Effect of Sulfhydryl Group Modification on Erythrocyte Membrane Adenosine Triphosphatase* (33507)

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Studies on heart muscle microsomes by Schwartz and Laseter (1) have shown a selective inhibitory effect of sulfhydryl reagents on the (Na + K)-activated ATPase. Weed and Berg (2) treated erythrocyte ghosts with chlormerodrin and found that under certain conditions the ATPase activation of sodium and potassium ions was completely abolished and the residual ATPase partially inhibited. On the other hand Skou and Hilberg (3) observed that blocking of sulfhydryl groups in ox brain microsomal preparations had a greater inhibitory effect on the ATPase in the absence of added alkali metal ions. These observations appear to indicate that in different membrane preparations there are different types of sulfhydryl groups with respect to their effects on membrane ATPase. We have used DTNB,[1] a reversible sulfhydryl-modifying reagent, on the intact red cell and its membrane fragments to study its effect on membrane ATPase and to localize the essential sulfhydryl groups. Preliminary results were reported earlier (4).

Methods. Human erythrocyte membranes were prepared according to the procedure of Dodge *et al.* (5). Hemoglobin-free membranes were subsequently fragmented and washed with 0.5 m*M* EDTA (Tris) and 2.5 m*M* imidazole (HCl), pH 7.6 (6). Membrane sulfhydryl groups modified by DTNB were determined spectrophotometrically (7). ATPase was determined as previously reported (6).

Results and Discussion. Table I shows that varying amounts of sulfhydryl groups reacted with DTNB under different conditions. When membrane fragments were solubilized in 1% (w/v) SDS and treated with 10 m*M* DTNB, the reaction reached a plateau in about 10 min. A total of 60 mμmoles of sulfhydryl groups/mg of membrane protein were found. This is in agreement with the finding of Sutherland and Pihl (9).

When erythrocyte membrane fragments were treated with DTNB directly a little less than half of the total sulfhydryl groups were modified in 30 min, and the reaction thereafter was very slow.

When intact cells were treated with DTNB only about 5% of the total sulfhydryl groups reacted. Since prolonging the incubation from 30 to 60 min. and doubling the concentration of DTNB to 20 m*M* did not result in a significant increase in groups that reacted, the data indicate that erythrocyte membrane is not permeable to DTNB under these conditions. This confirms the findings of VanSteveninck *et al.* (8) that only a small fraction of erythrocyte membrane sulfhydryl groups are accessible to reagents in the extracellular medium.

Table II shows that the ATPase in the control preparation and the preparation from DTNB-treated cells were both activated of sodium and potassium ions and in each case this activation was abolished by ouabain. It appears that the integrity of the "outer" sulfhydryl groups (about 5% of the total in the membrane) was not essential for the membrane ATPase. In contrast Table III shows that when membrane fragments were treated directly with DTNB the (Na + K)-activated ATPase was completely inhibited while ATPase in the absence of added sodium and potassium ions was not diminished. When the sulfhydryl modifying groups were removed by DTT, 80% of the (Na + K)-activated ATPase was recovered. Dick *et*

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[1] Abbreviations used are: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; PCMB, *p*-chloromercuribenzoate; and DTT, dithiothreitol.

TABLE I. Erythrocyte Membrane Sulfhydryl Groups Reacted with DTNB.

Condition	Sulfhydryl groups reacted (mμmoles/mg membrane protein)
Membrane fragments + 1% SDS + 10 m*M* DTNB (30 min)[a]	60
+ 10 m*M* DTNB (30 min)[b]	26
+ 10 m*M* DTNB (90 min)[b]	30
Erythrocytes + 10 m*M* DTNB (30 min)[c]	3.2
+ 20 m*M* DTNB (60 min)[c]	3.3

[a] Membrane fragments were solubilized in 1% SDS and then treated with DTNB (adjusted to pH 7.6 with Tris) in 25 m*M* Tris (HCl) buffer, pH 7.6, at 25°.

[b] Membrane fragments were treated with DTNB in the same buffer at 37°.

[c] Ten ml of washed erythrocytes were suspended in an equal volume of an isotonic solution containing 120 m*M* NaCl, DTNB and Tris (HCl) buffer, pH 7.6, and incubated at 37°. Excess reagent was eliminated by washing with 155 m*M* NaCl. Membrane fragments were prepared from DTNB-treated cell and the sulfhydryl groups that reacted with DTNB were determined as described in the text.

al. (10) have also observed complete loss of (Na + K)-activated ATPase in HK sheep erythrocyte membranes treated with cystine and other disulfides and the reversal of this inhibition by DTT.

It may be concluded that the sulfhydryl groups in the erythrocyte membrane which are specifically essential for the (Na + K)-activated ATPase are not accessible to DTNB in the extracellular medium. These groups can be modified by the reagent only subsequent to the fragmentation of the membrane. This is consistent with the findings of Sutherland *et al.* (11) in their studies on erythrocyte membrane sulfhydryl groups and cation permeability. They found that when only the "outer" groups were reacted with PCMB, there was no detectable change in erythrocyte cation transport. However, after several hours of incubation, PCMB slowly entered the cell and reacted with some of the less accessible membrane sulfhydryl groups and this resulted in a decrease of intracellular potassium. It is likely that membrane sulfhydryl groups which are essential for (Na + K)-activated ATPase are also indispensable for cation transport in the erythrocyte.

Summary. When intact human erythrocytes were treated with DTNB, about 5% of total membrane sulfhydryl groups were

TABLE II. ATPase in Membrane Preparations from DTNB-Treated Erythrocytes.

Erythrocyte DTNB treatment		ATPase (μmoles P_i released/mg of membrane protein per hr)[a]		
DTNB (m*M*)	Incubation time (min)	No addition	+ NaCl and KCl	+ NaCl, KCl, and ouabain
10	0	0.27	0.55	0.21
	30	0.33	0.54	0.20
	60	0.26	0.57	0.22
0	60	0.19	0.52	0.16
20	60	0.16	0.53	0.15

[a] Membrane fragments were prepared from the DTNB-treated cells and assayed for ATPase activity in a medium containing 1.25 m*M* ATP (Tris), 2.5 m*M* $MgCl_2$, 50 m*M* imidazole (HCl) buffer, pH 7.6, and additions in a final volume of 2.0 ml. When additions were made NaCl was 100 m*M*, KCl 25 m*M*, and ouabain 0.1 m*M*. ATPase incubation was at 37° for 60 min.

TABLE III. Reversible Effect of DTNB.

Expt.	Membrane preparation	ATPase (μmoles P_i released/mg of membrane protein per hr)[c]		
		No addition	+ NaCl and KCl	+ NaCl, KCl, and ouabain
1[a]	A (incubated with 10 m*M* DTNB)	0.18	0.17	0.16
	B (incubated without DTNB)	0.18	0.61	0.16
2[b]	A treated with DTT	0.14	0.52	0.12
	B treated with DTT	0.23	0.62	0.17

[a] Membrane fragments were treated with DTNB as described in Table I for 30 min. Excess reagent was removed by washing with EDTA-imidazole buffer.

[b] Both preparations A and B were treated with 10 m*M* DTT in 25 m*M* imidazole (HCl), pH 7.6, at 37° for 30 min. Excess reagent was removed by washing with EDTA-imidazole buffer.

[c] ATPase determination was described in Table II.

modified. No detectable changes were observed in the ATPase of the membrane fragments prepared from DTNB-treated cells. However, when membrane fragments were reacted directly with DTNB, about 50% of total sulfhydryl groups were modified and the (Na + K)-activated ATPase was completely abolished, while cation-independent ATP ase remained unchanged. About 80% of the (Na + K)-activation could be recovered by removing the sulfhydryl modifying groups with DTT.

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