J. Membrane Biol. 7, 285–312 (1972)

Modification of the Erythrocyte Membrane by Sulfhydryl Group Reagents

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Received 21 May 1971

Summary. In this study, the consequences of modification of human erythrocyte membrane sulfhydryl groups by N-ethyl maleimide (NEM), 5,5′dithiobis-(2-nitrobenzoic acid) (DTNB) and *p*-hydroxymercuriphenyl sulfonate (PHMPS) were investigated. These reagents differ in chemical reactivity, membrane penetrability and charge characteristics.

Results of sulfhydryl modification were analyzed in terms of inhibitory effects on activities of five membrane enzymes; Mg^{++}- and Na^+, K^+-ATPase, K^+-dependent and independent *p*-nitrophenyl phosphatase (NPPase) and DPNase. Structural considerations involved in the sulfhydryl-mediated inhibition were evaluated by studying the changes in susceptibility to sulfhydryl alteration produced by shearing membranes into microvesicles and by the addition of the membrane modifiers, Mg^{++} and ATP.

Conclusions from the data suggest that the effects of NEM appeared to result from modification of a single class of sulfhydryls; DTNB interacted with two different sulfhydryl classes. Increasing concentrations of PHMPS resulted in the sequential modification of many types of sulfhydryls, presumably as a result of increasing membrane structural disruption. DTNB and PHMPS caused solubilization of about 15% of membrane protein at concentrations giving maximal enzyme inhibition.

In contrast to the usually observed parallels between Na^+, K^+-ATPase and K^+-dependent NPPase, activities of Mg^{++}-ATPase, Na^+, K^+-ATPase and K^+-dependent NPPase varied independently as a result of sulfhydryl modification. We suggest complex structural and functional relationships exist among these components of the membrane ATP-hydrolyzing system.

Our studies indicate that the effects of sulfhydryl group reagents on these membrane systems should not be ascribed to sulfhydryl modification *per se*, but rather to the resulting structural perturbations. These effects depend upon the structural characteristics of the particular membrane preparation studied and on the chemical characteristics of the sulfhydryl group reagent used.

Sulfhydryl group modification has been shown to profoundly alter the biological properties of membranes including the permeability characteristics of heart mitochondria, fat cells and erythrocyte membranes [7, 15, 17,

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23, 27, 30] as well as the activity of membrane bound ATPases [31] and a respiratory-chain linked NADH dehydrogenase [10]. Despite the number and variety of such studies, the mechanism by which sulfhydryl reagents disrupt these membrane functions has thus far remained elusive, although the implicit conclusion has been that sulfhydryl group integrity is somehow required for biological function [11].

The interpretation of experiments based on sulfhydryl modification is subject to complexities arising from several sources. It has been shown, for example, that the extent of sulfhydryl modification of even a well characterized protein such as hemoglobin in aqueous solution is dependent upon the specificity of the particular reagent used as well as the experimental conditions. Under the same conditions, N-ethyl maleimide (NEM) modifies two sulfhydryl groups, *p*-chloromercuribenzene sulfonate reacts with 2–3, chlormerodrin modifies 6–7 groups, and inorganic mercury reacts with 8.5 [30]. A second factor is related to complexities which arise from the highly structured and compartmentalized nature of membrane systems. The observed effect of a given sulfhydryl reagent is the resultant of considerations relating to permeability and accessibility factors, introduction of charged groups within the membrane, and even gross alterations in membrane structure. Sulfhydryl group heterogeneity, which is presumed to determine the diverse effects of various sulfhydryl reagents on membrane properties even exists within classes of sulfhydryls reacting with the same reagent. Thus, it has been shown that a spin label closely related to NEM reacts with at least two different classes of sulfhydryls in bovine erythrocyte membranes [20]. Finally, it is clear that the differential reactivity of membrane sulfhydryls and the physiochemical consequences of their modification will depend in a crucial way upon the characteristics of the particular membrane preparation studied. It is therefore not surprising that the published data concerning the modifying effects of sulfhydryl reagents on membrane ATPase activities tend to be widely diverging and sometimes apparently contradictory, particularly regarding the relative susceptibilities of Mg^{++}-ATPase and Na^+, K^+-ATPase to inactivation [4, 22, 24, 28].

Despite the complexities involved, membrane sulfhydryl modification would offer a useful probe with which to investigate structural and functional aspects of membrane organization particularly if it were possible to separate those effects which are the result of generalized alterations in membrane structure from more selective or localized membrane perturbations at the level of specific enzyme activities. Study of the comparative behavior of various membrane-associated enzymes possessing greater or

lesser requirements of membrane organization might serve to dissociate specific enzyme attack from gross membrane perturbations.

In this study, the effects of three sulfhydryl reagents [NEM, *p*-hydroxymercuriphenyl sulfonate (PHMPS), and 5,5′dithiobis-(2-nitrobenzoic acid) (DTNB)] on enzyme activities associated with hemoglobin-free human erythrocyte membranes were investigated. These sulfhydryl reagents differed with respect to their membrane penetrability and in their mechanism of interaction with sulfhydryl groups. The enzyme activities studied were: Mg^{++}-ATPase and Na^+, K^+-ATPase, both of which require a high degree of membrane organization [1]; K^+-dependent NPPase, which is presumed to reflect the terminal K^+-dependent dephosphorylation step in Na^+ and K^+ mediated ATP hydrolysis, and which appears to be somewhat less dependent on overall membrane organization than ATPase [21]; and finally, nonspecific (K^+-independent) NPPase and DPNase, the activities of which persist following extensive membrane alteration [8, 9]. The effects of sulfhydryl modification on these enzymic activities were compared in both intact membranes and membrane vesicles produced by shearing whole membranes. These vesicles, of diameter less than 0.06 μ, were made from the intact membrane by shearing under pressure [21]. In such sheared membrane preparations, effects resulting from structural inhomogeneities in the intact membrane should be minimized. Studies were carried out in the presence and absence of the known membrane modifiers, ATP [2, 29] and divalent cations [29], to assess the role of overall membrane conformation in the observed effects. Finally, the effects of inhibitors were studied over a wide range of concentrations to permit a kinetic analysis of the resulting membrane effects, an approach which has not been extensively used in the study of sulfhydryl modification in membrane systems and which could yield valuable information regarding the complex mechanisms of interaction of inhibitors with membranes.

Materials and Methods

Erythrocyte Membrane Preparation

Hemoglobin-free erythrocyte ghosts were prepared from outdated human blood stored in acid-citrate-dextrose by the technique of a stepwise hypotonic lysis as already described [9]. Preparations had protein contents of 3 to 4.5 mg/ml as determined by the Lowry method [14] using 5 × recrystallized bovine serum albumin (Armour) as standard.

Membranes were sheared using a chilled Aminco French Press at a hydraulic pressure setting of 500 lb/in^2. This treatment resulted in the formation of microvesicles 0.06 μ or less in diameter [21].

Enzyme Assays

The ATPase and NPPase assays have been described in detail elsewhere [9]. Specific activities of ATPase (expressed as μmoles inorganic phosphate liberated per mg membrane protein/hr) were in the range of 0.5 to 0.7 for Mg^{++}-ATPase, and 0.3 to 0.5 for Na^+, K^+-ATPase. The specific activity of K^+-dependent NPPase was in the range of 30 to 50 nmoles *p*-nitrophenol liberated per mg membrane protein/hr. The K^+-independent NPPase activity was in the same order of magnitude as the K^+-dependent component. DPNase was assayed by the method of Kaplan [12] wherein residual DPN is measured as the cyanide ion complex.

Inhibition Studies

Preliminary experiments indicated that the reaction of NEM and PHMPS with the erythrocyte membrane was virtually immediate, whereas the DTNB reaction was only complete after 15 min at 23 °C. Therefore, all inhibitors were preincubated with erythrocyte membranes (containing 0.35 to 1.0 mg membrane protein in a volume of 1.0 ml) for 15 min at room temperature. Inhibitor and preincubation solutions were made up in unbuffered aqueous solution except in the case of DTNB. With this inhibitor, no reaction occurred below pH 7, so the inhibitor was made up and the incubation carried out in 0.1 M imidazole-glycylglycine, pH 7.4. DTNB is a particularly desirable reagent with which to study sulfhydryl modification, since the extent of reaction with sulfhydryls may be measured colormetrically. The mechanism of interaction of DTNB with free sulfhydryl groups proceeds via the formation of a mixed disulfide between the susceptible sulfhydryl group and 5-thio-2-nitrobenzoic acid (TNB), with simultaneous release of a molecule of free TNB into the medium [5]. The absorbance of the liberated TNB at 412 nm gives a measure of the extent of sulfhydryl modification.

In samples to be assayed for NPPase activity, it was necessary to first remove the TNB since this product had an absorption maximum in the same region as *p*-nitrophenol. It was found that removal of the TNB prior to assaying for membrane ATPase activity had no effect on the level of inhibition of ATPase activity resulting from DTNB attack. Therefore, following the preincubation with DTNB (in a 1.0 ml volume) 9 ml of cold 5 mM $MgCl_2$ were added and the membrane material sedimented by centrifugation at $30{,}000 \times g$ for 10 min. The addition of $MgCl_2$ minimized the solubilization of membrane components which may occur in low ionic strength solutions. The supernatant was carefully removed by aspiration, and the pellet was resuspended in 1.0 ml H_2O. Following the preincubation, 2.0 ml of the ATPase or NPPase assay medium were added [9], and samples were incubated for 60 min at 37 °C (with shaking). One ml of cold 20% trichloroacetic acid was added to stop the reaction and samples were centrifuged at $30{,}000 \times g$ for 5 min. Supernatants were decanted and assayed colorimetrically for inorganic phosphate or *p*-nitrophenol.

Analysis of Inhibition Data

Since the interaction of NEM and DTNB with the membrane resulted in covalent bond formation with membrane sulfhydryls, the usual mass action parameters for inhibition were not applicable. However, it was found that for each of the inhibitors studied, the maximal level of inhibition attained was determined by the concentration of inhibitor in the incubation medium. Therefore, since time dependence of inhibition by two of the three reagents (NEM and PHMPS) could not be conveniently measured

because of their rapidity of binding, the interaction of the three sulfhydryl group reagents with the membrane was measured in terms of the maximal inhibition (relative to appropriate controls) of various enzyme activities as a function of inhibitor concentration. In each case, the interaction was characterized by the I_{50} value (the concentration of inhibitor giving 50% inactivation of the activity in question) and the characteristics of the modified Hill plot, obtained by plotting the $\log \left[\frac{\text{percent residual activity}}{\text{percent inhibition}}\right]$ against log [inhibitor concentration]. Loftfield and Eigner [13] have shown that such plots may yield information regarding the number of different sites involved in the inactivation process and may also be indicative of the role of cooperative phenomena. It has been shown by Loftfield and Eigner that the interaction of irreversibly acting inhibitors such as iodoacetate may be analyzed in terms of such modified Hill plots.

A further complication derives from the fact that sulfhydryl group reagents interact with and bind to the membranes thereby reducing the amount of free reagents in solution. The use of total concentration of inhibitor added in kinetic equations would not be justified in such cases. In operational terms, relatively small amounts of NEM were bound and a constant fraction of PHMPS was bound (*see* Table 12). However, at the lowest concentrations of DTNB, virtually all of the inhibitor was membrane bound, and this observation was considered in the analysis of DTNB attack [*see* Table 8(a)].

It should be emphasized that because membrane preparations are known to vary, each of the inhibition studies was performed on a minimum of three sequential membrane preparations and a representative observation was not included in the Results section unless there was uniform conformation of experimental points and graphic analyses.

Solubilization of Membrane Protein

In a typical experiment, an aliquot of the erythrocyte membrane suspension (containing between 0.5 and 1.0 mg membrane protein) was preincubated with a given sulfhydryl inhibitor in a total volume of 1.0 ml for 15 min at 23 °C. The mixture was then centrifuged at $30{,}000 \times g$ for 5 min, and a 0.5-ml aliquot of the supernatant was assayed for protein content by the Lowry method [14]. Knowing the original quantity of protein added, it was possible to calculate the percentage of the total protein which was solubilized.

N-ethyl maleimide, *p*-hydroxymercuriphenyl sulfonic acid (monosodium salt), 5,5′-dithiobis (2-nitrobenzoic acid), Tris ATP and DPN were all obtained from the Sigma Chemical Company, and *p*-nitrophenyl phosphate (sodium salt) was obtained from Nutritional Biochemicals Corporation.

Results

Inhibition of N-Ethyl Maleimide (NEM)

Preincubation of erythrocyte membranes with NEM resulted in inhibition of ATPase activity with the Mg^{++}-dependent ATPase being somewhat more susceptible to inactivation than the Na^+, K^+-dependent ATPase, particularly in the low NEM concentration range [Table 1(a)]. The inactivation of the K^+-dependent NPPase paralleled that of the Mg^{++}-ATPase. The shearing of whole membranes into microvesicles approximately 0.06 μ in diameter resulted in an increase in susceptibility of Na^+, K^+-

Table 1. (a) Inhibition [a] of erythrocyte membrane ATPase and NPPase by NEM

Concentration of NEM in preincubation [b] ($\times 10^{-5}$ M)	Whole membranes (% inhibition)				Sheared membranes (% inhibition)			
	Mg^{++}-ATPase	Na^+, K^+-ATPase	K^+-depend. NPPase	K^+-independ. NPPase	Mg^{++} ATPase	Na^+, K^+-ATPase	K^+-depend. NPPase	K^+-independ. NPPase
10	28	22	19	33	13	22	33	41
20	18	31	38	46	26	20	44	41
30	39	30	45	50	40	41	48	45
50	50	29	55	71	46	44	56	53
100	60	32	64	68	56	52	61	61
200	79	42	66	83	76	63	63	75
300	83	49	64	75	79	73	65	70
400	83	56	61	85	79	78	69	87
500	84	60	68	81	77	80	69	83
1000	84	74	86	90	76	82	84	83

[a] Percent inhibition in all tables refers to $\left[\frac{\text{inhibited activity}}{\text{control activity}}\right] \times 100$.

[b] 15 min at 23 °C.

(b) NEM inhibition constants for ATPase and NPPase

	Whole membranes				Sheared membranes			
	Mg^{++}-ATPase	Na^+, K^+-ATPase	K^+-depend. NPPase	K^+-independ. NPPase	Mg^{++}-ATPase	Na^+, K^+-ATPase	K^+-depend. NPPase	K^+-independ. NPPase
I_{50} ($\times 10^{-5}$ M)	50	320	45	35	60	100	40	40
Hill interaction coefficient	0.9	0.8	0.7	0.6	0.9	0.8	0.6	0.4

Table 2. Effect of sulfhydryl modification on apparent K_m of membrane ATPase for ATP

Inhibitor	Concentration in preincubation [a]	K_m app (mM)			
		Mg^{++}-ATPase		Na^+, K^+-ATPase	
		(control) [b]	(inhibited)	(control)	(inhibited)
NEM	50×10^{-5} M	0.45	0.51	0.43	0.59
PHMPS	1.5×10^{-5} M	0.57	0.36	0.38	0.46
DTNB	2.0×10^{-5} M	0.40	0.62	1.25	1.43

[a] 15 min at 23 °C. Inhibitor concentrations approximating I_{50} values were chosen.
[b] Control preincubations for NEM and PHMPS carried out in aqueous solution, while the control for DTNB was preincubated in 0.15 M imidazole-glycylglycine buffer, pH 7.5.

ATPase to inactivation by NEM but had virtually no effect on the NEM inactivation of the Mg^{++}-ATPase and K^+-dependent NPPase activities. [Table 1(a) and (b)]. NEM modification did not appear to affect the affinity of either Mg^{++}- or Na^+, K^+-ATPase for ATP, since K_m apparent values for the substrate were not grossly changed as a result of NEM treatment (Table 2).

Inhibition Characteristics. The inhibition constants for the inactivation of Mg^{++}- and Na^+, K^+-ATPase and K^+-dependent NPPase are summarized in Table 1(b). The I_{50} values for Mg^{++}-ATPase and K^+-dependent NPPase activities were the same. However, the lesser susceptibility of the Na^+, K^+-ATPase to inhibition by NEM was reflected in higher I_{50} values in the whole preparation [Table 1(b)]. The inactivation of Mg^{++}-ATPase and Na^+, K^+-ATPase was characterized by a Hill plot of slope approximately equal to unity, while the inhibition of the K^+-dependent NPPase was characterized by a Hill interaction coefficient which was consistently lower, being in the order of 0.5.

Nonspecific Enzymes. It was found that the effects of NEM modification on the activity of K^+-independent NPPase essentially paralleled those on K^+-dependent NPPase [Table 1(a) and (b)]. In contrast to the behavior of K^+-independent NPPase, a concentration of NEM (375×10^{-5} M) giving rise to greater than 90% inhibition of Mg^{++}-ATPase resulted in only a 17% inhibition of the membrane DPNase (Table 3).

Effects of Membrane Modifiers. In the presence of 1 mM ATP, the extent of inactivation of Na^+, K^+-ATPase by NEM was essentially unchanged while the inhibition of Mg^{++}-ATPase (particularly at low concentrations of inhibitor) was decreased (Table 4). Substrate variation was not a factor

Table 3. Effect of sulfhydryl modification on DPNase activity of erythrocyte membranes [a]

Inhibitor	Concentration	Mg^{++}-ATPase [d]	DPNase [e]
Control [b]	—	1.24	69
NEM	375×10^{-5} M	0.09	57
PHMPS	15×10^{-5} M	0.07	63
Control [c]	—	1.27	69
DTNB	40×10^{-5} M	0.29	57

[a] Inhibitors were preincubated with erythrocyte membranes in a total volume of 4.0 ml at 23 °C for 15 min. Aliquots were then removed and assayed for ATPase and DPNase activity.
[b] Preincubated in aqueous solution.
[c] Preincubated in 0.15 M imidazole-glycylglycine buffer, pH 7.4.
[d] ATPase activities expressed as μmoles inorganic phosphate liberated per mg protein per hr.
[e] DPNase activities expressed as nmoles DPN degraded per mg protein per hr.

Table 4. Effect of ATP on inactivation of ATPase by NEM

Concentration of NEM in preincubation [a] ($\times 10^{-5}$ M)	% Inhibition			
	Mg^{++}-ATPase		Na^+, K^+-ATPase	
	(no ATP)	(1 mM ATP)	(no ATP)	(1 mM ATP)
10	15	6	17	20
20	30	16	27	23
50	54	39	37	31
100	70	56	35	33
500	79	70	53	48
1000	83	75	63	61

[a] 15 min at 23 °C.

Table 5. Effect of $MgCl_2$ (100 mM) on inhibition of Mg^{++}-ATPase and Na^+, K^+-ATPase

Inhibitor	I_{50} Value			
	Mg^{++}-ATPase		Na^+, K^+-ATPase	
	(no $MgCl_2$)	(100 mM $MgCl_2$)	(no $MgCl_2$)	(100 mM $MgCl_2$)
NEM	50×10^{-5} M	50×10^{-5} M	240×10^{-5} M	200×10^{-5} M
DTNB	0.8×10^{-5} M	0.8×10^{-5} M	3.7×10^{-5} M	1.7×10^{-5} M
PHMPS	1.8×10^{-5} M	2.4×10^{-5} M	1.7×10^{-5} M	1.5×10^{-5} M

in these experiments since the empirically determined optimal concentration of Mg^{++}-ATP (2 mM) was added to each tube during the enzyme assay. High concentrations of magnesium (100 mM) did not affect the relative

extent of inactivation of Mg^{++} or Na^+, K^+-ATPase (Table 5). It should be mentioned that the controls for this type of experiment containing 100 mM magnesium exhibited a substantial (60 to 70%) decrease in both Mg^{++}-ATPase and Na^+, K^+-ATPase activities (Table 5).

Inhibition by 5,5'Dithiobis-(2-Nitrobenzoic Acid) (DTNB)

The inactivation of membrane Mg^{++}- and Na^+, K^+-ATPase and K^+-dependent NPPase activity by DTNB occurred at concentrations one hundredth of those required to produce NEM inhibition (Tables 5 and 6). The Mg^{++}-ATPase was again more susceptible to inactivation as compared with the Na^+, K^+-ATPase. In this case, however, the inactivation of K^+-dependent NPPase paralleled that of the Na^+, K^+-ATPase instead of the Mg^{++}-ATPase [Table 6(a)]. Inhibition of Na^+, K^+-ATPase (Fig. 1) and K^+-dependent NPPase levelled off at approximately 50% inhibition whereas the Mg^{++}-ATPase could be inhibited over 90% by similar high concentrations of DTNB [Table 6(a)]. The difference in behavior between Mg^{++}-ATPase and Na^+, K^+-ATPase was also exhibited by a sheared microvesicle preparation. DTNB modification was not considered to cause any sub-

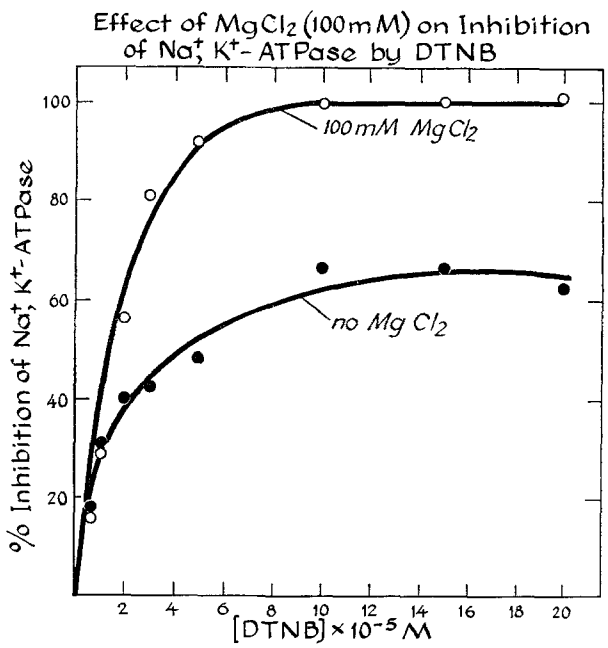

Fig. 1. The relative inhibition of Na^+, K^+-ATPase by increasing concentrations of DTNB is shown for whole membrane preparations preincubated with and without 100 mM $MgCl_2$

Table 6. (a) Inhibition of erythrocyte membrane ATPase and NPPase by DTNB

Concentration of DTNB in preincubation [a] ($\times 10^{-5}$ M)	Whole membranes (% inhibition)				Sheared membranes (% inhibition)	
	Mg^{++}-ATPase	Na^+, K^+-ATPase	K^+-depend. NPPase	K^+-independ. NPPase	Mg^{++}-ATPase	Na^+, K^+-ATPase
0.2	17	8	5	5	6	1
0.4	37	11	7	9	22	12
0.5	47	16	8	10	34	14
1.0	60	24	13	15	57	22
2.0	70	36	20	21	64	21
5.0	82	48	32	32	75	27
10.0	86	57	41	39	80	38
20.0	92	56	48	40	85	37

[a] 15 min at 23 °C.

(b) DTNB inhibition constants for ATPase and NPPase

	Whole membranes				Sheared membranes	
	Mg^{++}-ATPase	Na^+, K^+-ATPase	K^+-depend. NPPase	K^+-independ. NPPase	Mg^{++}-ATPase	Na^+, K^+-ATPase
I_{50} ($\times 10^{-5}$ M)	0.9	3.2	6.2	5.4	0.8	3.9
Hill interaction coefficients:						
First slope	1.5	0.8	0.7	0.7	1.8	0.4
Second slope	0.7	—	—	—	1.0	—

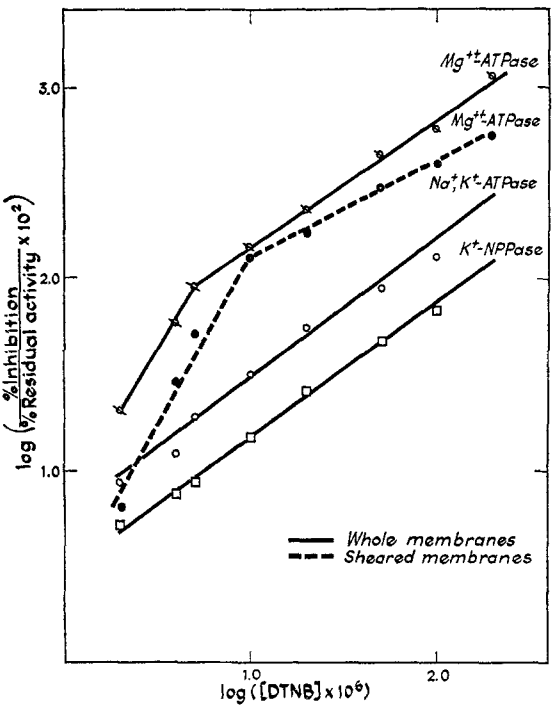

Fig. 2. Hill plot analysis of DTNB inhibition of Mg^{++}-ATPase, Na^+, K^+-ATPase and K^+-dependent NPPase in whole and sheared membranes

stantial alteration in the apparent K_m of Mg^{++}-ATPase or Na^+, K^+-ATPase for ATP (Table 2).

Inhibition Characteristics. Examination of the DTNB inhibition constants [Table 6(b)] indicated that the affinity of the Mg^{++}-ATPase for inhibitor was greater than the affinity of either the Na^+, K^+-ATPase or the K^+-dependent NPPase. The Hill plot (Fig. 2) for inactivation of Mg^{++}-ATPase consisted of two linear portions with a segment of decreasing slope occurring as the concentration of DTNB was increased. In contrast, the inhibition of Na^+, K^+-ATPase and K^+-dependent NPPase was characterized by a single line corresponding in slope to that characterizing the modification of Mg^{++}-ATPase activity at high concentrations of DTNB (Fig. 2).

Nonspecific Enzymes. The inhibitory effects of DTNB on the activity of K^+-independent NPPase paralleled the behavior of the K^+-dependent activity [Table 6(a) and (b)]. However, as with NEM, modification of membrane sulfhydryls by DTNB had a minimal effect on the activity of DPNase (Table 3).

Table 7. Effect of ATP on inactivation of ATPase by DTNB

Concentration of DTNB in preincubation[a] ($\times 10^{-5}$ M)	% Inhibition			
	Mg^{++}-ATPase		Na^+, K^+-ATPase	
	(no ATP)	(1 mM ATP)	(no ATP)	(1 mM ATP)
0.5	48	25	18	40
1.0	63	35	30	56
2.0	79	46	40	56
3.0	82	52	42	65
5.0	89	58	48	74
10.0	90	67	66	78
15.0	91	72	66	78
20.0	95	77	62	74

[a] 15 min at 23 °C.

Effects of Membrane Modifiers. In the presence of ATP, the DTNB-induced inactivation of Mg^{++}-ATPase was considerably decreased, while the inhibition of Na^+, K^+-ATPase was increased (Table 7).

The inactivation of Mg^{++}-ATPase by DTNB was unaltered by the presence of 100 mM $MgCl_2$ in the medium, whereas the inhibition of Na^+, K^+-ATPase by DTNB was altered in a striking manner in the presence of $MgCl_2$, as is shown in Fig. 1. The effects of $MgCl_2$ (100 mM) on the I_{50} values for inhibition of both Mg^{++}-ATPase and Na^+, K^+-ATPase by DTNB are shown in Table 5. In contrast to the pronounced effect of $MgCl_2$ on the inhibition of Na^+, K^+-ATPase, the inhibition of K^+-dependent NPPase by DTNB was not affected by 100 mM $MgCl_2$. The order in which membranes, $MgCl_2$ and DTNB were combined was found to be unimportant to the final effect on enzyme activity. This effect of $MgCl_2$ was also apparent at concentrations of 25 and 50 mM, but was optimal at 100 mM, the concentration used in all of these studies.

Since the interaction of DTNB with the membrane could be measured directly in terms of moles of sulfhydryl groups modified (*see* Materials and Methods), it was of interest to compare the modification of sulfhydryls in the absence and presence of Mg^{++}.

In the absence of Mg^{++}, modification of sulfhydryls increased with DTNB concentration [Table 8(a)]. Recalculation of these data in terms of moles DTNB added relative to moles incorporated indicated that, at low inhibitor concentrations, almost all the added DTNB was bound by the membrane. At intermediate DTNB concentrations, approximately 20 to

Table 8. (a) Modification of erythrocyte membrane sulfhydryl groups with DTNB in absence of magnesium

Column 1 Concentration of DTNB in preincubation [a] ($\times 10^{-5}$ M)	Column 2 nmoles TNB liberated per mg membrane protein	Column 3 nmoles TNB released by 2-ME [b]	Column 4 Stoichiometry of DTNB attack [c]
0.1	5.9	0.4	0.06
0.2	7.1	0.9	0.13
0.3	9.1	1.9	0.23
0.6	9.5	1.7	0.18
1.0	11.2	2.1	0.19
2.0	13.3	3.6	0.27
4.0	15.0	5.1	0.34
6.0	16.3	6.7	0.41
10.0	18.9	8.7	0.46
20.0	22.8	11.7	0.51
30.0	25.1	13.6	0.54

[a] 15-min preincubation at 23 °C.

[b] 2-ME: 2-mercaptoethanol.

[c] Expressed as the ratio of $\frac{\text{2 ME labile TNB}}{\text{initially liberated TNB}}$.

(b) Modification of erythrocyte membrane sulfhydryl groups with DTNB in presence of $MgCl_2$ (100 mM)

Column 1 Concentration of DTNB in preincubation [a] ($\times 10^{-5}$ M)	Column 2 nmoles TNB liberated per mg membrane protein	Column 3 nmoles TNB released by 2-ME	Column 4 Stoichiometry of DTNB attack [b]
0.1	6.1	1.4	0.23
0.2	6.7	1.7	0.25
0.3	7.5	1.8	0.24
0.6	8.8	1.9	0.22
1.0	12.7	3.7	0.29
2.0	18.9	5.9	0.31
4.0	26.3	11.5	0.44
6.0	28.7	14.9	0.52
10.0	34.3	18.5	0.54
20.0	39.0	23.7	0.61
30.0	41.6	26.5	0.64

[a] 15-min preincubation at 23 °C.

[b] Expressed as the ratio of $\frac{\text{2 ME labile TNB}}{\text{initially liberated TNB.}}$.

(c) DTNB modification of membrane sulfhydryl groups in the presence of divalent cations

Concentration of DTNB in preincubation [a]	Cation	Concentration	nmoles TNB liberated/ mg protein
2×10^{-5} M	None	—	21.3
2×10^{-5} M	Mg^{++}	50 mM	31.8
2×10^{-5} M	Ca^{++}	50 mM	32.8
2×10^{-5} M	Ba^{++}	50 mM	32.8

[a] 15-min preincubation at 23 °C.

40% was bound, and at high concentrations, 3 to 7% of the added DTNB was bound to the membranes.

In the presence of $MgCl_2$ (100 mM) the modification of membrane sulfhydryls at low concentrations of DTNB (less than 1.0×10^{-5} M) was the same as that in the absence of Mg^{++}. However, at concentrations of DTNB in excess of 2.0×10^{-5} M, the addition of $MgCl_2$ resulted in a substantial increase in the susceptibility of sulfhydryls to attack by DTNB [Table 8(a) and (b)].

Treatment of DTNB-modified membranes with a thiol such as 2-mercaptothanol would be expected to liberate one molecule of TNB for each TNB initially liberated as a result of DTNB attack [5]. In the absence of the $MgCl_2$, stoichiometry of DTNB attack (expressed as the ratio of TNB released from the DTNB-treated membrane by 2-mercaptothanol, to the TNB initially released as a result of DTNB attack on the membrane) was consistently less than 1 [Table 8(a)].

In the presence of Mg^{++}, the increased DTNB attack on membrane sulfhydryls was paralleled by an increase in the incorporation of 2-mercaptoethanol-labile TNB into the membrane [Table 8(b)]. We therefore decided to test the hypothesis that in the presence of Mg^{++}, DTNB reacted with membrane sulfhydryls predominantly in 1:1 stoichiometry.

The increase in TNB liberation on treatment of membranes with DTNB in the presence of 100 mM $MgCl_2$ was plotted against the Mg^{++}-stimulated increase in incorporation of 2-mercaptoethanol labile TNB into the membrane. A line with a slope of 1 at concentrations of DTNB greater than 1.0×10^{-5} M was obtained (Fig. 3). The increased modification of membrane sulfhydryls by DTNB in the presence of Mg^{++} did not appear to result

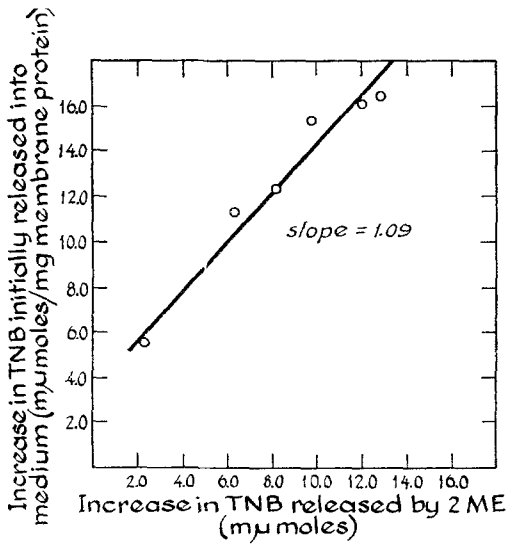

Fig. 3. The effect of addition of 100 mM $MgCl_2$ on the increase in TNB initially liberated by DTNB attack on erythrocyte membranes (nmoles/mg membrane protein) is plotted against the Mg^{++}-induced increase in incorporation of 2-mercaptoethanol-labile TNB into membranes. Concentration range of DTNB used was 2.0 to 30.0×10^{-5} M. The data for the abscissa were obtained by subtracting values in Table 8(a), Column 3, from corresponding values in Table 8(b), Column 3. Similarly, the data for the ordinate were obtained by subtracting values in Table 8(a), Column 2, from corresponding values in Table 8(b), Column 2

from specific ionic interaction, since essentially the same effect was observed with two other divalent cations [Ca^{++} and Ba^{++}, Table 8(c)] at equivalent concentrations.

Inhibition by PHMPS

The inhibition of membrane Mg^{++}- and Na^+, K^+-ATPase and K^+-dependent NPPase activities by PHMPS exhibited a complexity not encountered with the other two inhibitors studied. While the extent of inhibition of Mg^{++}-ATPase by PHMPS was consistent in all erythrocyte membrane preparations studied, the pattern of inhibition of Na^+, K^+-ATPase was extremely variable. This variability was particularly pronounced at concentrations of inhibitor below 3×10^{-5} M where apparent stimulation of Na^+, K^+-ATPase was occasionally observed. When the extent of inhibition of Na^+, K^+-ATPase was plotted against increasing concentrations of PHMPS, the points visually appeared to fall into two categories [Fig. 4(*a*), curves *1* and *2*] which coincided at high concentrations of inhibitor. Shearing of membranes into microvesicles appeared to resolve these two apparent

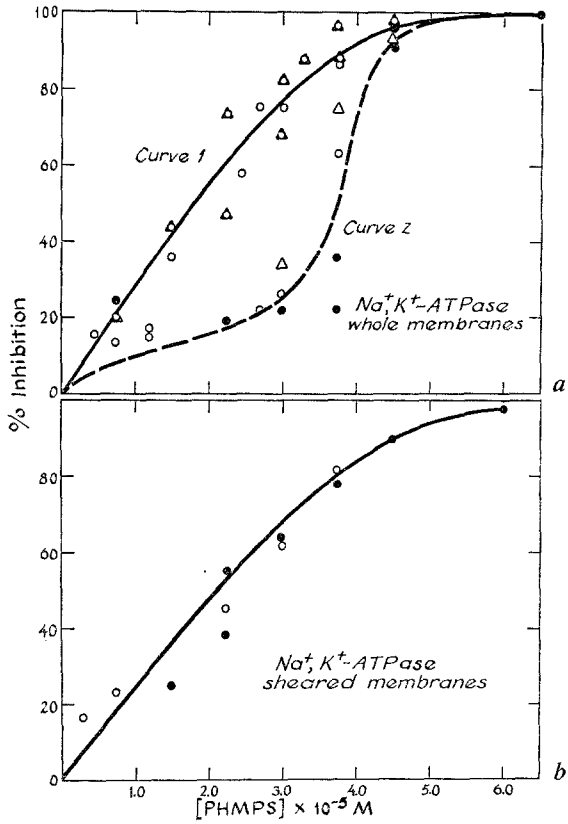

Fig. 4. (*a*) Inhibition by PHMPS of Na^+, K^+-ATPase in several whole membrane preparations. (Each symbol denotes a different membrane preparation.) (*b*) Effect of shearing on inhibition of Na^+, K^+-ATPase by PHMPS. [Symbols the same as in (*a*).]

patterns of Na^+, K^+-ATPase of unsheared membranes [Fig. 4(*a*), curve *1*]. In contrast, shearing had no effect on the susceptibility of Mg^{++}-ATPase to inhibition by PHMPS [Table 9(a)]. The K_m apparent values of Mg^{++}- and Na^+, K^+-ATPase for ATP were not altered by PHMPS modification (Table 2).

Inhibition Characteristics. A plot of reciprocal percent residual Mg^{++}-ATPase activity versus concentration of PHMPS [Fig. 5(*a*)] was linear at low concentrations of inhibitor (extrapolating to an I_{50} value of 1.5×10^{-5} M); however, as inhibitor concentration was increased, a sharply inflecting curve of increasing slope was obtained. Hill plot analysis [Fig. 5(*b*)] suggested initial attack of inhibitor at a single type of membrane site (slope = 1.1) followed by more extensive attack of a second inhibitor

Table 9. (a) Inhibition of Mg^{++}-ATPase and NPPase by PHMPS

Concentration of PHMPS in preincubation[a] ($\times 10^{-5}$ M)	Whole membranes (% inhibition)			Sheared membranes (% inhibition)		
	Mg^{++}-ATPase	K^+-depend. NPPase	K^+-independ. NPPase	Mg^{++}-ATPase	K^+-depend. NPPase	K^+-independ. NPPase
0.30	8	5	14	8	6	12
0.75	19	29	22	20	29	21
1.50	51	31	41	52	29	39
2.25	68	48	49	68	59	44
3.00	80	55	73	87	44	63
3.75	90	78	92	87	60	90
4.50	95	90	98	87	87	99

[a] 15-min preincubation at 23 °C.

(b) PHMPS inhibition constants for ATPase and NPPase

	Whole membranes				Sheared membranes			
	Mg^{++}-ATPase	Na^+, K^+-ATPase[a]	K^+-depend. NPPase	K^+-independ. NPPase	Mg^{++}-ATPase	Na^+, K^+-ATPase[b]	K^+-depend. NPPase	K^+-independ. NPPase
I_{50} ($\times 10^{-5}$ M)	1.5	1.6	2.3	2.3	1.5	2.1	2.3	2.6
Hill interaction coefficients:								
First slope	1.1	1.2	1.3	1.0	1.2	1.4	1.3	1.0
Second slope	2.0	2.2	2.1	1.6	1.9	2.1	2.1	1.6
Third slope	4.0	4.4	8.0	8.3	2.4	5.2	8.0	8.3

[a] These values for Na^+, K^+-ATPase were derived from curve 1, Fig. 4(*a*).
[b] These values for Na^+, K^+-ATPase were derived from Fig. 4(*b*).

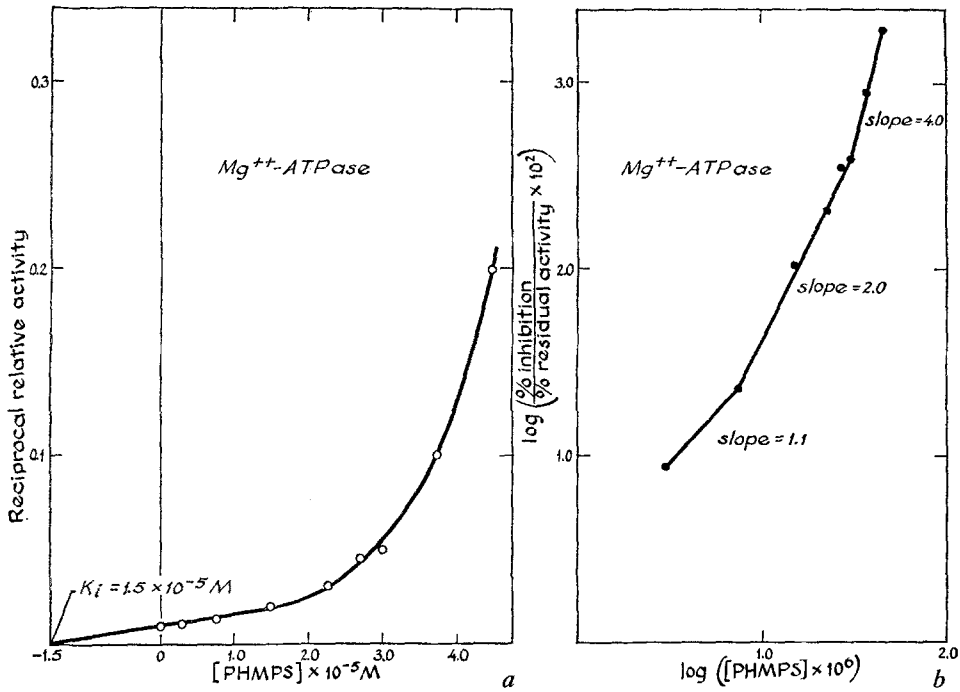

Fig. 5. (*a*) Concentration dependence of PHMPS-induced inhibition of whole membrane Mg^{++}-ATPase. Reciprocal Mg^{++}-ATPase activity is plotted against inhibitor concentration [Table 9(a)]. (*b*) Hill plot analysis of whole membrane Mg^{++}-ATPase inhibition by PHMPS [Table 9(a)]

Table 10. Reversal of PHMPS inhibition by 2-mercaptoethanol

Concentration of PHMPS in preincubation[a] ($\times 10^{-5}$ M)	% Inhibition total ATPase	% Reversal by 1 mM 2-mercapto-ethanol
0.30	12	100
0.75	25	100
1.50	31	88
2.25	37	77
3.75	78	56

[a] 15 min at 23 °C.

resulting ultimately in complete inactivation of enzyme. Parallel behavior was exhibited by Na^+, K^+-ATPase and K^+-dependent NPPase [Table 9(b)].

The reversibility of PHMPS inactivation of ATPase by 2-mercapto-ethanol at various levels of inhibition was examined (Table 10). At lowest

Table 11. Effect of ATP on inactivation of ATPase by PHMPS

Concentration of PHMPS in preincubation[a] ($\times 10^{-5}$ M)	% Inhibition			
	Mg^{++}-ATPase		Na^+, K^+-ATPase	
	(no ATP)	(1 mM ATP)	(no ATP)	(1 mM ATP)
0.75	12	25	20	30
1.50	43	49	44	48
2.25	63	72	73	64
3.00	78	75	82	87
3.75	88	95	88	95

[a] 15 min at 23 °C.

concentrations of inhibitor, the inhibition was fully reversible on incubation with 2-mercaptoethanol (1 mM). At higher concentrations of inhibitor, where Hill plot slopes exceeded unity, the enzyme inactivation was no longer completely reversible so that an approximately inverse relationship existed between percent inhibition and percent reversibility.

Nonspecific Enzymes. As with the other two inhibitors studied, the pattern of inactivation of K^+-independent NPPase paralleled the inhibition of the K^+-dependent component [Table 9(a) and (b)], and under conditions where Mg^{++}-ATPase was more than 90% inactivated as a result of PHMPS treatment, the activity of the membrane DPNase was minimally affected (Table 3).

Effect of Membrane Modifiers. Neither the presence of ATP (Table 11) nor of $MgCl_2$ (Table 5) had any significant effect on the inactivation of Mg^{++}-ATPase and Na^+, K^+-ATPase by PHMPS.

Sulfhydryl Modification and Protein Solubilization

Since DTNB exhibits a high degree of selectivity for interaction with sulfhydryl groups, the sulfhydryl titer of membranes treated with NEM and PHMPS was measured using the DTNB method in order to show that indeed NEM and PHMPS were reacting with sulfhydryl groups in the present system. The results shown in Table 12 indicate, as expected, a decreasing sulfhydryl content with increasing concentrations of both inhibitors.

The data in Table 12 could be used to calculate the proportion of added inhibitor bound to the membrane at each particular concentration, as was done for DTNB using the data in Table 8(a). In the case of NEM, the proportion of bound NEM relative to the total concentration was

Table 12. (a) Membrane sulfhydryl content following treatment with NEM

Concentration of NEM in preincubation [a] ($\times 10^{-5}$ M)	Sulfhydryl content [b] nmoles/mg protein
0	13.0
1	10.0
2	8.4
5	5.2
10	4.7
20	4.5
50	4.2
100	4.0
200	4.2
500	3.8
1000	4.2

(b) Membrane sulfhydryl content following treatment with PHMPS

Concentration of PHMPS in preincubation [a] ($\times 10^{-5}$ M)	Sulfhydryl content [b] nmoles/mg protein
0	13.9
0.30	13.3
0.75	12.2
1.50	10.6
2.25	9.6
3.00	7.4
3.75	6.5
4.50	4.0

[a] 15 min at 23 °C.
[b] Measured using DTNB colorimetric assay (*see* Materials and Methods).

always low, amounting to no more than 15% at the lowest NEM concentrations used in the inhibition studies, to 0.05% at the highest NEM concentration. The linearity of all Hill plots for NEM inhibition over the entire range of inhibitor concentrations tested indicates that these effects do not affect the kinetics of inhibition. On the other hand, with PHMPS, it was found that the proportion of inhibitor bound at each concentration represented a constant fraction ($15 \pm 1\%$) of the total concentration of inhibitor added. This effect, which stood in marked contrast to the behavior of NEM and DTNB, may perhaps be best explained by a

Table 13. Solubilization of erythrocyte membrane protein by NEM, DTNB and PHMPS

Inhibitor	Concentration in preincubation [a] ($\times 10^{-5}$ M)	Solubilization of membrane protein (%)	
NEM	10	0	
	20	0	
	50	0	
	100	0.9	
	200	0.7	
	500	0	
	1000	0.7	
		No Mg	100 mM Mg
DTNB	0.20	0	0
	0.50	0	0
	1.00	1.2	2.2
	2.00	0.8	1.2
	5.00	3.1	4.7
	10.00	8.2	9.5
	20.00	14.5	15.1
PHMPS	0.30		0.1
	0.75		0.7
	1.50		2.3
	2.25		3.7
	3.00		7.3
	3.75		10.5
	4.50		12.4

[a] 15 min at 23 °C.

partition of PHMPS between the outer aqueous phase and the inner membrane phase, such that the amount of PHMPS within the membrane matrix represents a constant fraction of the concentration of reagent in the outer phase. However, since this represented a constant component at each inhibitor concentration, no effect on the Hill plot behavior would be expected.

Sulfhydryl modification by DTNB and PHMPS would be assumed to result in enhancement of negative charge in certain regions of the membrane. Therefore, it was possible that such treatment could give rise to membrane instability, resulting in the solubilization of membrane protein fragments. Increasing concentrations of DTNB and PHMPS were found to result in progressive solubilization of membrane protein, whereas the neutral NEM molecule did not appear to exhibit such behavior in the concentration range studied (Table 13).

Discussion

The net effect of a given sulfhydryl-modifying reagent on the properties of a complex macromolecular structure such as the erythrocyte membrane will be the resultant of the combination of at least three factors: (1) the accessibility of the reagent to the various potential sites of attack. This is determined by chemical and stereochemical factors, as well as the ability of the reagent to penetrate the membrane; (2) the chemical reactivity of the molecule, and finally, (3) the perturbation induced in the structure of the membrane as a result of the modification. In the present studies, sulfhydryl group reagents of differing chemical characteristics were chosen to evaluate these different aspects of sulfhydryl modification.

Role of Gross Membrane Structure

The variability of action of sulfhydryl inhibitors arising from gross membrane accessibility features can be analyzed by comparing the consequences of sulfhydryl modification on the inactivation of membrane enzymes in whole and sheared membranes. The inactivation of Mg^{++}-ATPase and K^+-dependent NPPase by the three sulfhydryl reagents studied were unaffected by shearing into microvesicles. However, the inactivation of Na^+, K^+-ATPase by NEM and PHMPS was enhanced by shearing [Table 1(a) and Fig. 4] while the DTNB-induced inhibition of this activity was unaffected by shearing [Table 6(a)]. Since PHMPS and DTNB are both aromatic anions of low membrane penetrability [3, 11], the differing effects of these two reagents were somewhat surprising. This observation, together with the finding that sheared membranes are more susceptible to NEM attack despite the fact that erythrocyte membranes have been shown to be freely permeable to NEM [11] suggests that considerations other than gross accessibility factors determine the effects of some sulfhydryl agents.

Chemical Aspects of Sulfhydryl Modification

The different inhibitory patterns exhibited by each of the three sulfhydryl reagents studied here suggest that these effects are related to the structure and chemical reactivity of each molecule. The affinities of DTNB and PHMPS (as estimated from I_{50} values for inhibition of ATPase or NPPase) were of the order of 10^{-5} M, whereas the affinity for NEM was 100-fold lower (Table 5). This difference may arise in part from the fact

that while both DTNB and PHMPS are aromatic anions, NEM is neutral and non-aromatic. However, since the interaction of DTNB (and NEM) with membrane sulfhydryls presumably involves formation of a covalent linkage, considerations of chemical reactivity may also be important to an understanding of the comparative behavior of the three inhibitors. Hill plot analysis of the effects of sulfhydryl modification on membrane enzyme activities suggests that a different type of interaction mechanism was operative for each of the inhibitors studied.

The modification of membrane enzyme activities by NEM appeared to be associated with a single type of interaction, since Hill plots for Mg^{++}-ATPase and Na^+, K^+-ATPase and K^+-dependent NPPase were linear over a 100-fold concentration range [Table 1(b)]. It is known that the specificity of NEM for sulfhydryl groups is not absolute, and reaction with amino and imidazole groups may also occur under special conditions [26]. Our observation that increasing concentrations of NEM led to a parallel decrease in sulfhydryl groups detectable by DTNB [Table 12(a)] is consistent with, but does not prove, the involvement of sulfhydryl groups in the effects of NEM on ATPase and NPPase.

In contrast, the Hill plot for inactivation of Mg^{++}-ATPase by DTNB was biphasic, consisting of an initial linear segment of slope 1.5 at low concentrations of inhibitor, followed by a second segment of slope 0.7 at higher concentrations of DTNB (Fig. 2).

The avid binding of DTNB at low inhibitor concentrations [as calculated from the data in Table 8(a)] suggests the involvement of high affinity membrane sulfhydryl sites. These sites would be characterized by an I_{50} (for Mg^{++}-ATPase inhibition) of approximately 10 μM. Another category of sites, associated with inhibition of Na^+, K^+-ATPase and NPPase (K^+-dependent and independent) as well as Mg^{++}-ATPase at higher DTNB concentrations, would be characterized by I_{50} values between 30 and 60 μM, and would account for the approximately equivalent Hill plot slopes associated with all these inhibitory processes. It might be argued that the deviation at low concentrations of DTNB in the Hill plot for inhibition of the Mg^{++}-ATPase in both sheared and unsheared membranes could in some way result from the fact that most of the DTNB is bound to the membranes and little would remain free in solution. However, this would appear unlikely since no deviation is observed in the inhibition of Na^+, K^+-ATPase and NPPase at these same low concentrations of DTNB (Fig. 2). Therefore, it would seem most reasonable to explain the biphasic nature of the Mg^{++}-ATPase inhibition Hill plots in terms of different types of sulfhydryl sites. Further evidence for two distinct types

of sites came from the striking differences in stoichiometry of DTNB attack at high and low inhibitor concentrations. Thus, modification of sulfhydryls at low concentrations of DTNB was characterized by a low recovery of TNB presumably incorporated into the membrane [Table 8(a)]. This finding could be explained by attack of two molecules of DTNB on a pair of proximal sulfhydryls, accompanied by liberation of two molecules of TNB [6, 18]. At higher concentrations of DTNB, the increased incorporation of TNB into the membrane presumably reflected more usual DTNB modification involving the formation of stable mixed disulfides at single sulfhydryl sites [5].

In sharp contrast to the effects of DTNB, the modification of Mg^{++}-ATPase, Na^+, K^+-ATPase and K^+-dependent NPPase activities by PHMPS involved sequential modification of two types of sulfhydryl sites, and this was followed by extensive modification of membrane sulfhydryl sites [Table 9(b) and Fig. 5(*b*)]. Modification of the initial sulfhydryl site was associated with minimal disruption of membrane structure, since the process was completely reversible with 2-mercaptoethanol. Incorporation of subsequent inhibitor molecules was associated with increasingly irreversible membrane structural alterations (Table 10). The question arose as to whether this lack of reversibility might reflect reaction of PHMPS with groups other than sulfhydryls. However, the DTNB detectable content of membrane sulfhydryls decreased following exposure of membranes to increasing concentrations of PHMPS [Table 12(b)], and when the reciprocal of this sulfhydryl content was plotted against concentration of PHMPS, a curve identical in shape to the I_{50} plot in Fig. 5(*a*) was obtained, wherein at concentrations of inhibitor greater than 1.5×10^{-5} M exponential deviation from linearity was seen.

Therefore, while the effects of NEM, DTNB and PHMPS on membrane ATPase and NPPase activities all appeared to be due primarily to sulfhydryl modification, the mechanism of interaction of each inhibitor with the membrane was distinctly different.

Parallelisms between Na^+, K^+-ATPase and K^+-Dependent NPPase

It has been proposed that the K^+-dependent NPPase activity associated with Na^+, K^+-ATPase preparations reflects the terminal K^+-dependent dephosphorylation of an enzyme acyl phosphate intermediate during ATP hydrolysis [1]. As such, it was of interest to study the parallel behavior of these two activities and of the Na^+- and K^+-independent component

of ATPase activity (Mg^{++}-ATPase) as a result of sulfhydryl modification. In the presence of DTNB, Na^+, K^+-ATPase and K^+-dependent NPPase were inactivated to the same extent and to a lesser degree than Mg^{++}-ATPase [Table 6(a) and (b)]. However, in the case of PHMPS, Mg^{++}-ATPase, Na^+, K^+-ATPase and K^+-dependent NPPase were all inactivated in parallel fashion [Table 9(a) and (b)]. Surprisingly, the inhibition of K^+-dependent NPPase by NEM did not parallel Na^+, K^+-ATPase but rather Mg^{++}-ATPase inactivation [Table 1(a) and (b)]. These results might have some bearing on the proposal of Banerjee and Sen [3] that discreet types of sulfhydryl groups are involved in phosphorylation and dephosphorylation. More generally, our findings are suggestive of complex interrelationships between the components of the membrane ATP hydrolyzing system.

Generalized Membrane Alterations

The activities of nonspecific NPPase and DPNase, for which no biological function within the membrane has been proposed, were affected to different extents by sulfhydryl modification. DPNase activity was minimally affected by the three reagents studied (Table 3). Since the loss of this enzyme activity has been used as a measure of gross membrane disruption [8], it is clear that the structural perturbations induced by sulfhydryl modification are relatively selective in nature. It was interesting to find that the behavior of nonspecific (K^+-independent) NPPase paralleled exactly that of K^+-dependent NPPase (Tables 1, 6 and 9) with all the sulfhydryl inhibitors studied. This contrasted strikingly with the effects of carbodiimide modification of the erythrocyte membrane, which selectively inactivated the K^+-dependent component of NPPase activity [9]. The apparently similar inactivation of K^+-dependent and K^+-independent NPPases as a result of sulfhydryl modification is suggestive of generalized membrane effects resulting from perturbation of discreet regions within the membrane which may bear some relationship to *p*-nitrophenylphosphate binding sites.

The solubilization of membrane protein by DTNB and PHMPS but not NEM may have been due to the enhancement of negative charges at certain strategic sites on the membrane (Table 13) and as such would bear a relationship to the solubilization of membranes by chemical agents such as succinic anhydride [19]. The recent findings of Smith and Verpoorte [25], who showed that *p*-chloromercuribenzoate treatment and high pH also give rise to erythrocyte membrane protein solubilization are also consistent

with this hypothesis. These solubilization studies offer direct evidence of the disruptive effect of certain sulfhydryl group reagents on membrane structure.

Effects of Membrane Modifiers – $MgCl_2$ and ATP

In contrast to the gross alterations in membrane structure induced by shearing, the effects of membrane modifiers such as Mg^{++} and ATP are of a more subtle nature. Mg^{++} and ATP have been shown to play a complex and incompletely understood role in determining such membrane properties as deformability and susceptibility to trypsin digestion [16, 29]. The modifying effects of ATP and $MgCl_2$ on the inactivation of ATPase by sulfhydryl reagents depended upon the particular sulfhydryl reagent used, and there was a surprising independence of effects on Mg^{++}-ATPase and Na^+, K^+-ATPase. Thus, while addition of ATP did not alter the inhibition of Na^+, K^+-ATPase by the three inhibitors, the inactivation of Mg^{++}-ATPase by DTNB was substantially decreased (20 to 30%, Table 7).

On the other hand, the addition of 100 mM $MgCl_2$, strikingly increased the extent of inactivation of Na^+, K^+-ATPase by DTNB (Table 5 and Fig. 1) and this effect was associated with an enhanced incorporation of TNB into the membrane [Table 8(a) and (b)]. The effects of NEM and PHMPS were unaltered by the presence of $MgCl_2$ (Table 5). The rather selective membrane alteration induced by DTNB in the presence of 100 mM $MgCl_2$ probably resulted in perturbation of certain membrane components in some way involved in Na^+ and K^+ transport, and as such would warrant further study.

Thus, it is clear from our studies that the effects of sulfhydryl group reagents on membrane systems cannot be simply ascribed to sulfhydryl modification *per se*, but also to the disruptive effects of such modification on membrane structure. This would suggest that the native structure or configuration of the unmodified membrane should also determine the effect of a given sulfhydryl group reagent on membrane properties. The present investigations appear to indicate that this is indeed the case, and offer an explanation for the difficulties which have been encountered in attempting to interpret in a meaningful way the results of sulfhydryl modification studies wherein a variety of sulfhydryl reagents and membrane preparations were employed. Until a deeper understanding of membrane structure in terms of functional properties is obtained, the use of sulfhydryl modification as a probe to investigate molecular aspects of membrane pheonomena may well be of limited value. However, the comparative study of sulfhydryl-modifying agents with various chemical specificities, utilizing similar

analytical procedures as have been described here, could provide a useful tool to investigate the particular biochemical characteristics of a given membrane enzyme system (such as ATPase) as it occurs in a variety of functionally specialized tissues.

We gratefully acknowledge the helpful suggestions made by Drs. George Ellmar and George Stark in the preparation of this manuscript. D.V.G. is a postdoctoral fellow supported by the National Research Council of Canada.

This work was supported by the National Institutes of Health Grant AM 13682.

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Errata

M. Kasai and J.-P. Changeux. *In Vitro* Excitation of Purified Membrane Fragments by Cholinergic Agonists. *J. Membrane Biol.*, Vol. 6, pp. 1–88, 1971.

p. 38: The value for the permeability of the liposomes of 3.3×10^{-13} has been measured with K not with Na as indicated on the Table.

p. 60, line 16: should read 8.1×10^{-8} moles of AcChE *per liter* instead of per g protein.

p. 74, line 29: should read 10 to 4×10^5 ml/sec instead of ml · sec.

p. 77: the second equation should read

$$\langle S \rangle \left(\frac{k_S - k_T}{k_T + k_L} \right) \quad \text{instead of} \quad \langle S \rangle \left(\frac{k_S - k_T}{k_T + k_L} \right) - 1.$$