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Changes in erythrocyte membranes during preparation, as expressed by ATPase activity

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SUMMARY

During the preparation of membranes from human erythrocytes by osmotic lysis in 20 imosM Tris buffer at pH 7.6 there is a decrease in total ATPase activity and concomitantly a progressive alteration of the membrane (maximal at the fourth wash), as indicated by the exposure of (Na^+,K^+,Mg^{2+})-ATPase activity. These changes are pronounced at pH 7.6 but occur to a much lesser extent at pH 5.8.

Examination of calf and goat erythrocytes showed that these membranes are maximally altered (as indicated by ATPase activity) in the first hypotonic wash in the 20 T buffer, pH 7.6. Again a loss of total ATPase activity (units) was noted.

These results emphasize some of the problems encountered in correct depiction of the characteristics of a membrane and provide an interesting avenue to further study on factors involved in the stability and behavior of these membranes.

The mammalian erythrocyte has become a popular choice for study of membrane structure and behavior by virtue of its ease of preparation and isolation and the lack of any subcellular components. One approach to isolation of membranes from these cells has been through use of osmotic lysis, at very low osmolarities, of the intact erythrocytes. It was early recognized[1,2] that this technique, though giving hemoglobin-free preparations with retention of total lipid, at least in the case of the human erythrocyte, led to the loss of non-hemoglobin protein. As recently discussed by Fairbanks *et al.*[3] this fact has been confirmed in several different laboratories. Of interest, Steck *et al.*[4] have described a method wherein inside-out vesicles could be obtained from human erythrocyte membranes, prepared essentially by the technique of Dodge *et al.*[1], by incubation of the membranes for 1 h at pH 8 and then extrusion through a small capillary needle. In this procedure, the configuration of the membrane apparently changed as indicated by a decrease in the

availability of sialic acid residues to sialidase action. Thus, the surface sialoglycopeptides were considered turned inward in the form of vesicles. No mention was made of the enzymatic activities associated with these preparations. This was of interest, since our laboratory has had a long-standing involvement in elucidating the structure and behavior of the erythrocyte membrane, and we have found over the past two years evidence that there are significant alterations in membrane-associated enzyme activities during preparation of these membranes.

The (Na^+,K^+,Mg^{2+})-ATPase system has been considered a prime example of a membrane-bound enzyme. This enzyme activity has been used as a marker in a study designed to explore the biochemical nature of the erythrocyte membrane. It has been found that on preparation of membranes from human, calf and goat erythrocytes there is a substantial and significant change in the level, nature and behavior of the ATPase enzyme system. Obviously the membrane is altered and active sites are being exposed without resort to use of treatments such as freeze thaw or sonication. Recently, Bramley *et al.*[5], in an excellent article, reported that human erythrocyte membranes (ghosts) prepared at osmolarities below 20 imosM (bicarbonate buffer) showed a high (Na^+,K^+,Mg^{2+})-ATPase activity, without necessity of exposure to sonication or detergent. On the other hand, if the membranes were prepared at higher osmolarities, *e.g.* 80 imosM bicarbonate, there was a low (Na^+,K^+,Mg^{2+})-ATPase activity (exposure of these membranes to sonication or detergent was necessary to produce a high ATPase activity). This communication in part confirms the observations of Bramley *et al.*[5] and in part presents new data on the changes in erythrocyte membranes during preparation.

Blood was collected into heparinized Vacutainer (Becton–Dickinson) tubes, centrifuged and the plasma and buffy coat removed. Cells were washed with an equal volume of 0.172 M Tris buffer, pH 7.6, three times and the cells spun down each time at nearly $1000 \times g$, in a Sorvall RC-2 centrifuge, for 30 min at 4°. Cells were resuspended in the same buffer to approximately 50% hematocrit. Healthy adult male and female humans were used as donors with no attempt at differentiation of particular blood groupings. Blood was obtained by jugular vein tap from Holstein calves ranging from a few hours to several weeks old; the very kind cooperation of Dr. Gerald Stott, Department of Dairy Sciences, is deeply appreciated. Blood from goats of the Angora and Spanish strain, males and females, respectively, were obtained from the University of Texas Medical School at San Antonio through the courtesy of Dr. B.D. Fremming of the Department of Laboratory Animal Medicine.

Membranes were prepared from the washed erythrocytes essentially by the procedure of Dodge *et al.*[1] using 20 imosM Tris buffer at the desired pH (at ratio of 1 vol. of cells to 7.5 vol. of buffer). In the discussion of the data obtained in the experiments, the first contact of the intact cells with low ionic strength or hemolyzing buffer is called the first hemolysis and the membranes obtained by centrifugation from this treatment are often noted as "Wash I membranes". Subsequent washes are then numbered in succession. Usually by the third wash at pH 7.6 the membranes are only very lightly pink and most often by the fourth wash nearly colorless. Occasionally a small button of erythrocytes remain along with the white stroma, but this varies considerably. Quite consistently, though, a small clot is found and this is removed after the last wash. Membranes were prepared at pH 5.8 using a maleic acid buffer, and the same procedure as above was

followed. The membranes were pink to red in color.

The ATPase assay was conducted essentially by the technique outlined by Brewer *et al.*[8], using ouabain as an inhibitor of the (Na^+,K^+,Mg^{2+})-ATPase (sensitive) activity. A freeze thaw technique for expression of ATPase activity was that outlined by Garrahan *et al.*[9]. Activities are expressed as μmoles P_i released per ml packed cells per 2 h. Ca^{2+}, Mg^{2+}, K^+ and Na^+ were assayed by atomic absorption spectroscopy. Ouabain octahydrate (strophanthin C), Trizma base (Reagent Grade), and adenosine triphosphate (disodium salt) were purchased from Sigma Chemical Company (St. Louis, Mo.). Total lipid was isolated by methanol–chloroform extraction of the intact cells or membranes. An aliquot was then evaporated, transferred with chloroform to assay tubes and analysed for total cholesterol[6]. Another portion of the original extract was assayed for phosphorus[7] after passage through a Sephadex G-25 column which had been packed in chloroform–methanol–water (3:4:1, v/v/v).

Experimentally, membranes of human erythrocytes were examined at various stages of their preparation and it was noted that there was a significant and progressive loss in total ATPase activity during successive washing procedures. Concomitantly, there was a progressive change in the membrane, as indicated by increased (Na^+,K^+)-activated ATPase activity without any freeze thaw treatment. Data on these changes are shown in Table I. It should be noted that the human erythrocytes (intact, original) must be made permeable to ATP in order to elicit any (Na^+,K^+,Mg^{2+})-activated ATPase activity. This is accomplished through use of the technique described by Garrahan and Glynn[9]. Thus, on the basis of the evidence presented in Table I, membranes prepared at pH 7.6 in 20 imosM Tris buffer show a definitive loss of total ATPase activity, and concomitantly exhibit an "opening" of the membrane, as indicated by the (Na^+,K^+,Mg^{2+})-ATPase activity. It is important to emphasize that this latter activity is expressed *without* resort to any freeze thaw treatment. These observations compare closely with those of Bramley *et al.*[5] who examined membranes only after 5 to 6 hypotonic washes and noted development of latent ATPase activity. On the other hand, however, human erythrocyte membranes prepared at pH 5.8 do not undergo the same change, at least with respect to the (Na^+,K^+,Mg^+)-ATPase. The data in Table I show that after four hemolysis washes in pH 5.8, 20 imosM maleate buffer, the membrane appears to be closed. Of considerable interest, treatment of these pH 5.8 membranes with pH 7.6, 20 imosM Tris buffer produced an altered membrane. Further examination of the data in Table I showed that the membranes obtained in the first wash at pH 7.6 and also at 5.8 had a considerably reduced total ATPase (even after freeze thaw) than did membranes obtained in later washes. It is obvious from a simple calculation of the volumes of cells and buffer used that the first wash is not at 20 imosM, but closer to 60 imosM. Of importance, though, is the observation that short-term sonication (5 to 10 sec at 4°) of the first-wash membranes (20 T non-frozen) increased the total as well as (Na^+,K^+,Mg^{2+})-ATPase activity by some 3-fold, as compared to non-sonicated, non-frozen (control) membranes.

Provocative as these results were, it was of considerable importance to establish whether this phenomenon was associated with the human erythrocyte only, or whether it might be a rather general property of membranes prepared in this manner. Consequently, studies were extended to include the calf and the goat.

Previous observations in this laboratory showed that the adult cow erythrocyte membrane was quite fragile and unless calcium or magnesium salts were present during

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TABLE I

ATPase ACTIVITY OF HUMAN ERYTHROCYTE MEMBRANES AT VARIOUS STAGES OF PREPARATION IN 20 imosM BUFFER AT pH 7.6 AND 5.8★.

Erythrocytes were examined for total and (Na^+,K^+,Mg^{2+})-ATPase activity at different washing stages with 20 imosM Tris buffer. ATPase activity expressed as μmoles P_i released per ml packed cells per 2 h. Sensitive refers to that activity inhibitable by ouabain. NF, non-frozen; FT, frozen thawed. M.H. and D.M. are the particular subjects used in these experiments.

(A) pH 7.6

ATPase	*Original cells in isotonic Tris at pH 7.6*		*Membranes from*											
					Wash I				*Wash II*		*Wash IV*			
	NF		*FT*		*NF*		*FT*		*NF*	*FT*	*NF*		*FT*	
	M.H.	*D.M.*	*M.H.*	*D.M.*	*M.H.*	*D.M.*	*M.H.*	*D.M.*	*M.H.*	*M.H.*	*M.H.*	*D.M.*	*M.H.*	*D.M.*
Total	4.16	3.64	Not run	19.0	3.18	1.71	5.17	4.78	3.65	7.95	7.65	5.87	9.15	7.55
Insensitive	3.66	2.98	Not run	11.6	1.69	1.27	1.71	3.12	1.69	2.20	1.64	2.98	2.30	4.70
Sensitive	0.50	0.66	Not run	7.4	1.49	0.44	3.46	1.66	1.96	5.75	6.01	2.89	6.85	2.85

(B) pH 5.8 (M.H. only)

ATPase	*Original cells in isotonic Tris at pH 5.8*		*Membranes from*					
			Wash I		*Wash II*		*Wash IV*	
			NF	*FT*	*NF*	*FT*	*NF*	*FT*
Total	4.22	16.0	1.00	4.48	1.44	10.6	1.96	10.9
Insensitive	3.66	6.1	0.79	1.81	0.73	3.26	0.70	3.23
Sensitive	0.56	9.9	0.21	2.67	0.71	7.34	1.26	7.67

★Retention of total cholesterol and lipid phosphorus was very good in individual membrane preparations at pH 7.6, ranging in one typical experiment from 2.66 μmoles cholesterol and 4.2 μmoles lipid phosphorus per ml packed cells in the intact erythrocytes to 2.66 μmoles cholesterol and 3.7 μmoles lipid phosphorus in membranes obtained on a fourth hypotonic wash. Preliminary evidence on the pH 5.8 membranes showed good recovery of lipid.

TABLE II

ATPase ACTIVITY OF CALF ERYTHROCYTE MEMBRANES AT VARIOUS STAGES OF PREPARATION IN 20 imosM BUFFER AT pH 7.6 AND 5.8

These erythrocytes were obtained from Holstein calves and examined in a manner similar to that described for human erythrocytes in Table I (see text for further details). NF, non-frozen; FT, frozen and thawed. Wash I refers to the membranes obtained (by centrifugation) after suspending the intact erythrocytes in 20 imosM Tris buffer (20 T); Wash II refers to those membranes obtained after two such washes (and centrifugations) with 20 imosM buffer, *etc.*

(A) Age of animal, 7 days

ATPase activity	*Intact cells suspended in 20 T*		*pH 7.6*				*pH 5.8*			
			Wash I		*Wash IV*		*Wash I*		*Wash IV*	
	NF	*FT*	*NF*	*FT*	*NF*	*FT*	*NF*	*FT*	*NF*	*FT*
Total	8.10	10.30	6.70	7.15	6.56	5.80	5.30	6.40	3.50	3.42
Insensitive	3.02	4.90	1.97	2.96	1.85	1.47	1.51	2.86	1.40	1.52
Sensitive	5.08	5.40	4.73	4.19	4.71	4.33	3.79	3.54	2.10	1.90

(B) Age of animal, 14 days (pH 7.6 only)

ATPase activity	*Intact cells suspended in isotonic Tris*		*Wash I*		*Wash IV*	
	NF	*FT*	*NF*	*FT*	*NF*	*FT*
Total	2.10	11.0	6.22	6.36	6.30	4.87
Insensitive	1.94	5.47	2.67	2.85	2.37	2.40
Sensitive	0.16	5.53	3.55	3.51	3.93	2.47

(C) Age of animal, 28 days (pH 7.6 only)

ATPase activity	*Intact cells suspended in isotonic Tris*		*Wash I*		*Wash IV*	
	NF	*FT*	*NF*	*FT*	*NF*	*FT*
Total	1.47	8.62	3.65	3.65	3.50	3.52
Insensitive	1.26	6.17	2.10	2.44	1.86	2.31
Sensitive	0.21	2.45	1.55	1.21	1.68	1.21

hypotonic hemolysis, there was irreversible damage to the membrane with concomitant fragmentation[10]. The adult cow erythrocyte is a high Na^+-containing cell and has no detectable (Na^+,K^+,Mg^{2+})-activated ATPase. It was of some import then to see if the calf erythrocyte was different than the adult cow and consequently the ATPase activity in these cells was followed from birth of the calf until it reached 12 weeks of age. During the early part of the this study, Brewer *et al.*[8] presented preliminary evidence in a study on K^+ polymorphism in sheep erythrocyte ATPase that there was a high (Na^+,K^+,Mg^{2+})-ATPase activity in the newborn calf and negligible levels in the adult cow. This observation is certainly confirmed here. Even though our results on the developmental changes will be presented in detail elsehwere (D.J. Hanahan and J. Ekholm, unpublished observations), it should be noted that the erythrocytes of the newborn calf contained a high (Na^+,K^+,Mg^{2+})-ATPase activity, whereas the erythrocytes in the 11–12-week-old calf were essentially devoid of any (Na^+,K^+,Mg^{2+})-ATPase activity. A particularly fascinating result was that the (very young) calf cells are maximally altered on the first hemolysis wash, *i.e.* a sensitive ATP activity was obtained without freeze thawing of the membrane (Table II). Examination of the adult cow total ATPase activity showed that it was some 20-fold less than the calf and that there was absence of any (Na^+,K^+,Mg^{2+})-ATPase. Further, the data in Table II show that the calf cells are "open" (by the ouabain-sensitive ATPase activity) on the first pH 5.8, 20 imosM Tris buffer wash in contradistinction to that observed with human cells under similar conditions. Goat erythrocytes treated exactly the same as the human and calf gave results comparable to those observed for the calf. Data on the goat cells are presented in Table III.

These results and those of Bramley *et al.*[5] emphasize an important facet of membrane biochemistry, to which many investigators interested in solving the structure of the membrane, have apparently paid little attention. Specifically, the erythrocyte membrane obtained by osmotic lysis must be considered, at best, a first derivative of the intact erythrocyte membrane and that depending on the manner in which they are prepared, various configurations of membranes may result. Thus, membrane model building is an excellent approach if one is certain of the characteristics of the membrane under

TABLE III

ATPase ACTIVITY IN GOAT ERYTHROCYTE MEMBRANES AT VARIOUS STAGES OF PREPARATIO IN 20 imosM BUFFER, pH 7.6

Conditions and details are the same as described in Table II. All operations were done at pH 7.6.

	ATPase activity	*Intact cells in isotonic Tris*		*Wash I (20 T buffer)*		*Wash II (20 T buffer)*	
		NF	*FT*	*NF*	*FT*	*NF*	*FT*
Spanish (females)	Total	1.03	7.06	3.28	4.86	Not run	4.69
	Insensitive	0.79	2.88	1.51	1.73		1.64
	Sensitive	0.24	4.18	1.77	3.13		3.05
Angora (males)	Total	0.95	7.58	3.44	5.34	Not run	4.74
	Insensitive	0.68	3.02	1.27	1.61		1.53
	Sensitive	0.27	4.56	2.17	3.13		3.21

investigation and how it may relate to membrane preparation of other investigators. Our results and those of Bramley *et al.*[5] indicate some fascinating new dimensions to interpretation of the behavior and structure of the erythrocyte membrane and illustrate some of the problems associated with this type of investigation.

These studies also suggest the provocative possibility that hemoglobin may exert some effect, conceivably conformational in nature, on the behavior of these membranes. Further studies are underway to explore this idea.

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