MOLECULAR PHARMACOLOGY, 9, 802-819

Studies on the Membrane-Perturbational Effects of Drugs and Divalent Cations Utilizing Trinitrobenzenesulfonic Acid

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(Received May 16, 1973)

SUMMARY

GODIN, DAVID V., AND WAN NG, THERESE: Studies on the membrane-perturbational effects of drugs and divalent cations using trinitrobenzenesulfonic acid. *Mol. Pharmacol.* **9**, 802-819 (1973).

This study represents an inquiry into the molecular mechanisms whereby the interaction of divalent cations and certain cationic drugs with the erythrocyte membrane produces a marked increase in the modification of membrane amino groups by trinitrobenzenesulfonate (TNBS). A mechanism based on simple neutralization of membrane anionic sites by activator cations was untenable because the incorporation of picryl chloride, an uncharged analogue of TNBS, was also markedly increased in the presence of magnesium or chlorpromazine. Rather, it is suggested that the interaction of activators with membrane components induces configurational changes which alter the accessibility and/or reactivity of membrane protein and phospholipid amino groups. These configurational changes, which were found to require the integrity of both polar and nonpolar regions of phospholipids, may involve alterations in membrane hydration following charge neutralization. The specific membrane perturbations induced by individual activators have been analyzed in terms of effects on the activation energy for TNBS incorporation and also in terms of TNBS labeling patterns of membrane protein and lipid components characteristic of the particular cationic activator.

INTRODUCTION

Characterization of the effects of pharmacological agents at the level of membrane structural components is an essential prerequisite to the understanding of the molecular mechanisms by which drugs alter the functional properties of biological membranes. One approach to this problem involves the use of specific chemical reagents, whose rate and extent of incorporation into membranes is markedly influenced by pharmacological agents and divalent cations which presumably alter the configurational state of membrane components. We have recently shown that the incorporation of trinitrobenzenesulfonic acid, an amino group-specific reagent, into erythrocyte membranes is enhanced by divalent cations such as Mg^{++}, Ca^{++}, Sr^{++}, and Mn^{++}, and by a variety of pharmacological agents possessing local anesthetic activity, including tetracaine, chlorpromazine, and propranolol (1). The detailed molecular mechanisms whereby the interaction of drugs and divalent cations with the membrane leads to increased tri-

This work was supported by the Medical Research Council of Canada.

nitrobenzenesulfonate incorporation and the possible relevance of these effects to the pharmacological properties of the molecules in question have not yet been clarified.

It has been shown that stimulatory effects require the integrity of membrane anionic phospholipid sites (1). The fact that all agents thus far shown to be capable of increasing TNBS[1] incorporation are cationic and that the TNBS molecule itself is anionic suggests the possibility that enhancement of TNBS labeling may result from nonspecific charge neutralization, which would facilitate the access of TNBS to membrane sites of attack. Additionally, or alternatively, the binding of cationic activators to membrane anionic sites may induce configurational changes which increase the accessibility and/or reactivity of membrane amino groups toward TNBS. Such configurational changes might be triggered by alterations in the hydrational state of charged membrane sites following charge neutralization and could be mediated via nonpolar components of membrane lipids to membrane proteins. One of the objectives of the present study, therefore, was to examine the possible role of charge neutralization, hydrational alterations, and nonpolar membrane components in the mediation of the stimulatory effects of drugs and divalent cations on TNBS incorporation.

Finally, in view of the chromophoric properties and stability of incorporated trinitrophenyl groups derived from TNBS, the effects of activators on TNBS labeling patterns of membrane proteins and lipids were investigated as a chemical approach to the characterization of the perturbational effects of cationic activators on membrane structural components.

METHODS

Membrane preparation. Erythrocyte membranes were prepared from outdated blood bank blood stored in acid-citrate-dextrose by stepwise hypotonic lysis in NaCl followed by a wash with 10 mM Tris-HCl (pH 7.4), using the methods described earlier (2).

Measurement of TNBS incorporation. Experimental conditions for the determination of the rate and extent of TNBS incorporation into erythrocyte membranes were essentially the same as those described in detail in our previous study (1). Briefly, membranes were incubated at 37° ± 0.5° in the presence of 1.0 ml of 20 mM Tris buffer (pH 8.0) in a total volume of 2.9 ml. The reaction was initiated by the addition of 0.1 ml of 10 mM TNBS solution (pH 8.0). Reaction times for rate measurements in the presence of activators were usually 30–60 sec, while those for time course experiments and for rate measurements in the absence of activators were considerably longer (15–60 min). Reactions were terminated by the addition of 2.0 ml of a 1:1 mixture of 1 M HCl and 10 % sodium dodecyl sulfate, and the extent of trinitrophenylation was expressed as the absorbance at 335 nm per milligram of membrane protein.

Hexane extraction of membranes. Membrane suspension (5 ml, containing 15–20 mg of membrane protein) was freeze-dried, and the resulting dry residue was extracted with 5 ml of hexane at −20° for 5 min. The membranes were recovered by centrifugation, using a clinical centrifuge, and the extraction could be repeated as often as required to achieve the desired decrease in membrane cholesterol. After the final extraction and centrifugation, the membranes were freed of residual hexane under vacuum and resuspended in water at the original volume (5.0 ml) from which they were derived.

Enzymatic treatment of membranes. Treatment of membranes with phospholipase C (*Clostridium welchii*, Sigma) was carried out exactly as described previously (1).

Phospholipase A treatment of membranes was carried out as follows. A solution of phospholipase A (*Naja naja* venom, Sigma) (10 mg/ml) was heated for 10 min at 70° to destroy protease activity. Membranes (2.0 ml) were then incubated for 15 min at 37° ± 0.5° in the presence of the heated phospholipase A (0.1 ml), 8.0 ml of $CaCl_2$ (20 mM), and 2.0 ml of Tris (20 mM, pH 8.0). The reaction was terminated by the addition of a 10-fold excess of EDTA (4 mM, pH 7.0). Membranes were isolated by centrifugation at 30,000 × *g* and washed once with 20 mM Tris (pH 8.0). In experiments in which the

[1] The abbreviation used is: TNBS, trinitrobenzenesulfonic acid.

effect of removal of phospholipase A reaction products by bovine serum albumin was examined, phospholipase A-treated membranes were washed three times with 1% bovine serum albumin (fatty acid-free, type F, Sigma) at room temperature, and this was followed by three water washes to remove the bovine serum albumin.

Erythrocyte membranes were treated with trypsin 37° ± 0.5° under the following conditions. The membrane suspension (2 ml) was combined with 1.5 ml of a solution of trypsin (bovine pancreas, type III, twice crystallized, Sigma) (50 μg/ml), and the mixture was incubated for 15 min. Membranes were isolated by centrifugation as usual.

D_2O studies. Membranes (usually in 2.0- or 5.0-ml portions) were lyophilized to remove water as completely as possible. The dried material was then dissolved in D_2O (99.8% D, Stohler Isotope Chemicals, Azuza, Cal.) and incubated for 30 min at room temperature. Membrane material was isolated by centrifugation, washed once with D_2O, and finally brought up to the original volume (2.0 or 5.0 ml) with D_2O. Control membranes were prepared in exactly the same manner, except that H_2O was substituted for D_2O. In TNBS incorporation studies, the components of the reaction medium, including Tris and the various cationic activators, were made up in D_2O, with identical quantities of material being weighed out and dissolved in H_2O for comparative purposes. There was some difficulty in deciding whether the effects of H_2O and D_2O should be compared at equal "pH" values (that is, as indicated by the pH meter reading) or at a pH equal to the pD value, since it is known that pH and pD differ from each other by 0.4 unit (3). The problem was resolved by performing experiments under both conditions (that is, at pH values of 7.6 and 8.0, and at pD values of 8.0 and 8.4).

Solubilization and gel filtration of labeled membranes. To estimate the effects of activators on the distribution of trinitrophenylated derivatives among various membrane components, erythrocytes were labeled to approximately the same degree in both the presence and absence of activators of TNBS incorporation. This was achieved by varying reaction times and/or activator concentrations such that ultimately membranes with the same absorbance at 335 nm per milligram of protein were obtained whether or not activator was present, and regardless of the nature of the activator (Mg^{++}, Zn^{++}, or chlorpromazine). This procedure was adopted so that any differences in the nature and extent of labeling of membrane components could be attributed solely to the nature of the activator and not to the extent of incorporation of label.

Membranes were incubated with TNBS under the usual conditions, and when the desired degree of labeling had been attained the reaction was terminated by the addition of 1 M HCl. Membranes were isolated and washed free of unreacted TNBS by centrifugation. Labeled membranes were solubilized prior to gel filtration in one of two ways. In the first procedure, membranes were dissolved in 1% sodium dodecyl sulfate containing 0.02% sodium azide. Samples were then centrifuged at 40,000 × *g* for 10 min to remove residual undissolved material (the amount of which was usually slight). The supernatant fluid was then applied to a column containing Sephadex G-200; elution was carried out with 1% sodium dodecyl sulfate–0.02% sodium azide. Alternatively, membranes were solubilized by Oberly and Duncan's (4) modification of the method of Lenard (5), i.e., by extensive dialysis (36 hr) at 4° against 5 mM EDTA–5 mM mercaptoethanol, pH 7.5, followed by addition of sodium dodecyl sulfate (final concentration, 3%) to the dialysate, and finally heating at 100° for 3 min in order to complete disaggregation. Following centrifugation at 40,000 × *g* for 15 min, the supernatant fluid was applied to a Sephadex G-200 column and elution was performed with 1% sodium dodecyl sulfate–5 mM EDTA–0.05 M ammonium bicarbonate–0.02% sodium azide, pH 7.4.

Phospholipid extraction and analysis. Membranes (5.0 ml) were incubated with or without TNBS in the usual reaction medium until the desired degree of labeling had occurred in TNBS-containing samples. Following addition of 25 ml of 1 M HCl, samples were centrifuged at 30,000 × *g*, and the resulting pellets were washed twice with water and

isolated by centrifugation. The membranes then were extracted with 2-propanol (11 ml) for 1 hr at room temperature, and following this chloroform (5 ml) was added to the mixture. After 1 hr at room temperature, samples were centrifuged in a low-speed centrifuge, and the resulting supernatant solutions were washed twice with 50 mM KCl (6). Butylated hydroxytoluene was added to each supernatant fraction (final concentration, 0.01%), and samples were then concentrated to a final volume of 2.0 ml under vacuum at room temperature. The phospholipid mixture was resolved by thin-layer chromatography on coated silica gel F-254 plates (Brinkmann) activated for 30 min at 110° prior to use. The solvent system consisted of chloroform–methanol–concentrated ammonia (14:6:1). Spots were visualized either directly (in the case of TNBS derivatives, which were yellow) or following exposure to iodine vapor. Lipids containing amino groups were localized by spraying the plates with ninhydrin, and individual phospholipid spots were identified as described by Winterbourn and Carrell (7).

Chemical analyses. Protein contents were determined by the method of Lowry *et al.* (8), using bovine serum albumin (Armour) as standard. Phospholipid was estimated by Bartlett's method (9), and cholesterol by the method of Zak *et al.* (10). The proteolipid content of chloroform–2-propanol extracts of membranes was measured as described by Lees and Paxman (11).

RESULTS

Effects of membrane cholesterol depletion. In view of the apparent correlation which had previously been found between cation-stimulatory effects on TNBS incorporation and the phospholipid to cholesterol ratio in different membrane preparations (1), the effects of hexane extraction were examined. This solvent preferentially depletes membranes of cholesterol as compared with phospholipids. The results in Table 1 indicate that removal of up to 75% of the membrane cholesterol had virtually no effect on the stimulatory properties of Mg^{++}, Zn^{++}, or chlorpromazine with respect to TNBS incorporation.

TABLE 1

Effects of cholesterol extraction on incorporation of TNBS into erythrocyte membranes

Activator	Concentration	TNBS incorporation rate	
		Control[a]	Hexane-extracted[b]
	mM	Aμ/min/mg protein	
Mg^{++}	1	0.087	0.108
	5	0.372	0.359
	10	0.468	0.466
	20	0.727	0.740
	30	0.857	0.861
Zn^{++}	0.5	0.182	0.152
	1.0	0.273	0.238
	2.0	0.394	0.295
	5.0	0.403	0.462
Chlorpromazine	0.05	0.074	0.090
	0.10	0.134	0.152
	0.20	0.173	0.224
	0.40	0.610	0.682
	0.60	1.165	1.184

[a] Chemical composition: cholesterol, 259 μg/mg of protein; phospholipid, 616 μg/mg.

[b] Chemical composition: cholesterol, 66 μg/mg of protein; phospholipid, 581 μg/mg.

Phospholipase A studies. Treatment of erythrocyte membranes with phospholipase A, which is known to hydrolyze fatty acid ester linkages primarily at the β-position of phospholipids (12, 13), would be expected to cause extensive perturbation of the nonpolar regions of phospholipids. The effects of phospholipase A treatment on Mg^{++} and Zn^{++} stimulation of TNBS incorporation are shown in Fig. 1. The finding that the stimulatory properties of Zn^{++} were virtually unaltered, whereas those of Mg^{++} were markedly affected, is consistent with the results of our previous studies with phospholipase C, which showed that Mg^{++} stimulation (in contrast to the effects of Zn^{++}) required the integrity of membrane phospholipids (1).

The action of phospholipase A results in the formation of lysophosphatidyl compounds and free fatty acids, both of which tend to remain associated with the membrane (14). Such membrane-bound fatty acids might form a new class of membrane cation-binding sites. The possible role of such groups in the diminished responsiveness of

phospholipase A-treated membranes to Mg^{++} was examined. Figure 2 illustrates the effects on Mg^{++}-stimulated TNBS incorporation of removing membrane-bound free fatty acids by means of extensive washing with fatty acid-free bovine serum albumin (15). The phospholipid and cholesterol contents of phospholipase A-treated and bovine serum

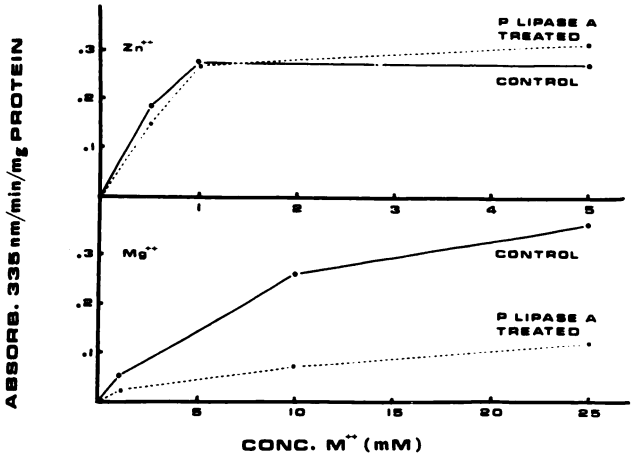

FIG. 1. *Effect of phospholipase A treatment on stimulatory effects of divalent cations on TNBS incorporation*

Two milliliters of membrane suspension, 0.1 ml of heated phospholipase A (P Lipase A) solution (10 mg/ml), 0.8 ml of $CaCl_2$ (20 mM), and 2.0 ml of Tris (20 mM, pH 8.0) were incubated at 37° ± 0.5° for 15 min. The reaction was terminated by the addition of 10 ml of EDTA (4 mM, pH 7.0), and the mixture was centrifuged. The pellet was washed once with 20 mM Tris (pH 8.0). The final pellet was resuspended in 2.0 ml of H_2O, and 0.2-ml aliquots were assayed for TNBS incorporation in the presence of Mg^{++} or Zn^{++}, as described in METHODS.

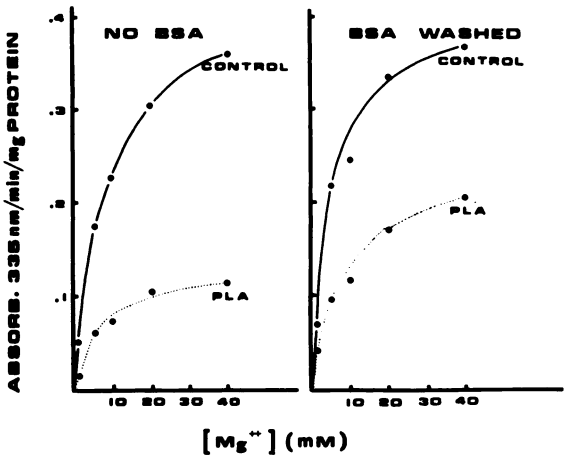

FIG. 2. *Treatment of control and phospholipase A-treated membranes with bovine serum albumin; effects on Mg^{++} stimulation*

Membranes were treated with phospholipase A (PLA) as indicated in Fig. 1. They were then washed three times with 1% defatted bovine serum albumin at room temperature, and finally three water washes to remove the bovine serum albumin were carried out. Membranes not treated with bovine serum albumin were subjected to the three water washes as well. After these washes all membrane samples were resuspended in the same volume of water, and aliquots were assayed for TNBS incorporation in the presence of varied concentrations of Mg^{++}.

TABLE 2

Chemical composition of phospholipase A-treated and bovine serum albumin-washed erythrocyte membranes

Details concerning phospholipase A treatment and BSA washing are given in the legend to Fig. 2.

Treatment	Phospholipid	Cholesterol
	μg/mg protein	
No BSA		
Control	28.6	201
Phospholipase A	26.7	216
BSA-washed		
Control	25.9	204
Phospholipase A	19.7	203

TABLE 3

Eadie plot analysis of Mg^{++} stimulation in phospholipase A- and bovine serum albumin-treated membranes

Data were obtained from Fig. 2. V_{max} and K_{diss} values were evaluated from the y intercept and slope, respectively, of regression lines derived from data expressed in the form of Eadie plots by means of a Compucorp 140 (Statistician) calculator.

Treatment	V_{max}	Apparent K_{diss}
	A_{335}/min/mg protein	*mM*
No BSA		
Control	0.417	7.4
Phospholipase A	0.127	6.0
BSA-washed		
Control	0.413	5.0
Phospholipase A	0.195	4.1

albumin-washed membranes are shown in Table 2. It is clear that minimal alterations in membrane composition accompanied these procedures. The data in Fig. 2 were converted to the form of Eadie plots (16), from which V_{max} and apparent K_{diss} values could be obtained. This information is summarized in Table 3. It indicates that the main effect of phospholipase A treatment was on the V_{max} value rather than on the apparent K_{diss}, and also that the effects of the phospholipase are probably not immediately attributable to the liberation of free fatty acids within the membrane.

Studies with picryl chloride. The fact that integrity of nonpolar lipid regions of the erythrocyte membrane seems to be required for Mg^{++} stimulation of TNBS incorporation provides evidence that the mechanism by which cations stimulate incorporation is probably more complex than simple charge neutralization resulting in enhanced accessibility of the anionic TNBS molecule. Additional information on this possibility was obtained by examining the behavior of picryl chloride, an uncharged analogue of TNBS, which also forms trinitrophenylated derivatives of membrane amino groups (17). However, unlike TNBS, the incorporation of picryl chloride should be independent of possible electrostatic barriers.

The effects of Mg^{++} and phospholipase C treatment on the incorporation of picryl chloride and TNBS into erythrocyte membranes were compared directly under identical experimental conditions. The data are summarized in Fig. 3. The reaction of picryl chloride with the membrane was more rapid than that of TNBS, presumably as the result of greater ease of permeation of picryl

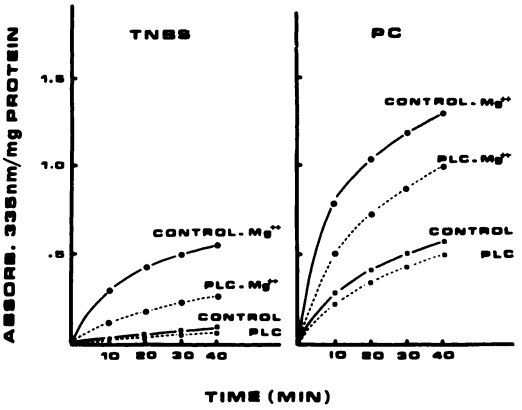

FIG. 3. *Incorporation of TNBS and picryl chloride (PC) into erythrocyte membranes; effects of Mg^{++} and phospholipase C (PLC)*

Reaction mixtures consisted of 0.2 ml of membrane protein, 1.0 ml of 20 mM Tris buffer (pH 8.0), divalent cation when indicated, and water to a final volume of 2.9 ml. Reactions were initiated by addition of 0.1 ml of TNBS (20 mM) in water or 0.1 ml of picryl chloride (20 mM) in ethanol. Ethanol was shown to have no measurable effect on the TNBS reaction, even at higher concentrations than those employed in the picryl chloride experiments. The reactions were stopped using HCl–10% sodium dodecyl sulfate, and absorbance at 335 nm was determined as described in METHODS.

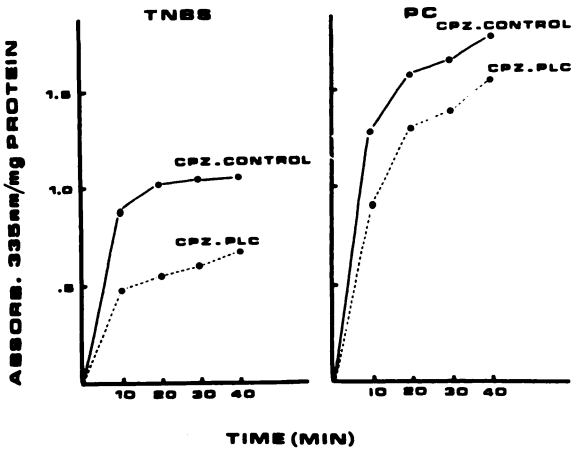

FIG. 4. *Incorporation of TNBS and picryl chloride (PC) into erythrocyte membranes; effects of chlorpromazine (CPZ) and phospholipase C (PLC)*

The methodology used in these experiments was exactly the same as that described in Fig. 3.

chloride (17). However, the effects of Mg^{++} and phospholipase C treatment (on both unstimulated and Mg^{++}-stimulated activities) were qualitatively similar for both TNBS and picryl chloride, although quantitatively these effects were most marked in the case of TNBS. The effects of chlorpromazine on incorporation of both substances were studied. The results in Fig. 4 show an enhancement by chlorpromazine in each case, and a decrease in this stimulatory effect following phospholipase C treatment.

D_2O studies. On the basis of the foregoing results with picryl chloride, it seemed that simple removal of electrostatic barriers to TNBS as a result of charge neutralization by cationic activators was inadequate to explain the enhancement in incorporation. The possibility that charge neutralization by activators might lead to structural rearrangements as the result of alterations in the hydrational state of membrane components seemed to merit investigation. This hypothesis was explored by studying the effect of substituting D_2O for H_2O on TNBS incorporation and its stimulation by cationic activators. An important consideration in such studies is the choice of the pH or pD value at which reactions are to be carried out, since pD = pH + 0.4, where pH is the pH meter reading (3). Because it was not clear whether comparisons should be made at equal pH values (as indicated by pH meter readings) or at equal pH and pD values, both comparisons were made.

TABLE 4

Effect of H_2O and D_2O on initial rate of TNBS incorporation

Aliquots (5.0 ml) of membrane suspension were freeze-dried to remove as much H_2O as possible. The dry residue was then suspended in D_2O, and the mixture was allowed to stand at room temperature for 30 min. Following centrifugation, the membranes were resuspended in D_2O (5.0 ml) and assayed for TNBS incorporation in a D_2O reaction medium containing 1.0 ml of Tris buffer in D_2O (20 mM) of appropriate pH as described in METHODS. Studies in H_2O were performed on membranes treated in an identical manner, except that H_2O replaced D_2O.

Sample	Solvent	pH	pD	Rate of TNBS incorporation
				A_{335}/mg protein/hr
Untreated	H_2O	8.0		0.094±0.019
Freeze-dried	H_2O	8.0		0.094±0.016
Untreated	H_2O	7.6		0.079±0.024
Freeze-dried	H_2O	7.6		0.072±0.012
Freeze-dried	D_2O	8.0[a]	8.4	0.083±0.021
Freeze-dried	D_2O	7.6[a]	8.0	0.082±0.026

[a] pH meter reading of D_2O–Tris buffer; the pD value was obtained from this reading by adding 0.4.

It is clear from the data in Table 4 that replacement of H_2O by D_2O had virtually

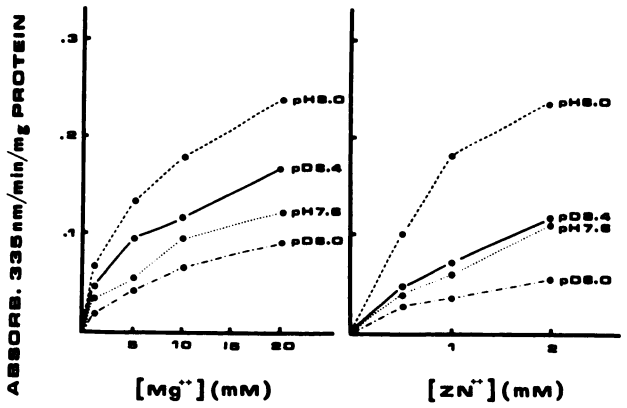

FIG. 5. *Effect of D_2O on cation-stimulated incorporation of TNBS into erythrocyte membranes*

In each graph the two upper curves correspond to pH meter readings of 8.0, and the two lower curves to pH meter readings of 7.6. Membranes were freeze-dried and resuspended in H_2O or D_2O as described in Table 4, and measurements of the rate of TNBS incorporation were made on the basis of 30-sec reaction times, as described in METHODS.

no effect on the time course of TNBS incorporation. However, the stimulatory effects of Mg^{++} and Zn^{++} on incorporation were altered in the presence of D_2O (Fig. 5). In both cases, whether effects were compared at equal pH values or at pH = pD, the substitution of D_2O for H_2O resulted in a decreased rate of incorporation. Eadie plot analysis of cation stimulation indicated that the effect of D_2O was to decreae the maximal velocity without altering the apparent dissociation constant for the cation (Table 5). The decrease in V_{max} produced by D_2O relative to H_2O for each cation appeared relatively independent of pH or pD, and seemed to be different for Mg^{++} and Zn^{++}.

Temperature dependence of TNBS incorporation. Another approach to studying the effects of activator cations on membrane configuration was to investigate their effects on the activation energy for TNBS incorporation into erythrocyte membranes. The results of such a study are shown in Table 6. Perturbation of membrane phospholipids by phospholipases A or C had only a minor effect on the temperature dependence of unstimulated TNBS incorporation. In contrast, activation of TNBS incorporation by magnesium or chlorpromazine was associated with a substantial decrease in activation energy relative to the unstimulated situation. This ability of magnesium and chlorpromazine to decrease the activation energy of TNBS incorporation was abolished by phospholipase C treatment of the membranes. The decrease in activation energy produced by magnesium seems particularly

TABLE 5

Eadie plot analysis of concentration dependence of cation stimulation of TNBS incorporation in presence of H_2O and D_2O

The data were obtained from Fig. 5. V_{max} and K_{diss} values were evaluated from regression lines derived from Eadie plots, using a Compucorp 140 (Statistician) calculator.

Conditions	Apparent K_{diss}	V_{max}	Decrease in V_{max} caused by D_2O
	mM	*A_{335}/min/ mg protein*	%
Cation: Mg^{++}			
pH 8.0	7.6	0.325	
pD 8.4	6.3	0.208	36
pH 7.6	15.5	0.219	
pD 8.0	11.7	0.140	36
Cation: Zn^{++}			
pH 8.0	1.4	0.411	
pD 8.4	1.6	0.196	53
pH 7.6	2.3	0.218	
pD 8.0	1.2	0.084	62

TABLE 6

Effects of phospholipases, activators, and solubilization on activation energy for TNBS incorporation

Temperature dependence studies were performed at five different temperatures in the range 17–45° on a minimum of two, but usually three, different membrane preparations, and in each case activation energy figures represent the average of six to eight separate experiments. Membranes were treated with phospholipases as described in METHODS, and solubilization of membrane proteins with butanol or pentanol was carried out as described in ref. 1. The final concentrations of activators were: $MgCl_2$, 1 mM; NaCl, 3 mM; and chlorpromazine, 0.05 mM. In each experiment slopes and y intercepts of Arrhenius plots were evaluated from the best-fitting line as determined by a Compucorp 140 (Statistician) calculator.

Membrane treatment	Activation energy
	kcal/mole
None	8.3 ± 0.7
Phospholipase C	8.2 ± 0.8
Phospholipase A	9.2 ± 1.3
Butanol extraction	6.2 ± 1.6
Pentanol extraction	6.8 ± 1.7
None + NaCl	8.0 ± 0.3
None + $MgCl_2$	6.1 ± 0.4
None + chlorpromazine	4.9 ± 0.5
Phospholipase C + $MgCl_2$	8.2 ± 1.4
Phospholipase C + Chlorpromazine	10.3 ± 3.2

significant, in view of the fact that the stimulatory effect of sodium chloride (at the same ionic strength as magnesium chloride) on TNBS incorporation was not associated with a decrease in activation energy relative to the unstimulated case (Table 6).

In addition to the slopes of Arrhenius plots, from which activation energies may be estimated, y intercepts of such plots also provide valuable information. These intercepts, which are numerically equal to the natural logarithm of the frequency factor A in the Arrhenius expression, may be considered to be proportional to the Eyring entropy of activation parameter, and hence they offer an indication of the extent to which the process under consideration depends upon structural or configurational factors (18, 19). In processes where alterations in solvation, particularly hydration, are important, a linear correlation between activation energy and ln A (corresponding to a correlation between Eyring enthalpies and entropies of activation) usually is observed (18). The data of the present temperature dependence experiments exhibited two striking correlations of this type (Fig. 6). Lines *1* and *2* depict the situation in the absence and presence of cationic activators, respectively. The relationship between ln A and activation energy E_a may be expressed as follows:

$$\ln A = \left(\frac{1}{R\beta_0}\right) E_a + \text{constant}$$

where R is the gas constant and β_0 is referred to as the isokinetic temperature (18). Under certain circumstances a correlation between ln A and E_a may be fortuitous, arising from errors in the measurement of activation energies. When this is the case, β_0 is found to be equal to the mean temperature of the ex-

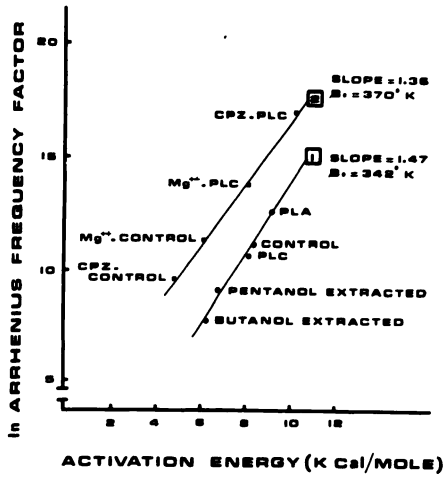

FIG. 6. *Relationship between Arrhenius parameters (frequency factor and activation energy) for TNBS incorporation into erythrocyte membranes*

The data represented here were derived from the Arrhenius plots of the experiments described in Table 6. Activation energies are those listed in Table 6, and ln frequency factor terms are equal in each case to the corresponding y intercept of the Arrhenius plot in question. PLA = phospholipase A; PLC-phospholipase C; CPZ = chlorpromazine.

periment (20). Here the values of β_0 for lines *1* and *2*, 342° and 370°K, respectively, differ substantially from 304°K, the mean experimental temperature in the present experiments. Therefore these results provide strong evidence for the involvement of hydrational effects in membrane amino group modification by TNBS and indicate that the relationship between membrane hydration and TNBS incorporation is different in the presence and absence of cationic activators.

Effects of activators on distribution of the trinitrophenyl label in membrane components. In an attempt to characterize somewhat more concretely the effects of cationic activators on the labeling of membrane moieties, membranes were labeled with TNBS in the absence or presence of activators, and this was followed by solubilization and gel filtration on Sephadex G-200 in order to determine the distribution of label in each case. In each of these experiments membranes were compared at the same degree of total TNBS incorporation relative to membrane protein (absorbance at 335 nm per milligram of membrane protein). This was achieved by appropriate manipulation of incubation time and TNBS concentration.

The results of a series of such experiments are shown in Fig. 7. In each case two major TNBS-labeled components (peaks I and II) are apparent. Peak I invariably coincided with a high molecular weight protein band, whereas peak II occurred in the region where both phospholipids and cholesterol were eluted (data not shown). It therefore appears reasonable to assume that peak II primarily reflected labeling by TNBS of the amino groups of membrane phospholipids. Although all four samples exhibited similar general characteristics, the extent of protein labeling (absorbance at 335 nm relative to protein content in peak I) and of phospholipid labeling relative to protein labeling (as reflected in the ratio of peak II to peak I) varied, depending upon the absence or presence of an activator and upon the nature of the activator. The information summarized in Fig. 8 has been derived from the data in Fig. 7. The interaction of magnesium or chlorpromazine with the membrane apparently resulted in increased labeling of phospholipids relative to the control, and there was a corresponding

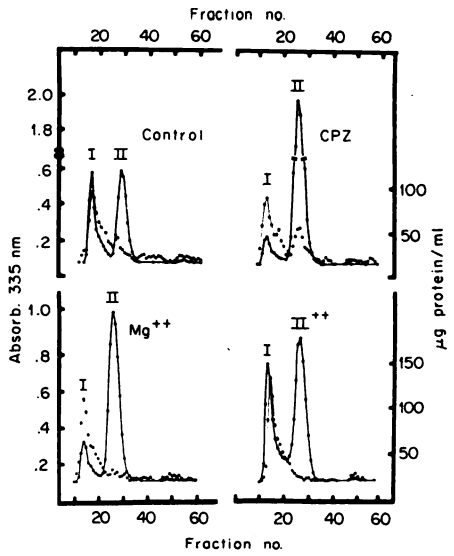

FIG. 7. *Gel filtration chromatography of TNBS-labeled erythrocyte membranes on Sephadex G-200*

Membrane aliquots (2 ml) were labeled to a final TNBS incorporation of approximately 0.75 absorbance units at 335 nm per milligram of protein in the absence or presence of Mg^{++}, chlorpromazine (CPZ), or Zn^{++} by adjustment of incubation time and TNBS concentration. Each sample was solubilized by the method of Oberley and Duncan (4) and applied to a 1.5 × 90 cm Sephadex G-200 column with 1% sodium dodecyl sulfate–5 mM EDTA–0.05 M ammonium bicarbonate–0.02% sodium azide as eluent (flow rate, 2 ml/hr). Fractions of 50 drops were collected, and each fraction was analyzed for absorbance at 335 nm and protein.

decrease in protein labeling under these same conditions. The stimulatory effects of zinc, on the other hand, resembled the situation in the absence of activator, and were associated with preferential labeling of protein rather than phospholipid components.

In order to confirm and extend the foregoing observations, the incorporation of TNBS into protein and phospholipid components was examined separately. Membranes were again labeled to the same degree of total TNBS incorporation in the presence or absence of activators, followed by extraction of the membrane lipids with chloroform–2-propanol (6), as described under METHODS, producing a lipid-depleted protein residue and a lipid extract.

The protein residues were solubilized and

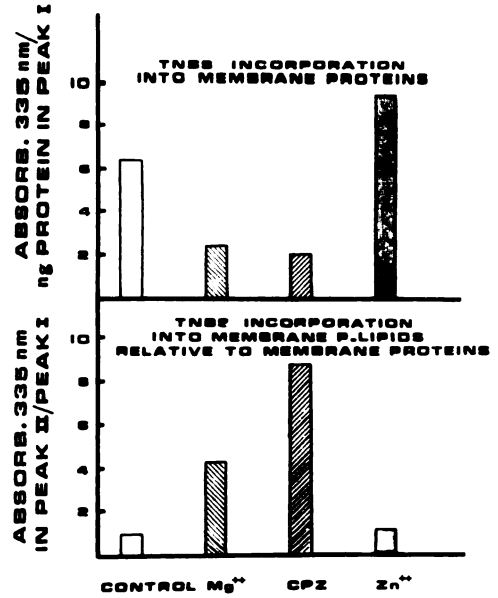

FIG. 8. *Relative incorporation of TNBS into protein and lipid fractions of erythrocyte membranes*

These data were derived directly from the results presented in Fig. 7. P-lipids = phospholipids; CPZ = chlorpromazine.

subjected to Sephadex G-200 gel filtration. The results are summarized in Table 7A. In each case two peaks of TNBS-labeled material were resolved. The major peak of TNBS incorporation invariably was associated with the major peak of membrane protein and, in the presence of magnesium or chlorpromazine, the TNBS incorporation per nanogram of protein was markedly decreased, in agreement with the results shown in Fig. 8. The second (minor) peak of TNBS incorporation did not appear to be associated with substantial amounts of membrane protein on the basis of the Lowry protein assay. The large decrease in this peak (compared to the corresponding peak II in Fig. 7) which resulted from lipid extraction procedures suggests that this minor component represents labeled phospholipid material which escaped extraction into the chloroform–2-propanol medium, possibly as a result of intimate association with some protein component.

Thin-layer chromatographic analysis of the chloroform–2-propanol extract indicated the presence of two classes of trinitrophenylated derivatives: a major component with an R_F value of 0.83 and a lesser component with

TABLE 7

Analysis of isolated protein and lipid components of TNBS-labeled membranes

A. *Lipid-depleted protein fraction.* Membranes were labeled as described previously, and washed by centrifugation to remove excess TNBS. Following extraction with 2-propanol–chloroform (see METHODS), the membranes were dissolved in 1% sodium dodecyl sulfate and applied to a 1.6 × 40 cm Sephadex G-200 column eluted with 1% sodium dodecyl sulfate–0.02% sodium azide. Fifty-drop fractions were collected.

Membranes	Major component		Minor component
	A_{335}	A_{335}/ng *protein*	A_{335}
Control	1.63	6.9	0.20
Mg^{++}	0.87	2.2	0.48
Chlorpromazine	0.45	1.4	0.37
Zn^{++}	1.36	3.0	0.41

B. *Lipid extract.* Following washing with 50 mM KCl and addition of butylated hydroxytoluene (see METHODS), lipid extracts were concentrated under vacuum to a minimal volume, and volumes were then adjusted to 2.0 ml with chloroform–methanol (2:1). Aliquots of each extract were spotted on an activated (30 min at 110°) silica gel plate (Brinkmann), and development was carried out with chloroform–methanol–ammonia (14:6:1 by volume). The two major yellow spots in each sample were scraped off the plate and eluted with methanol (final volume, 2.0 ml). Aliquots of each methanol extract were used for the determination of absorbance at 335 nm.

R_F	Sample	A_{335}
0.83	Control	0.055
	Mg^{++}	0.058
	Chlorpromazine	0.152
	Zn^{++}	0.099
0.71	Control	0.028
	Mg^{++}	0.027
	Chlorpromazine	0.057
	Zn^{++}	0.018

an R_F of 0.71. Also, in the control, magnesium-, and particularly the chlorpromazine-treated samples, an additional minor component was present, which had an R_F of 0.6. However, this component was not investigated further here. The extent of TNBS incorporation into each of the two major

TABLE 8

Analysis of phospholipid content of untreated and TNBS-treated erythrocyte membranes

Membranes (5 ml) were labeled with TNBS in the presence or absence of activators, and a parallel series of membranes containing only activator, but no TNBS, was also run. Following isolation and washing of the membranes, extraction with 2-propanol (11 ml) and later with chloroform (5 ml) was carried out (see METHODS). Extracts were washed twice with 50 mM KCl, and butylated hydroxytoluene (final concentration, 0.01%) was added to prevent autoxidation. Extracts were concentrated to a low volume under vacuum and applied to previously activated silica gel F-254 plates (Brinkmann). Development was carried out with chloroform–methanol–ammonia (14:6:1 by volume). Spots were visualized by exposure to iodine vapors and analyzed for phosphorus content by Bartlett's method (9). Data from untreated membranes (with or without activator) were pooled, and mean values ± standard deviations are given. Phosphatidylserine was not well resolved from other (minor) components near the origin, so that this material is referred to as the phosphatidylserine fraction to indicate its heterogeneity.

Membranes	Phospholipid content			
	Phosphatidyl-ethanolamine	Phosphatidyl-choline	Sphingomyelin	Phosphatidyl-serine fraction
	μg P	*μg P*	*μg P*	*μg P*
Untreated	0.49 ± 0.12	0.53 ± 0.16	0.59 ± 0.22	0.52 ± 0.15
TNBS—control	0.16	0.47	0.52	0.48
TNBS—Mg^{++}	0.19	0.40	0.48	0.42
TNBS—chlorpromazine	0.15	0.46	0.47	0.45
TNBS—Zn^{++}	0.15	0.37	0.50	0.45

components varied with the nature of the activator (Table 7B). The presence of magnesium did not appear to be associated with increased labeling of either component, whereas chlorpromazine produced an increase in labeling of both components. Zinc in one case produced an increase and in the other a decrease in labeling relative to the control. The formation of these two groups of lipid-soluble TNBS derivatives was associated with a marked decrease in the phosphatidylethanolamine (but not phosphatidylserine) content of the membranes (Table 8). The fact that the R_F values of the two TNBS-labeled components were substantially greater than that of phosphatidylethanolamine (0.48 in this system) is consistent with the formation of less polar trinitrophenyl derivatives of the amine function of this phospholipid. It may be that the differences in the extent of labeling of these two components present in the lipid extract reflect differences in the effects of activators at the level of membrane phospholipid accessibility and/or reactivity. However, one cannot eliminate the possible contribution to labeling of small amounts of proteolipid (11) which are soluble in lipid extraction media. In fact, the presence of protein material which may be proteolipid in nature was demonstrated in unfractionated lipid extracts (Table 9). Further large-scale resolution of the individual RNBS-labeled components present in chloroform–2-propanol extracts will be required before a complete understanding of the role of membrane protein and lipid components

TABLE 9

Proteolipid content of lipid extracts of erythrocyte membranes

The chloroform–2-propranol extracts described in Table 8 were concentrated under vacuum and brought to a final volume of 2.0 ml with chloroform–methanol (2:1 by volume). Aliquots (0.02 ml) of this solution were analyzed for proteolipid, using bovine serum albumin as standard (with the appropriate correction factor) as described by Lees and Paxman (11).

Membranes	Proteolipid	
	No TNBS	TNBS-treated
	mg/ml	*mg/ml*
Control	3.66	2.90
Mg^{++}	2.87	2.60
Chlorpromazine	2.34	3.21
Zn^{++}	2.84	4.39

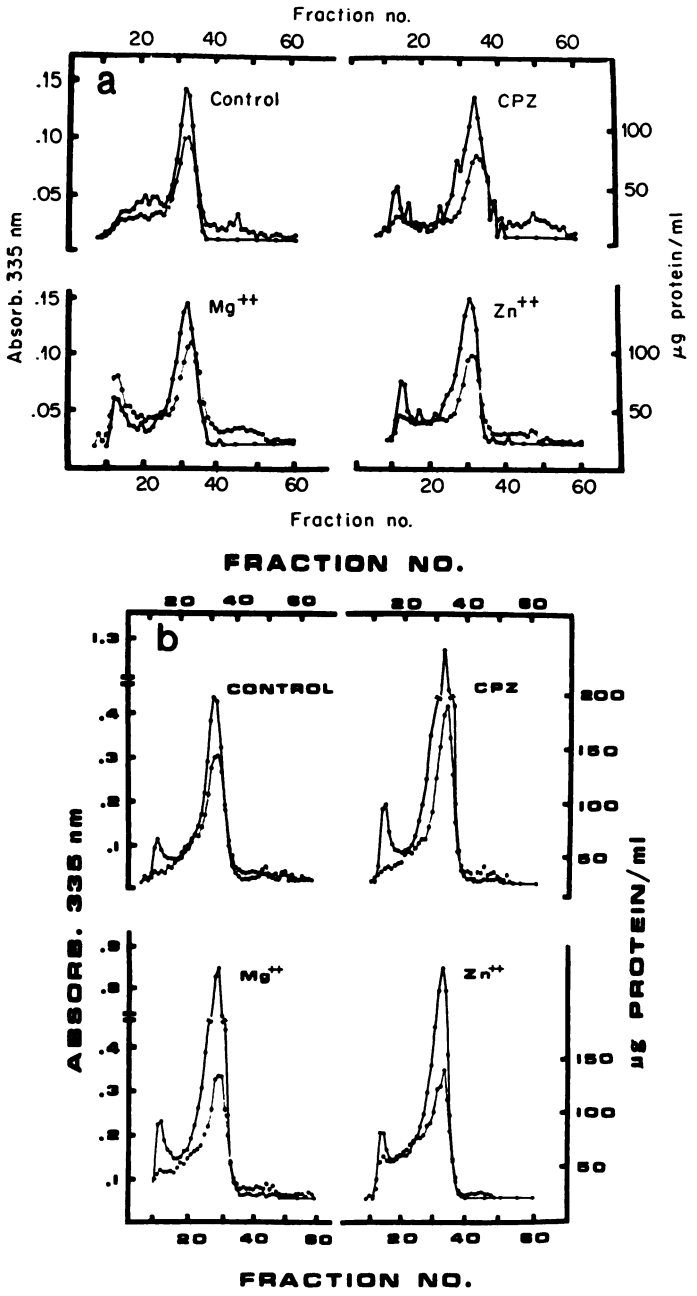

FIG. 9. *TNBS incorporation into membrane components solubilized by trypsin (A) and into insoluble residue following trypsin digestion (B)*

Two-milliliter aliquots of membranes were labeled with TNBS as described in the experiments illustrated in Fig. 7. Following the labeling, membranes were washed once with 20 mM Tris buffer (pH 8.0) and were then treated with 1.5 ml of trypsin (50 µg/ml) for 15 min at 37° ± 0.5°. Supernatant and particulate fractions resulted from centrifugation of the mixture following incubation; 0.5-ml aliquots of each supernatant fraction were applied to a 1 × 27 cm Sephadex G-200 column, and elution was carried out with 1% sodium dodecyl sulfate–0.02% sodium azide. Fractions of 15 drops were collected. The residues following centrifugation were solubilized in 1.0 ml of elution medium, and 0.5-ml aliquots were subjected to Sephadex gel filtration in the same manner as the supernatant fractions. CPZ = chlorpromazine.

as sites of TNBS incorporation in both the absence and presence of activators can be achieved.

In the final series of experiments, preliminary attempts were made to elucidate the membrane localization of components susceptible to TNBS attack in the absence or presence of cationic activators. Membranes were labeled to the same degree of total TNBS incorporation in the absence or presence of magnesium, chlorpromazine, or zinc, and the unlabeled membranes were then treated with trypsin to remove peripherally located proteins susceptible to proteolytic attack (21). The Sephadex G-200 elution patterns and TNBS labeling of the proteins rendered soluble by trypsin were compared with those of the residue remaining following trypsin treatment. The results are shown in Fig. 9A and B, respectively. The most readily interpretable information is obtained from the major peaks of TNBS incorporation, which in each case corresponded with the regions of maximal protein content. Variations in the extent of labeling of these major components (absorbance at 335 nm per nanogram of protein) were minimal with the supernatant fractions, but considerably more variation was found in the labeling of the residual fractions in each case. This information is summarized in Fig. 10, and is consistent with the view that the effects of activators may be most pronounced on less peripherally located membrane moieties. A study of the effects of cationic activators on the extent of TNBS incorporation into individual, well-characterized membrane proteins of known location within the membrane matrix would constitute a more rigorous approach to this problem.

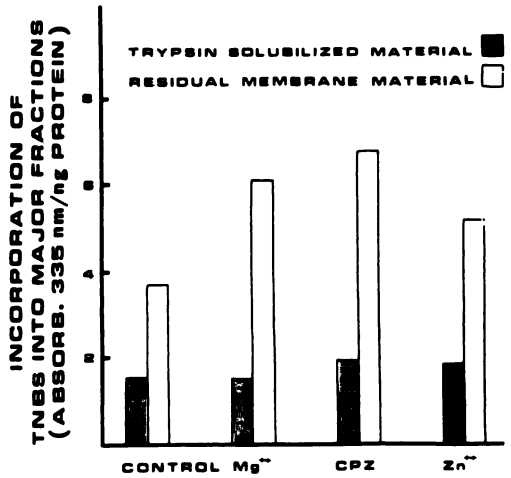

FIG. 10. *Relative labeling by TNBS of particulate and soluble fractions of erythrocyte membranes treated with trypsin*

Data were derived directly from Fig. 9A and B. CPZ = chlorpromazine.

DISCUSSION

The studies described here provide evidence that the stimulatory effects of drugs and divalent cations on TNBS incorporation into erythrocyte membranes reflect perturbations of both polar and nonpolar regions of membrane lipids and also membrane proteins; that these perturbations may involve alterations in the hydrational state of membrane components; and that it may be possible to describe the specific perturbational effects of individual drugs or divalent cations in terms of characteristic changes in the distribution of the chromophoric trinitrophenyl groups, derived from TNBS.

The role of nonpolar regions of membrane lipids in determining cation-stimulated TNBS incorporation was investigated by examining the effects of cholesterol depletion or of phospholipase A-induced hydrolysis of β-fatty acid moieties of membrane phospholipids (22) on incorporation. Extensive depletion of membrane cholesterol had virtually no effect on the stimulatory properties of Mg^{++}, Zn^{++}, or chlorpromazine on TNBS incorporation (Table 1). This was somewhat unexpected, since phospholipid integrity is required for the stimulatory effects of Mg^{++} and chlorpromazine (1), and cholesterol markedly influences the physical state of phospholipid fatty acid chains (23) and may contribute to bilayer stability (24). It may be that the cholesterol which remains associated with the membrane following hexane extraction (25% of the total cholesterol) may be sufficient to maintain membrane structural and functional integrity (25, 26). The influence of phospholipase A treatment (involving removal of a nonpolar phospholipid moiety) on cation-stimulated TNBS incorporation into membranes was

quite analogous to the effect of treatment with phospholipase C (involving removal of a dipolar phospholipid moiety) (1). Both enzyme treatments reduced the stimulatory effects of Mg^{++}, but produced little change in the stimulatory activity of Zn^{++} (Fig. 1). This suggests that integrity of both charged and nonpolar segments of phospholipids is necessary for the stimulatory effects of Mg^{++}, and this in turn may result from a functional interdependence of polar and nonpolar regions of membrane phospholipids. The effects of phospholipase A treatment on membrane structural and functional properties have been attributed primarily to the hydrolysis of β-fatty acid moieties (22) rather than to the action of reaction products—fatty acids and lysophosphatidyl derivatives, which remain membrane-associated (14). This assumption seems reasonable on the basis of the work of others (22, 27, 28) but it is difficult to verify. Experiments designed to test the effect of removing fatty acid products (Fig. 2 and Table 2) showed that only a small degree of restoration was obtained following extensive washing with bovine serum albumin (Table 3). Had greater effects been obtained, they would have been difficult to interpret unequivocally, since it has been shown that removal of membrane-associated fatty acids liberated as a result of phospholipase A action leads to further alterations in membrane structure, which as a result is even more removed from the native state (12, 27).

One must now inquire into possible mechanisms whereby interaction of cationic substances with membranes increases TNBS incorporation. One straightforward explanation would be that simple charge neutralization might serve to decrease coulombic repulsions between anionic membrane sites and trinitrobenzenesulfonate anions, thereby enhancing TNBS incorporation. Such an interpretation was offered by Rubalcava *et al.* (29) for the cationic enhancement of aminonaphtholsulfonic acid binding to erythrocyte membranes. However, the stimulatory effects of Mg^{++} and chlorpromazine on TNBS incorporation are also observed with picryl chloride, a neutral analogue of TNBS (Figs. 3 and 4). The impairment of Mg^{++} stimulation of TNBS incorporation by prior treatment with phospholipase A was also inconsistent with an explanation based on simple charge neutralization.

Since cation-stimulatory effects are most strikingly manifested as an increase in phospholipid (mainly phosphatidylethanolamine) labeling, it is appropriate to consider the results obtained from studies concerning the modification of pure phospholipid dispersions by TNBS (summarized in ref. 30). It was found that the reactivity of phosphatidylethanolamine toward TNBS was enhanced by factors which tend to disrupt ion pairing between the ammonium function and adjacent phosphate groups (31). However, the results of Carraway and Huggins (32) on the modification of phosphatidylethanolamine and phosphatidylserine, using acetic anhydride, suggest that the situation is considerably more complex. It is shown that both positively and negatively charged detergents increase the reactivity of phosphatidylethanolamine and phosphatidylserine dispersions; furthermore, with isolated erythrocyte membrane lipids, while positively charged detergents still increase amino group reactivity, negatively charged substances decrease reactivity. It would seem, therefore, that the susceptibility of amino groups of pure phospholipid aggregates to modification depends on the modifying reagent and also on the configuration of the phospholipid array, which in an intact membrane is probably affected to some degree by the presence of protein. Certainly there is evidence that the configuration of some membrane proteins depends upon the nature and configurational state of membrane lipids (22, 27).

Although the initial interaction of divalent cation and phospholipid is presumably electrostatic in nature, cooperative alterations may occur in nonpolar segments (33, 34), and this could trigger configurational changes in the lipid phase (35). The interaction of local anesthetics with membrane phospholipids is believed to involve both electrostatic and nonpolar types of interactions, the latter being particularly important in determining local anesthetic activity per se and also local anesthetic potency (36). As with divalent cations, the interaction of both general and local anesthetics with membranes can pro-

duce alterations in the ordering and fluidity of nonpolar regions of membranes (37).

Variations in the hydration of critical groups may furnish a common basis for the functional interdependence of polar and nonpolar segments of membrane lipids and the modifying effects of drugs and divalent cations on membrane lipoprotein components. Polar regions of phospholipids contain appreciable amounts of motionally restricted water (38), and this hydration affects the organization of nonpolar hydrocarbon regions of membrane lipids (39, 40). Furthermore, it has been suggested that variations in membrane hydration caused by cholesterol (41–43), drugs (44–46), and ions (47–50) may be intimately related to the membrane-modifying properties of these agents. There is evidence that the solvational and configurational characteristics of erythrocyte membrane components are altered when membranes are transferred from an aqueous medium to one containing D_2O (51). In the present study, extensive replacement of medium H_2O by D_2O decreased (although to somewhat different extents) the stimulatory effects of Mg^{++} and Zn^{++} (Fig. 5) without appreciably affecting TNBS incorporation in the absence of cations (Table 4). The decreased stimulatory effectiveness of both cations was associated with a decreased V_{max} value rather than an increased apparent K_{diss} (Table 5), suggesting that configurational changes following binding, rather than binding per se, were altered in the presence of D_2O. The recently observed inhibitory effects of D_2O on mixed-function oxidases of rat hepatic microscomes (52) exhibited analogous characteristics (decreased V_{max} without a change in K_m) and were likewise interpreted in terms of configurational alterations in membrane components consequent to hydrational changes.

As an approach to the molecular characterization of the postulated membrane configurational changes associated with alterations in TNBS incorporation in the presence or absence of activators, and to investigate further the role of hydrational effects, temperature dependence studies of TNBS incorporation were undertaken (Table 6). In the presence of Mg^{++} or chlorpromazine, the activation energy for TNBS incorporation was decreased, and this decrease was prevented by prior phospholipase C treatment. The finding that activation energy (E_a) for incorporation also decreased as progressive perturbation of native membrane structure by butanol and pentanol extraction occurred (1) was consistent with the involvement of activator-induced membrane configurational alterations, which alterations would increase accessibility and/or reactivity of membrane amino groups. Linear relationships between E_a and ln frequency factor (ln A), which are formally analogous to Eyring enthalpies and entropies of activation, respectively (18, 19), were demonstrated for TNBS incorporation in the presence and absence of activators (Fig. 6). Such linearity (or compensation) between enthalpy and entropy terms has been observed for a number of chemical (53), enzymatic (54), and membrane (18, 46) processes occurring in aqueous media, and in each case has been taken as evidence for an important role of hydrational effects in the process. Although the amount of data and the range of E_a values obtained here are small, our results suggest that TNBS incorporation may be affected by the nature and extent of hydration of membrane components and that the effects of activators may, either directly or indirectly, involve alterations in the state of membrane-bound water. On this basis, in the absence of cationic activators (Fig. 6, line *1*), phospholipase A treatment increases the structuring of membrane-associated water molecules (there is an increase in both ln A and E_a terms relative to the control), whereas phospholipase C, pentanol, and butanol treatments progressively disrupt membrane hydration. TNBS incorporation in the presence of Mg^{++} or chlorpromazine is characterized by a second linear relationship between ln A and E_a (Fig. 6, line *2*). It is apparent from these data that phospholipase C treatment, which impairs the ability of both molecules to stimulate TNBS incorporation (1), does not abolish the interaction of these activators with the membrane (data points fall on line *2* rather than on line *1*) but instead appears to decrease, relative to membranes where phospholipids are

intact, the degree of hydrational disruption which results from this interaction.

Our preliminary experiments to analyze, in structural terms, the effects of activators on TNBS labeling have indicated that the presence of Mg^{++} or chlorpromazine results in a marked increase in the labeling of membrane phospholipids relative to membrane proteins, whereas the stimulatory effects of Zn^{++} are not associated with a preferential increase in phospholipid labeling (Figs. 7 and 8). TNBS incorporation into membrane phospholipids was associated exclusively with phosphatidylethanolamine, and no evidence for phosphatidylserine labeling was found (Table 8). This low reactivity of membrane phosphatidylserine toward amino group-modifying reagents has been observed by others (55, 56), and has been taken as evidence for an asymmetrical distribution of phosphatidylserine in membrane bilayer structures, such that most, if not all, would be present on the inner surface (56). While our results are consistent with this interpretation, other explanations are possible. The interaction of TNBS with amino groups is extremely sensitive to the local environment of the amino group, so that negative charges in the vicinity of the reaction site cause a decrease, and positive charges an increase, in reactivity (57). Thus the decreased modification of phosphatidylserine by TNBS may be attributable to the presence in the molecule of an additional negative charge relative to phosphatidylethanolamine. This strong influence of local environmental factors on amino group modification by TNBS may determine the sensitivity of TNBS incorporation to the configurational state of the membrane.

The present study has described the effects of cationic activators on the gross labeling characteristics of membrane proteins and phospholipids by TNBS. More subtle information concerning the relationship between activator binding and the enhancement of TNBS labeling will require the characterization of activator-binding sites and of the spatial configuration within the membrane matrix of components whose reactivity toward TNBS is altered by activator binding. Preliminary experiments involving trypsin digestion of labeled membranes indicate that the incorporation of TNBS into peripherally located proteins liberated by trypsin is not appreciably aflected by the presence of Mg^{++}, Zn^{++}, or chlorpromazine (Figs. 9 and 10). Finally, studies currently in progress are attempting to correlate effects of various pharmacological agents on the labeling of membrane structural components with their effects on membrane enzymatic activities. It may eventually be possible to attribute functional significance to the perturbational effects of drugs on the configurational characteristics of membrane structural components, using such an approach.

ACKNOWLEDGMENT

We are most grateful to Dr. J. G. Foulks for his interest in this work and for his many helpful suggestions during the preparation of the manuscript.

REFERENCES

1. D. V. Godin and T. Wan Ng, *Mol. Pharmacol.* **8**, 426–437 (1972).
2. D. V. Godin and S. L. Schrier, *Biochemistry* **9**, 4068–4077 (1970).
3. P. K. Glasoe and F. A. Long, *J. Physiol. Chem.* **64**, 188–190 (1960).
4. T. D. Oberley and J. L. Duncan, *Biochem. Biophys. Res. Commun.* **48**, 1339–1347 (1972).
5. J. Lenard, *Biochemistry* **9**, 1129–1132 (1970).
6. H. G. Rose and M. Oklander, *J. Lipid Res.* **6**, 428–431 (1965).
7. C. C. Winterbourn and R. W. Carrell, *Brit. J. Haematol.* **23**, 499–505 (1972).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265–275 (1951).
9. G. R. Bartlett, *J. Biol. Chem.* **234**, 466–468 (1959).
10. B. Zak, R. C. Dickenman, E. G. White, H. Burnett, and P. J. Cherney, *Amer. J. Clin. Pathol.* **24**, 1307–1315 (1954).
11. M. B. Lees and S. Paxman, *Anal. Biochem.* **47**, 184–192 (1972).
12. S. Gul and A. D. Smith, *Biochim. Biophys. Acta* **288**, 237–240 (1972).
13. D. J. Hanahan, H. Brockerhoff, and E. J. Barron, *J. Biol. Chem.* **235**, 1917–1923 (1960).
14. E. Weidekamm, D. F. H. Wallach, and H. Fischer, *Biochim. Biophys. Acta* **241**, 770–778 (1971).
15. G. Meissner and S. Fleischer, *Biochim. Biophys. Acta* **255**, 19–33 (1972).
16. G. S. Eadie, *J. Biol. Chem.* **146**, 85–93 (1942).
17. J. J. Arrotti and J. E. Garvin, *Biochim. Biophys. Acta* **255**, 79–90 (1972).

18. M. F. Coldman and W. Good, *Biochim. Biophys. Acta* **150**, 194–205 (1968).
19. M. F. Coldman and W. Good, *Biochim. Biophys. Acta* **150**, 206–213 (1968).
20. O. Exner, *Nature* **201**, 488–490 (1964).
21. T. Okuda, A. Ito, and R. Sato, *J. Biochem. (Tokyo)* **71**, 333–342 (1972).
22. H. Simpkins, S. Tay, and E. Panko, *Biochemistry* **10**, 3579–3585 (1971).
23. J. E. Rothman and D. M. Engelmann, *Nature New Biol.* **237**, 42–44 (1972).
24. J. C. Hsia and J. M. Boggs, *Biochim. Biophys. Acta* **266**, 18–25 (1972).
25. B. Dueticke and C. Zöllner, *Biochim. Biophys. Acta* **266**, 726–731 (1972).
26. R. A. Cooper, M. Diloy-Puray, P. Lando, and M. S. Greenberg, *J. Clin. Invest.* **51**, 3182–3192 (1972).
27. E. Weidekamm, D. F. H. Wallach, and H. Fischer, *Biochim. Biophys. Acta* **241**, 770–778 (1971).
28. G. L. Scherphof, A. Scarpa, and A. Van Toorenenbergen, *Biochim. Biophys. Acta* **270**, 226–240 (1972).
29. B. Rubalcava, D. Martinez de Muñoz, and C. Gitler, *Biochemistry* **8**, 2742–2747 (1969).
30. C. Gitler, *Annu. Rev. Biophys. Bioeng.* **1**, 51–92 (1972).
31. D. Papahadjopoulos and L. Weiss, *Biochim. Biophys. Acta* **183**, 417–426 (1969).
32. K. L. Carraway and J. W. Huggins, *Chem. Phys. Lipids* **8**, 56–64 (1972).
33. Y. K. Levine, N. J. M. Birdsall, A. G. Lee, and J. C. Metcalfe, *Biochemistry* **11**, 1416–1421 (1972).
34. J. Cerbón, *Biochim. Biophys. Acta* **102**, 449–458 (1965).
35. R. P. Rand and S. Sengupta, *Biochim. Biophys. Acta* **255**, 484–492 (1972).
36. H. Hauser, S. A. Penkett, and D. Chapman, *Biochim. Biophys. Acta* **183**, 466–475 (1969).
37. P. Seeman, *Pharmacol. Rev.* **24**, 583–655 (1972).
38. N. J. Salsbury, A. Darke, and D. Chapman, *Chem. Phys. Lipids* **8**, 142–151 (1972).
39. D. Chapman, R. M. Williams, and B. D. Ladbrooke, *Chem. Phys. Lipids* **1**, 445–475 (1967).
40. J. L. Rigaud, Y. Lange, C. M. Gary-Bobo, A. Samson, and M. Ptak, *Biochem. Biophys. Res. Commun.* **50**, 59–65 (1973).
41. A. Darke, E. G. Finer, A. G. Flook, and M. C. Phillips, *J. Mol. Biol.* **63**, 265–279 (1972).
42. C. Horwitz, L. Krut, and L. S. Kaminsky, *Chem. Phys. Lipids* **8**, 185–196 (1972).
43. N. L. Gershfeld and M. Muramatsu, *J. Gen. Physiol.* **58**, 650–666 (1971).
44. H. Schneider, *Biochim. Biophys. Acta* **163**, 451–458 (1968).
45. P. Seeman, R. I. Sha'afi, W. R. Galey, and A. K. Solomon, *Biochim. Biophys. Acta* **211**, 365–368 (1970).
46. M. F. Coldman, W. Good, and D. Swift, *Biochim. Biophys. Acta* **173**, 62–70 (1969).
47. J. M. Tobias, D. P. Agin, and R. Pawlowski, *Circulation* **26**, 1145–1150 (1962).
48. G. J. Leitch and J. M. Tobias, *J. Cell. Comp. Physiol.* **63**, 225–232 (1964).
49. J. A. Verpoorte and F. M. Smith, *Can. J. Biochem.* **50**, 177–185 (1972).
50. J. Cerbón, *Biochim. Biophys. Acta* **144**, 1–9 (1967).
51. J. M. Graham and D. F. H. Wallach, *Biochim. Biophys. Acta* **241**, 180–194 (1971).
52. J. L. Holtzman and M. L. Carr, *Mol. Pharmacol.* **8**, 481–489 (1972).
53. D. J. G. Ives, *J. Chem. Soc.* 649–676 (1965).
54. B. Belleau and J. L. Lavoie, *Can. J. Biochem.* **46**, 1397–1409 (1968).
55. J. Poensgen and H. Passow, *J. Membr. Biol.* **6**, 210–232 (1971).
56. S. E. Gordesky and G. V. Marinetti, *Biochem. Biophys. Res. Commun.* **50**, 1027–1031 (1973).
57. G. E. Means, W. I. Congdon, and M. L. Bender, *Biochemistry* **11**, 3564–3571 (1972).