 7.6. One set of aliquots was diluted with

*Equivalent to 310 imosM.

the respective isotonic buffer, while another set was washed first with an isotonic buffer and then treated with 20 imosM solutions of each respective buffer [6]. The membranes were recovered by centrifugation at 27 000 x *g* for 40 min at 4 °C in a Sorvall RC-2B centrifuge using an SS-34 rotor. In the low-osmolar treatment, the membranes were washed four successive times with the same volume of the hemolyzing buffer and then made to volume in the same buffer. A typical lipid extraction was conducted as follows: 1.0 ml of a 50% washed cell suspension was transferred to 5 ml methanol and 4 ml chloroform added. The sample was mixed, allowed to stand 30 min at 20 °C and then centrifuged at 1000 x *g* for 4 min at 20 °C. The supernatant was saved and the residue extracted with 5 ml methanol and 5 ml chloroform. The sample was recentrifuged and the supernatant combined with the previous one. This (supernatant) lipid extract was evaporated to dryness in vacuo and dissolved in chloroform—methanol—water (60:30:4.5 by vol.) and passed through a Sephadex G-25 column as described by Wells and Dittmer [7]. The eluate was collected and made to 10 ml. Aliquots were assayed for cholesterol [8] and (lipid) phosphorus [9]. Membranes were treated in a similar manner. These results are presented in Table I.

(B) This procedure essentially was that described by Turner and Rouser [1] in which EDTA was the only anticoagulant used, the cells washed with sodium phosphate buffer, pH 7.4 (Wash 1) [1] and hemolyzed in hypotonic sodium phosphate buffer (Wash 7) [1]. The membranes were pelleted by centrifugation for 30 min at 20 000 x *g* at 4 °C, and washed four times with the same hypotonic buffer. One aliquot of these hemoglobin-free membranes was extracted with organic solvents as described by Turner and Rouser [1]. Non-lipid contaminants were removed on Sephadex-G-25 by the method of Siakotas and Rouser [10]. The organic solvents were evaporated under a stream of N_2 and the chloroform-soluble components recovered. Another aliquot of these same membranes was subjected to lipid extraction and cholesterol and lipid phosphorus as described in Procedure A above. Acetylcholine esterase levels were determined essentially by the procedure of Ellman et al. [11]. These results on the distribution of lipid phosphorus, cholesterol and acetylcholine esterase levels in the cells and ghosts are given in Table II. Inasmuch as the phospholipids were not subjected to separation by thin-layer chromatography, a check was made to rule out the presence of other nonlipid phosphate compounds in the lipid extracts. The details are as follows.

An aliquot of the Sephadex G-25 eluant obtained in the two extraction procedures was distributed in a biphasic system of chloroform—methanol—0.9% NaCl. (4:2:3, by vol.). The lower chloroform-rich phase, containing the lipids, was washed one additional time with 40% methanol in 0.9% NaCl. In an unpublished study (Luthra, M.) it was found that the recovery of phospholipids (in the chloroform phase) was more than 98% and insignificant amounts were detected in the upper phase and at the interface; any nonlipid phosphate was found in the chloroform-poor layer.

Subsequently, in the current investigation, the chloroform-rich phase was evaporated and assayed for lipid phosphorus. Data obtained on the lipid distribution before and after this "biphasic" treatment showed that the lipid extracts obtained in all the cases were free of nonlipid phosphate-containing compounds (Table II).

In contrast to the results of Turner and Rouser [1], our results confirm the observations of Dodge et al. [2] and Weed et al. [5]. Turner and Rouser [1] analyzed the lipid extract after thin-layer chromatography so it is difficult to explain whether the lipid loss occurred during thin-layer chromatography or during the membrane preparation or both. Also, these authors did not present any data on the recovery of lipids in the hemolysate obtained during membrane preparation (after four washes with hypotonic buffer). In one experiment, the authors recovered less than 80% of lost lipids in the combined hemolysate obtained during hemolysis and 15 further washes (Wash 4) [1]. Thus, it is difficult to decide to what extent the loss was encountered during the membrane preparation and how much additional loss was obtained with the subsequent 15 washes.

Finally, in our study, acetylcholine esterase levels were used as an index of membrane recovery. In confirmation of earlier observations [3], the recovery of acetylcholine esterase in hemoglobin-free membrane was excellent (Table II). In the studies of Turner and Rouser [1], recovery of membranes was not checked at any stage of the experimental study. Hence, it is possible that the loss of lipids in their studies was due to the loss of membranes during hypotonic lysis since it is extremely easy to lose material from the loosely packed pellet obtained on centrifugation.

In summary, we conclude that lipid is not lost from membranes* carefully prepared by hypotonic hemolysis and this confirms our previous observations [2] and those of Weed et al. [5].

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*Bramley et al. [12] reported that only non-heme protein was lost in (human erythrocyte) membranes prepared in less than 40 imosM buffers and there was no loss of phospholipid or cholesterol. In the range of 15—5 imosM, these investigators noted fragmentation of the membranes with a selective loss of protein.