J. Membrane Biol. 28, 143 – 168 (1976)

Perturbational Effects of Inorganic Cations on Human Erythrocyte Membranes

David V. Godin and Maureen Garnett

Department of Pharmacology, Faculty of Medicine,
The University of British Columbia, Vancouver, Canada V6T 1W5

Received 26 September 1975; revised 12 January 1976

Summary. The perturbational effects of monovalent and divalent cations on human erythrocyte membranes were analyzed by examining their influence on kinetic and structural characteristics of trinitrobenzenesulfonic acid (TNBS) incorporation into the amino groups of protein and phospholipid structural components. The stimulatory effects of monovalent cations on TNBS incorporation, which were size-independent and attributed to nonspecific membrane alterations resulting from ionic strength factors, contrasted with the more pronounced stimulatory properties of divalent cations which were markedly size-dependent. These stimulatory effects of cations on TNBS incorporation were associated with alterations not only in rate but also in activation energy of incorporation. Changes in activation energy produced by divalent cations paralleled their ability to perturb membrane protein components and probably reflected changes in probe permeation. The rate of TNBS incorporation exhibited a dependence on divalent cation ionic radius which paralleled ion-induced perturbations in the labelling of the membrane amino phospholipid phosphatidylethanolamine. Divalent cations differed both in the relative extent and in the characteristics of protein and phospholipid perturbation. Alkaline earth cations behaved as a rather homogeneous group while Ni^{++}, Co^{++} and Mn^{++} constituted a second heterogeneous group. The influence of monovalent and divalent cations on the hemolytic behavior of intact erythrocytes paralleled their effects on TNBS incorporation into isolated membranes rather closely. It is suggested that TNBS incorporation may provide a valuable means of analyzing functionally relevant cation-induced alterations in biological membranes in general.

Considerable evidence now exists attesting to the crucial role of inorganic cations in determining both structural and functional characteristics of biological membranes [3, 4, 7, 22, 27, 34, 36]. This is well exemplified in excitable tissues, where electrical excitation and transmitter release are critically influenced by cation-membrane interactions [18, 24, 28, 30]. Studies of the molecular basis of inorganic cation-induced membrane perturbations, using a variety of physico-chemical approaches, including electron spin resonance [9, 19], fluorescent probes [11, 38, 41], and a combination of differential scanning calorimetry and fluorescence polar-

ization [21], have tended to focus on the ion-binding properties of phospholipids, particularly in experiments involving artificial membranes. Such investigations have underlined the importance of inorganic cations in determining the configuration of lipid arrays and have emphasized differences in the perturbational effects of monovalent vs. divalent cations – differences which may well be relevant to the roles of these ions in determining membrane excitability [18]. Furthermore, the ability of divalent cations to induce membrane fusion [33, 35] has provided a possible interpretation for their requirement in secretory processes involving exocytosis [8].

Despite the obvious advantages of investigating the behavior of inorganic cations in relatively simple artificial phospholipid systems as models of real biological membranes, such approaches preclude an analysis of the role of proteins in influencing both structural and functional alterations resulting from membrane-cation interaction. Not surprisingly, therefore, the high degree of specificity characterizing most cation-dependent biological processes is rarely achieved in artificial systems and cation effects have generally been interpreted in terms of simple charge neutralization or screening. On the other hand, in studies utilizing real biological membranes, limitations in available physico-chemical probes have often made it difficult to analyze in detail the relative contributions of lipid and protein membrane components to cation-induced structural alterations.

Recent reports from this laboratory [13, 15] have described the use of group-specific chemical probes in the analysis of drug- and cation-induced perturbations in biological membranes. In the present study, structural alterations induced in human erythrocyte membranes by monovalent and divalent cations have been characterized kinetically, in terms of effects on the accessibility and/or reactivity of membrane amino groups towards trinitrobenzenesulfonic acid (TNBS) and analyzed structurally by measuring the influence of cations on the incorporation of covalently bound, chromophoric trinitrophenyl groups into membrane proteins and phospholipids. Attempts have also been made to relate these cation-dependent structural alterations to corresponding membrane functional changes in intact erythrocytes, namely their susceptibility to hypotonic hemolysis. In view of the suggested analogy between the effects of pharmacological agents on the hemolytic behavior of erythrocytes and on the electrical properties of excitable membranes [37, 40], it was hoped that these investigations would provide some insight into molecular aspects of pharmacologically relevant membrane-cation interactions.

Materials and Methods

Butylamine Model Studies

Effects of temperature, cations and pH variation on the incorporation of trinitrobenzenesulfonic acid (TNBS) or picryl chloride (PC) into butylamine were examined in a 3.0 ml reaction mixture containing 1.0 ml Tris buffer (20 mM), at pH values ranging from 7.0 to 9.0 (in half unit increments); butylamine (0.2 ml of a solution consisting of 1 part butylamine diluted to 500 parts with double distilled water); TNBS (0.1 ml, 10 mM, pH 8.0) or PC (0.1 ml, 10 mM in ethanol); 0.1 ml ion solution in the cation studies (30 mM $MgCl_2$ or 300 mM NaCl) and sufficient double distilled water to make a final volume of 3.0 ml. Reactions were initiated by the addition of the TNBS or the PC, and reaction times varied from 30–60 sec in experiments at high pH to 12–24 min in experiments at low pH. Five reaction temperatures were selected in the range between 5–46 $\pm$ 0.5 °C for the construction of Arrhenius plots. Reactions were terminated by the addition of 2.0 ml 1:1 10% sodium dodecyl sulfate: 1 M HCl. Rates were expressed as absorbance at 335 nm per min and the slope of the linear relationship between ln rate and reciprocal absolute temperature was determined by regression analysis (using a Compucorp 140 statistical calculator) and used to evaluate Arrhenius activation energies.

Membrane Studies with TNBS—Effects of Cations

Human erythrocyte membranes were prepared from outdated blood stored in acid-citrate-dextrose by a stepwise hemolytic procedure [14] and contained 3–4 mg membrane protein per ml. Kinetic measurements of TNBS incorporation as a function of variations in temperature, pH and cation composition of the medium were performed under essentially the same experimental conditions as described for the butylamine model studies, except that membranes (0.2 ml) replaced the butylamine solution. In preliminary experiments it was found that reaction times of 30 min (in the absence of added cations) or 60 sec (in the presence of divalent cations) yielded sufficiently large absorbance increments for reliable measurement and also yielded linear reaction rates. It was also shown that activation energy values measured within the first 30 min (unstimulated) or the first 60 sec (stimulated) were independent of the reaction time chosen. In experiments using added monovalent cations (final concentration 10 mM), in order to test the effects of these ions under conditions exactly comparable (in terms of membrane concentration and absorbance increments) to those used in the divalent cation studies, it was necessary to utilize a reaction time (10 min) which did influence somewhat the magnitude of the measured rates and activation energies. Arrhenius plots were linear in all cases, however, and a reaction time of 10 min was felt to be a reasonable compromise between strict linearity of rate with time and impractically low absorbance values as a basis for comparing the stimulatory properties of these ions. The fact that all the monovalent cations exhibited virtually identical behavior at any given reaction time obviated the need for more rigorous quantitative comparisons.

Enzyme Treatment of Membranes

In a number of experiments the effects of prior treatment of erythrocyte membranes with neuraminidase, trypsin or phospholipase A on the unstimulated and cation-stimulated incorporation of TNBS were examined.

In the neuraminidase treatment, each ml membrane suspension utilized 3.0 ml sodium acetate buffer (50 mM, pH 5.5), 0.5 ml $CaCl_2$ (20 mM) and 20 µl neuraminidase (*Vibrio comma* (*cholerae*), Behringwerke, Germany). Incubations were carried out at 37 ± 0.5 °C for 30 min and membranes were isolated and washed with double distilled water by centrifugation at $30{,}000 \times g$. Membranes were assayed for sialic acid following a 30 min hydrolysis in 0.1 N H_2SO_4 at 80 ± 1 °C using the procedure of Warren [43]. This procedure removed about 70% of the membrane-associated sialic acid.

Trypsinization of membranes was performed by incubating 30 ml membrane suspension with 2.1 ml trypsin (Sigma, type III, 2× crystallized, 500 µg/ml) at 37 ± 0.5 °C for 15 min. Samples were centrifuged at $30{,}000 \times g$ and membranes were washed once with cold water by centrifugation and resuspended in a final volume of 18 ml.

Membranes were treated with a phospholipase A (*Naja naja* venom, Sigma) solution (10 mg per ml) which had previously been heated at 70 °C for 10 min. In a typical experiment, membranes (2.0 ml) were incubated at 37 ± 0.5 °C for 15 min in the presence of 4.0 ml Tris buffer (20 mM, pH 8.0), 1.0 ml $CaCl_2$ (20 mM) and 100 µl phospholipase A solution. Reactions were terminated by the addition of an equal volume of cold ethylenediamine tetraacetic acid (EDTA) (4 mM, pH 7.0). Samples were centrifuged at $30{,}000 \times g$, washed once by centrifugation with Tris buffer and resuspended in water.

Gel-Filtration Studies of TNBS-Labelled Membranes

Two ml aliquots of membrane suspension were labelled with TNBS by incubation for 1 hr at 37 ± 0.5 °C in a medium containing 7.0 ml Tris buffer (20 mM, pH 8.0), 0.7 ml TNBS (10 mM, pH 8.0) and divalent (1 mM final concentration) or monovalent (10 mM final concentration) cations as the chloride salts, all in a final volume of 20 ml. Reactions were terminated by the addition of 7.0 ml HCl (1 M) and membranes were isolated and washed once (with 10 ml Tris buffer) by centrifugation. Labelled membranes were resuspended in 1.5 ml water and dialyzed at 4 °C for 70 hr against 5 mM EDTA-5 mM 2-mercaptoethanol. Following dialysis, samples were combined with 0.4 ml 10% sodium dodecyl sulfate, boiled for 10 min and a 1.0 ml aliquot was applied to a Sephadex G-200 column (16 × 100 cm). Elution was carried out with 1% sodium dodecyl sulfate-0.02 % sodium azide-0.05 M ammonium bicarbonate [25]. Flow rates were of the order of 3 ml per hr and 50 drop (approximately 1.2 ml) fractions were collected. Each fraction was analyzed for absorbance at 335 nm as a measure of TNBS incorporation, protein [26], phospholipid [1] and sialic acid [43].

Miscellaneous Chemical Analyses

The phospholipid profiles of TNBS-labelled membranes were determined by extracting labelled membranes (derived from 1.0 ml membrane suspension) twice with 2 ml chloroform/methanol (2:1, v/v). Extracts were combined, washed three times with 0.75% sodium chloride and evaporated to near dryness under N_2. The residue was dissolved in 0.5 ml chloroform/methanol (2:1, v/v) and 40–60 µl aliquots were applied to precoated silica gel F 254 plates (0.25 mm thickness, Brinkman) which had previously been activated by heating at 110 °C for 30 min. Elution was performed with chloroform/methanol/ammonia 14:6:1 (v/v/v). Amino phospholipid spots were located with ninhydrin spray (Baker Chemical Company), while the remaining phospholipids were visualized following exposure to iodine vapor. Individual phospholipids were quantitated by extraction from the silica gel using methanol and phosphate analysis by the procedure of Bartlett [1].

Membrane sulfhydryl groups were estimated using 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB). The reaction medium contained membrane suspension (0.2 ml), 1.0 ml imidazole buffer (0.15 M, pH 7.4), 0.1 ml DTNB solution (0.3 mM in 0.15 M imidazole, pH 7.4)—with or without added cations, in a final volume of 3.0 ml. After a 30 min incubation at 37 ± 0.5 °C, samples were centrifuged at $30{,}000 \times g$ and the absorbance of supernatants at 412 nm was determined. The sulfhydryl group titer per mg membrane protein was calculated using a molar extinction coefficient value of 1.36×10^4 [10].

Hemolysis Studies

Blood (approximately 5 ml, 0 positive) was withdrawn from volunteers into heparinized (50 units heparin per ml of blood) syringes and centrifuged at top speed in a clinical centrifuge at 4 °C for 5 min to remove plasma and buffy coat material. The erythrocytes were washed three times with four volumes of a solution containing 0.25 M sucrose in 15 mM Tris, pH 7.0. Packed cells were resuspended in 32 ml of the washing medium (final hematocrit 6%). An aliquot (0.2 ml) of this cell suspension was added to 1.5 ml of a mixture containing divalent cation (1 mM) or monovalent cation (10 mM) as the chloride salts in 0.15 M sucrose-15 mM Tris (pH 7.0). After mixing, samples were incubated 15 min at room temperature and this was followed by a hypotonic challenge consisting of 2.3 ml ion solution (1 mM for divalent cations, 10 mM for monovalent cations) in 15 mM Tris (pH 7.0). Samples were again mixed, incubated 10 min at room temperature, and centrifuged at $30{,}000 \times g$ at 4 °C for 1 min. Supernatants were removed and the degree of hemolysis determined from the absorbance at 540 nm. Hemolysis was expressed as a percentage of the control where no cations were present.

Materials

Butylamine and 2-mercaptoethanol were obtained from Baker Chemical Co. The phosphorus standard solution, Trizma base, imidazole and sodium dodecyl sulfate were purchased from Sigma. Bovine serum albumin (fraction V from bovine plasma) used as Lowry protein standard was obtained from Armour Pharmaceuticals, picryl chloride from Matheson, Coleman and Bell, and ethylenediaminetetraacetic acid from Fisher Chemical Company.

Results

Model Studies Utilizing Butylamine

In order to focus attention on the membrane perturbational effects of inorganic cations as reflected in alterations produced in the reaction of TNBS and PC with membrane amino groups, it was essential to determine whether or not inorganic cations, at concentrations used in the erythrocyte membrane studies, influenced the reactivity of these probes towards membrane amino groups. Butylamine was chosen as a simple model amino compound and an extensive study of the effects

Table 1. Model studies on the incorporation of TNBS and picryl chloride into butylamine (a) Effect of Mg^{++} (1 mM) on the activation energy and rate of TNBS incorporation into butylamine and into membranes

	Activation energy (kcal/mole)		Rate (1n absorbance at 335 nm/min at 25 °C)	
	butylamine	membranes	butylamine	membranes
Unstimulated	11.5	7.2	−4.11	−6.34
Mg^{++} (1 mM)	11.8	4.9	−3.88	−2.95

(b) Effect of pH on the activation energy and rate of TNBS incorporation into butylamine and into membranes

pH	Activation energy (kcal/mole)			
	TNBS		Picryl chloride	
	butylamine	membranes	butylamine	membranes
9.0	8.1	9.1	10.9	6.6
8.5	9.8	8.1	12.0	7.3
8.0	11.5	7.2	12.8	6.5
7.5	12.4	6.7	13.3	7.8
7.0	12.3	5.3	12.0	7.7
	Rate (1n absorbance at 335 nm/min at 25 °C)			
9.0	−0.94	−6.18	−2.86	−2.73
8.5	−2.76	−6.19	−4.30	−3.12
8.0	−4.11	−6.34	−5.47	−3.58
7.5	−5.14	−6.72	−6.38	−4.28
7.0	−5.69	−7.18	−6.80	−4.70

of pH, temperature and cations on the trinitrophenylation of butylamine by TNBS or PC under conditions analagous to those used with erythrocyte membranes was carried out. The results of these experiments are briefly summarized in Table 1*a* and *b*. The data in Table 1*a* illustrate the lack of effect of Mg^{++} (1 mM) on the incorporation of TNBS into butylamine, as contrasted with the situation in membranes where very marked effects on both activation energy and rate are apparent. The important points to be noted in Table 1*b* are the following:

1. For both probes, decreasing pH predictably produced a decrease in the rate and an increase in the activation energy of butylamine modifi-

cation, both presumably reflecting the decreased reactivity of amino groups on protonation.

2. Modification of membrane amino groups by TNBS exhibited a much narrower range of variation in rate with pH than modification of butylamine amino groups, and although the rate of TNBS incorporation into membranes decreased with decreasing pH (as was the case with butylamine), activation energy also progressively decreased (the reverse of the situation with butylamine).

3. A comparison of the relative rates of modification of butylamine and membranes by TNBS or picryl chloride showed that, while picryl chloride reacted somewhat less readily with butylamine amino groups than TNBS, the reaction of membrane amino groups was very much more rapid with picryl chloride as compared with TNBS.

It was clear, therefore, that the behavior of TNBS and PC in the model butylamine system differed considerably from that in the erythrocyte membrane, and, in the studies to be described in the remainder of this work, these differences are analyzed in terms of factors relating to membrane structure, including probe permeability and ion-induced membrane structural perturbations.

Cation-Induced Membrane Structural Alterations

The influence of divalent or monovalent cation size (ionic radius) on the rate and activation energy for TNBS incorporation into erythrocyte membranes was examined. Rates of TNBS incorporation in the absence or presence of added cations were studied as a function of temperature and expressed in the form of Arrhenius plots (Fig. 1) from which activation energies and rates at 25 °C were determined. The properties of divalent cations (Fig. 2) which were size dependent contrasted with those of monovalent cations (Fig. 3) which were independent of ionic radius, suggesting differences in the underlying mechanisms whereby these two groups of cations influenced TNBS incorporation. Further, the different size-dependence relationships of divalent cations with respect to activation energy and rate would appear to indicate that these two measured quantities are reflecting at least partially distinct structural consequences of membrane-cation interaction. The following series of experiments represents an effort to explore the molecular basis of these observations in more detail.

Since the TNBS molecule exists predominantly as an anion in the

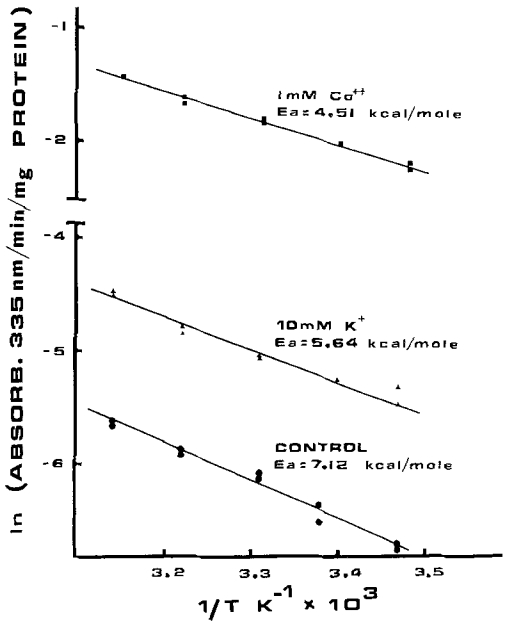

Fig. 1. Arrhenius plots for TNBS incorporation into erythrocyte membranes in the absence or presence of monovalent (K^+, 10 mM) and divalent (Co^{++}, 1 mM) cations. TNBS incorporation was measured as described in Materials and Methods at five temperatures in the range 5–45 °C using reaction times of 30 min, 10 min and 60 sec for control, 10 mM K^+ and 1 mM Co^{++} samples, respectively. Duplicate determinations were always performed. Regression lines of resulting Arrhenius plots were determined using a Compucorp 140 statistical calculator and activation energies (E_a) were evaluated by multiplying the slope by the gas constant R

slightly alkaline (pH 8.0) reaction medium and the incorporation of this anionic chemical probe is increased by cations, a question arises as to the extent to which the anionic character of TNBS influences the observed effects of cations on rate or activation energy. The external surface of the erythrocyte membrane is rich in anionic carboxylate groups derived from sialic acid [5, 42]. Such anionic groups might exert an effect on TNBS incorporation either under basal conditions or as modulated by variations in the pH or inorganic cation composition of the medium. Experiments to investigate these possibilities were performed using membranes treated with neuraminidase, which removed about 70% of the membrane-bound sialic acid. Neuraminidase treatment did not produce any appreciable alteration in the pH dependence or cation stimulation of TNBS incorporation (Fig. 4). These pH dependence studies again illustrated the lack of parallelism between rates and activation energies first seen in the divalent cation experiments. Thus, despite a downward

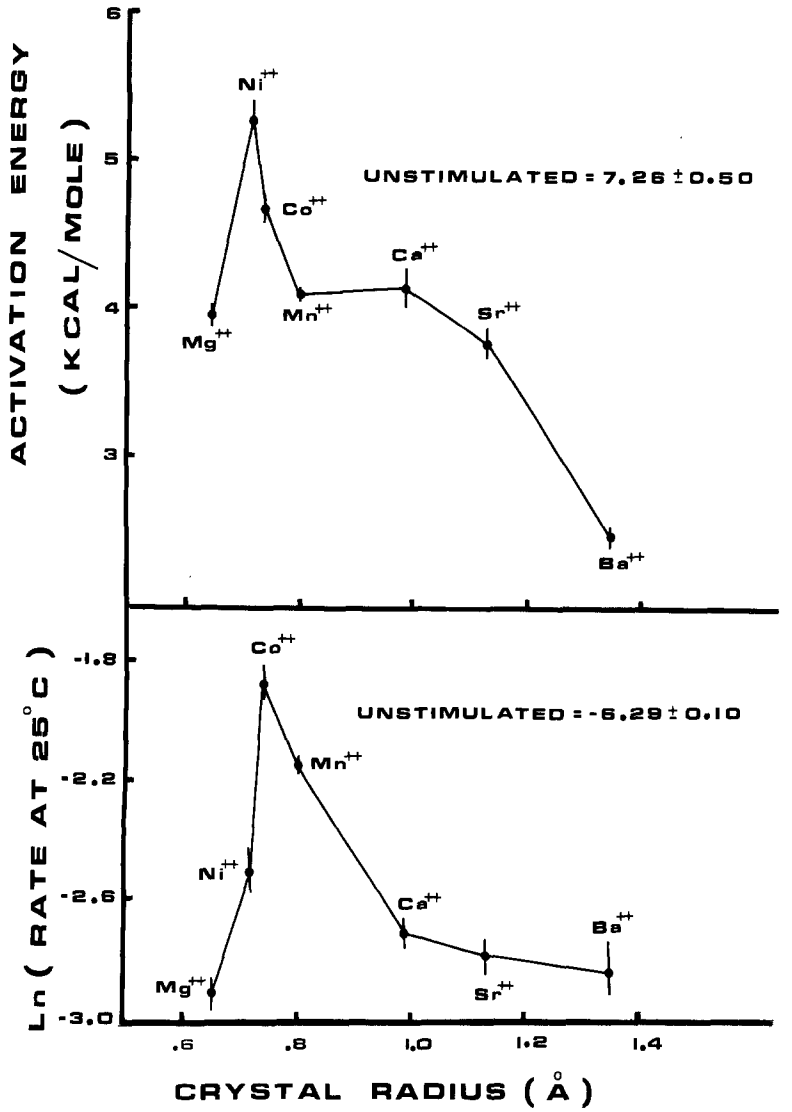

Fig. 2. Effect of divalent cation size on the activation energy and rate (at 25 °C) of TNBS incorporation into erythrocyte membranes. All cations were tested at a final concentration of 1 mM using reaction times of 60 sec. Activation energies and rates (at 25 °C) were evaluated from Arrhenius plots (*see* Fig. 1). Each point represents the mean ± SEM of duplicate experiments performed with three different membrane preparations. Activation energy and rate at 25 °C for samples in the absence of added divalent cations (unstimulated) using a reaction time of 30 min are included for comparison

trend in activation energy for TNBS incorporation with decreasing pH, rates of incorporation decreased as well. This behavior, which differed from the parallel increases in rate with decreases in activation energy

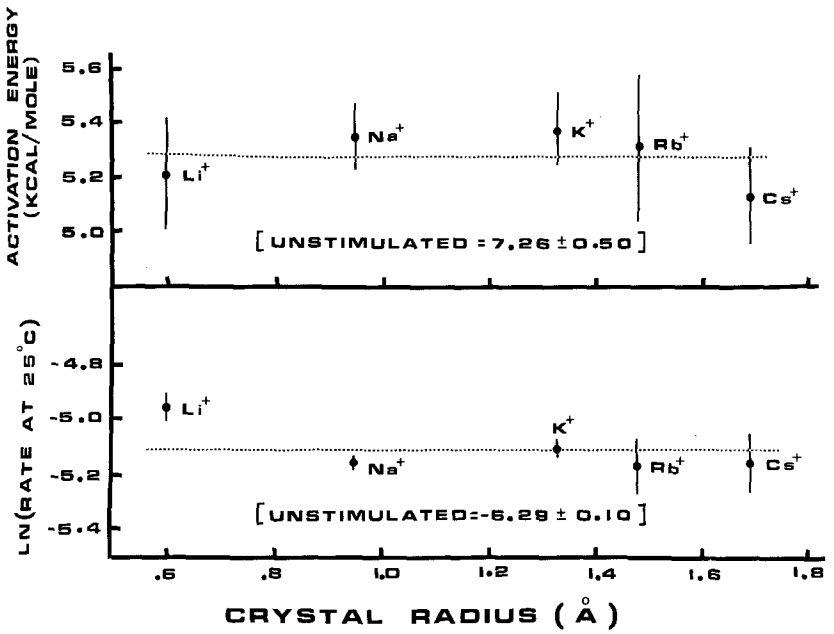

Fig. 3. Effect of monovalent cation size on the activation energy and rate (at 25 °C) of TNBS incorporation into erythrocyte membranes. All cations were tested at a final concentration of 10 mM. Activation energies and rates were evaluated as in Fig. 2. Each point represents the mean ± SEM of duplicate experiments using three different membrane preparations

seen previously in the butylamine model studies, was attributed to pH-dependent membrane structural alterations influencing amino group reactivity independently of the effects of bulk medium pH on amino group protonation.

The role of probe charge was analyzed more directly by examining the properties of picryl chloride (PC), a neutral trinitrophenylating analogue of TNBS. The unstimulated rate of incorporation of PC (Fig. 5) was approximately three orders of magnitude greater than that for TNBS (Fig. 2) despite rather similar activation energies in each case. All divalent cations, with the exception of Ni^{++}, produced a small, size-independent decrease in activation energy for PC incorporation relative to the control (unstimulated) conditions (Fig. 5). This behavior clearly differed both qualitatively and quantitatively from the situation with TNBS incorporation (Fig. 2) and suggested that changes in activation energy for TNBS incorporation produced by divalent cations primarily reflected membrane structural alterations influencing probe permeation. In contrast, the rate-ionic radius profile for PC incorporation (Fig. 5) was qualitatively very

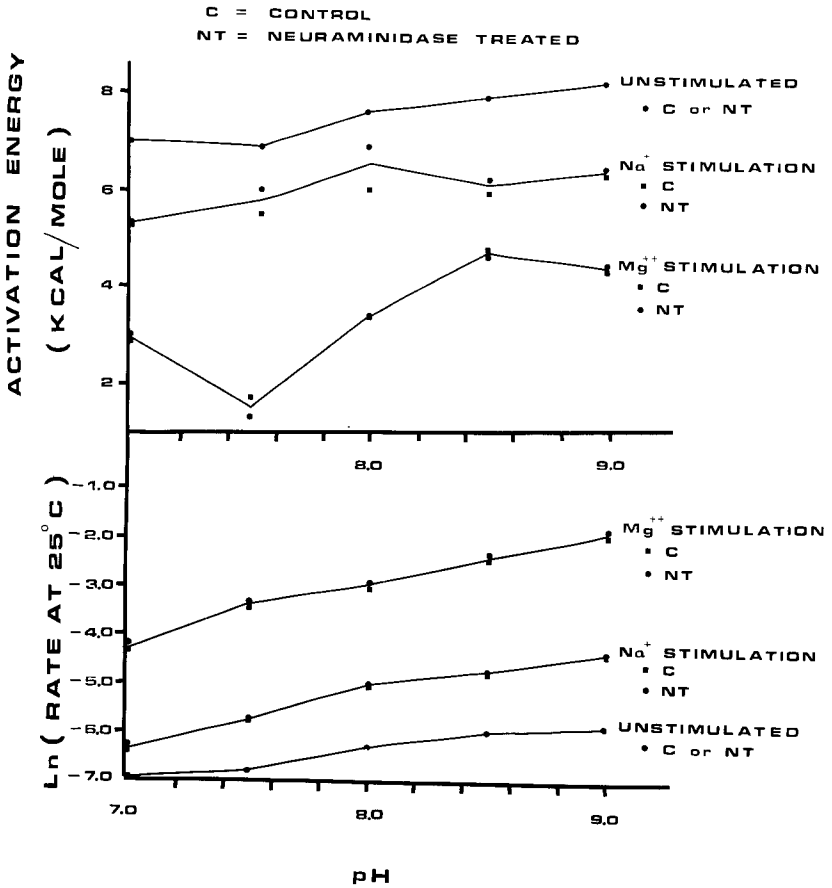

Fig. 4. Effects of neuraminidase treatment on the pH dependence of TNBS incorporation in the absence or presence of inorganic cations. Membranes were treated with neuraminidase and $CaCl_2$ in sodium acetate buffer (pH 5.5) as described in Materials and Methods. Control membranes were treated identically, except for the omission of neuraminidase. Activation energies and rates were determined as in Figs. 2 and 3

similar to that for TNBS incorporation (Fig. 2) with the exception of Ni^{++}. The anomalously high activation energy and low rate of PC incorporation may well be interrelated and attributable to the same type of molecular perturbation. However, the overall pattern of cation-membrane interaction characterized by a nonparallelism between effects of cations on activation energy and rate was similar for both TNBS and PC incorporation.

The effects of prior membrane modification by proteolytic or phospholipase enzymes on divalent cation-stimulated TNBS incorporation were examined. Activation energies for all cations except Ni^{++} and Co^{++} were increased by trypsinization (Fig. 6, *upper*). Phospholipase

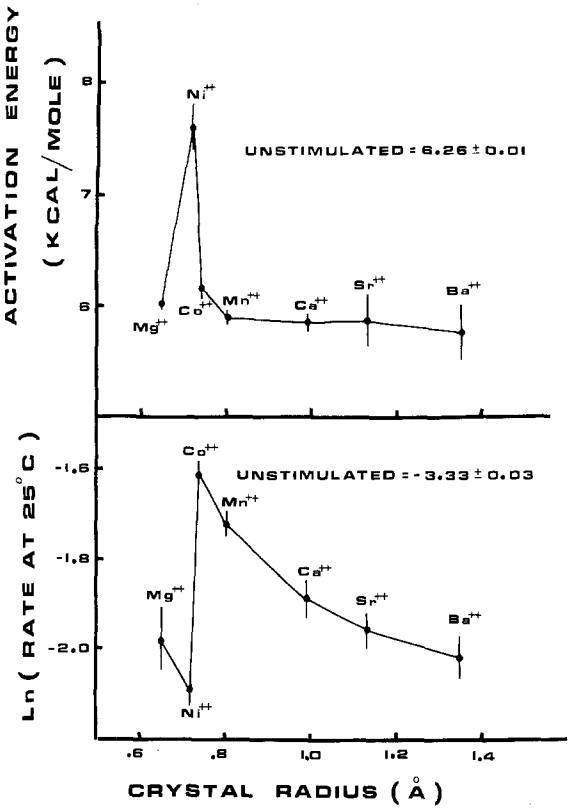

Fig. 5. Effect of divalent cation size on the activation energy and rate (at 25 °C) of PC incorporation into erythrocyte membranes. Experimental details were the same as in Fig. 2 except that PC dissolved in ethanol replaced TNBS. Reaction times of 60 sec were utilized as before. Each point represents the mean ± SEM of duplicate experiments performed with three different membrane preparations. Corresponding values in the absence of added divalent cations (unstimulated) using a reaction time of 2 min are included for comparison

A treatment, on the other hand, did not alter activation energies for Mg^{++}, Ca^{++} or Sr^{++}, but increased this quantity for the other ions. When the effects of enzyme pretreatment on rates of TNBS incorporation at 25 °C were examined (Fig. 6, *lower*), it was found that trypsinization only altered the rates for Ni^{+} and Co^{++}, while phospholipase A treatment markedly decreased incorporation rates for all ions, the largest decreases occurring with Mg^{++}, Ca^{++}, Sr^{++} and Ba^{++}. These results suggest an important role of membrane proteins in determining activation energy and of membrane phospholipids in determining rate of TNBS incorporation. It would also appear from these experiments that divalent cations differ appreciably in their ability to influence or be influenced by protein and phospholipid components of the membrane.

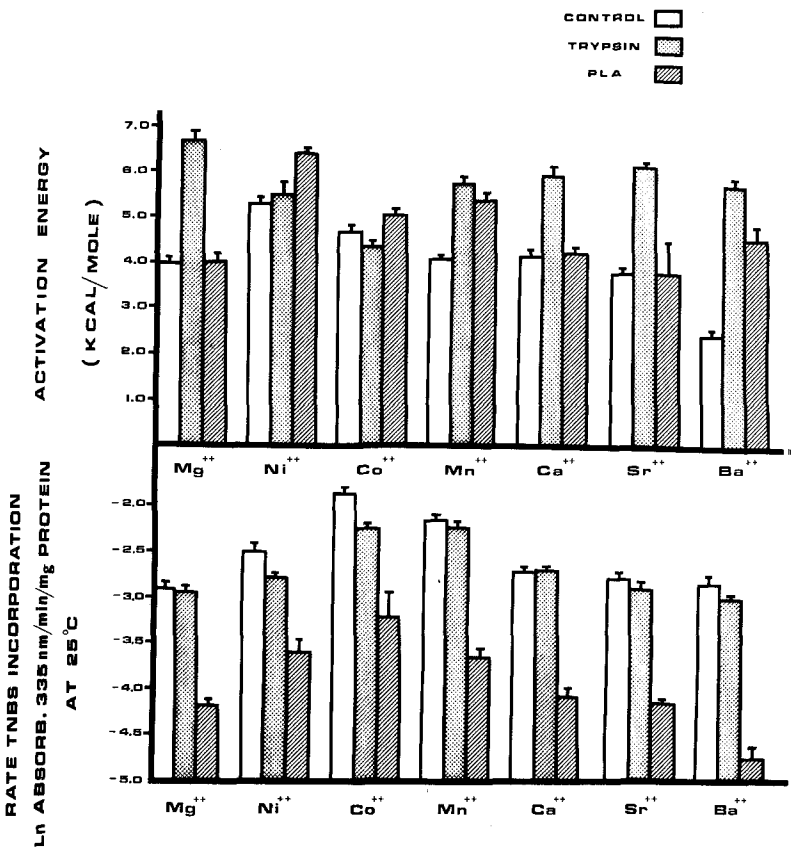

Fig. 6. Effects of divalent cations in control, trypsinized and phospholipase A-treated membranes. Membranes were pretreated with trypsin or phospholipase A as described in Materials and Methods. TNBS incorporation as a function of temperature (in the range 10–45 °C) was measured in the presence of divalent cations at a final concentration of 1 mM using a reaction time of 1 min for control or trypsinized membranes and 2 min for phospholipase A-treated membranes. Arrhenius plots for phospholipase A-treated membranes exhibited a discontinuity above 30 °C, so that only points below and including 30 ° were used in the determination of activation energies and rates. Each value represents the mean ± SEM of experiments using a minimum of two different membrane preparations

Membrane Labelling Studies

A more direct approach to the relative protein- and phospholipid-perturbing properties of divalent cations involved a study of their influence on the covalent incorporation of chromophoric trinitrophenyl groups derived from TNBS into membrane protein and phospholipid components. Membranes were labelled with TNBS in the absence or

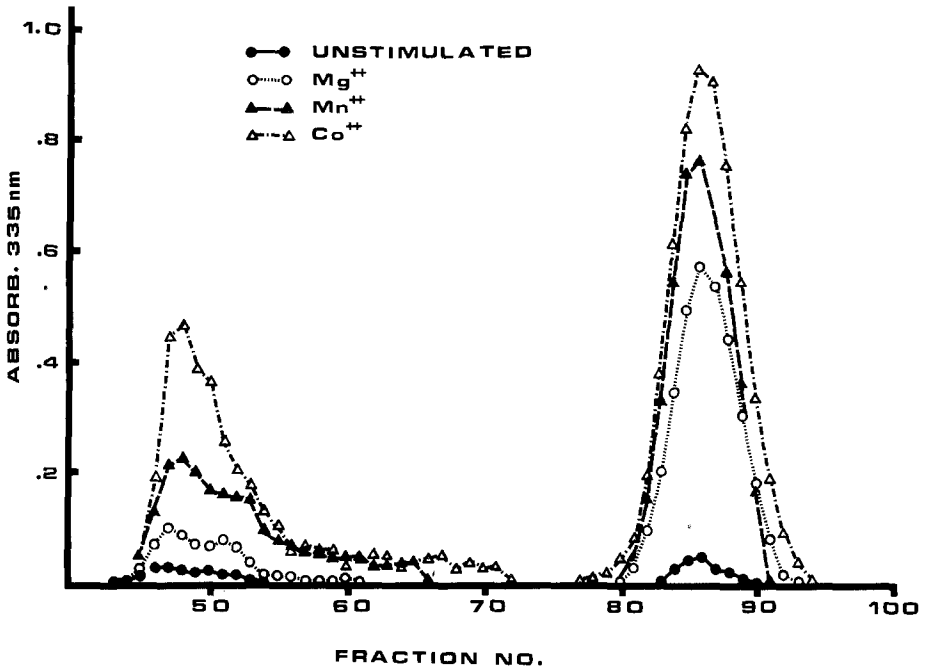

Fig. 7. TNBS labelling patterns of erythrocyte membranes in the absence or presence of divalent ions. Membranes (2.0 ml) were labelled in the absence or presence of divalent cations (at a final concentration of 1 mM), washed, and solubilized as described in Materials and Methods. 1.0 ml aliquots were applied to a 16 × 100 cm Sephadex G-200 column and eluted with 1% sodium dodecyl sulfate-0.02% sodium azide-0.05 M ammonium bicarbonate. 50 drop fractions were collected and absorbance at 335 nm monitored

presence of divalent cations, and the relative order of cation stimulatory potencies was the same whether determined following a 1 min incubation (the reaction time employed in the previously described kinetic studies), a 60 min incubation (as in the present labelling studies), or a 24 hr incubation (at which point maximal incorporation for each ion had been attained). This latter observation indicated that cation-induced structural perturbations affected not only rate but also the maximal level of incorporation. Labelled membranes were solubilized in sodium dodecyl sulfate and membrane protein and lipid components were resolved by gel filtration using Sephadex G-200. Proteins eluted in a rather complex pattern beginning at the void volume and were completely separated from phospholipids, which eluted considerably later. This completeness of separation allowed a detailed analysis of the effects of cations on protein and lipid components of the erythrocyte membrane as exemplified by the TNBS labelling patterns shown in Figure 7. Two observations are immediately apparent from these data. First, the presence of divalent

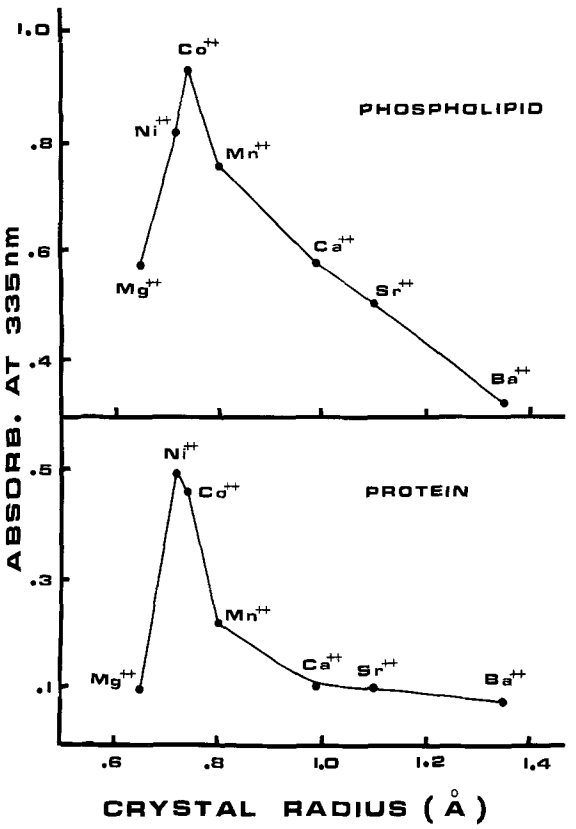

Fig. 8. Summary of divalent cation-induced incorporation of TNBS into resolved protein and phospholipid components of erythrocyte membranes. Each point represents the maximal absorbance at 335 nm for protein and phospholipid components resolved by Sephadex G-200 chromatography (*see* Fig. 7)

cations is associated with a marked stimulation of TNBS incorporation into both protein and lipid components; and secondly, cations differ in their ability to perturb protein and lipid components. Fig. 8 summarizes the results obtained with all the divalent cations studied and illustrates that the phospholipid- and protein-perturbational effects of these ions exhibit different size dependences. Particularly noteworthy was the parallel between the characteristics of phospholipid labelling and the rate-ionic radius profile for TNBS incorporation, both of which were maximal with cobalt ion (Fig. 2). Thin-layer chromatographic analysis of the phospholipids in labelled membranes revealed that TNBS labelling was almost entirely confined to phosphatidyl ethanolamine (PE), with marginal modification of the other major aminophospholipid phosphatidyl serine (PS) (Fig. 9). Protein labelling, on the other hand, exhib-

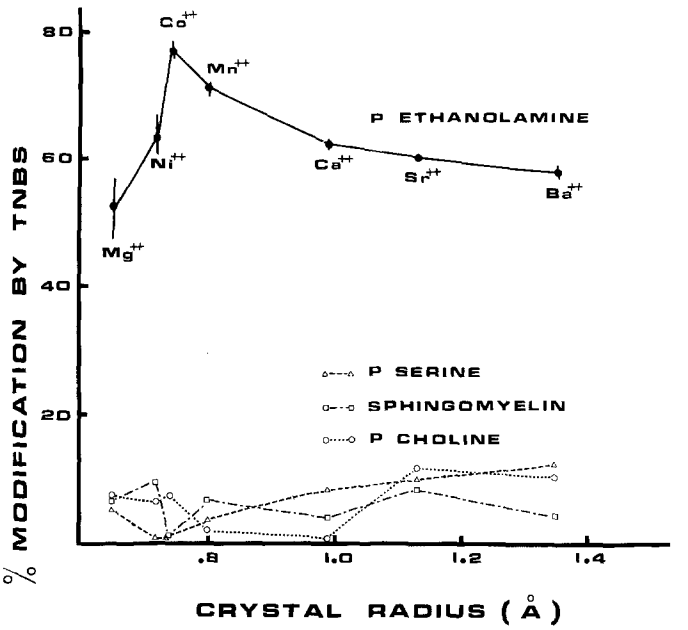

Fig. 9. Modification of membrane phospholipids by TNBS—effects of divalent cations. Membranes (2.0 ml) were labelled with TNBS (as in Fig. 7) and lipids extracted using chloroform/methanol (2:1, v/v). Extracts were washed with 0.75% sodium chloride, evaporated to near dryness under nitrogen and the residue dissolved in chloroform/methanol (2:1, v/v). Individual phospholipids were quantified by thin-layer chromatography using chloroform/methanol/ammonia (14:6:1, v/v/v) followed by elution of individual spots from the silica gel with methanol and phosphate analysis (*see* Materials and Methods). The per cent of each phospholipid modified was taken as 100 minus the percentage of total phosphorus in the extract the phospholipid comprised. Each point represents the mean ±SEM for three different membrane preparations

ited a different dependence on ion size, so that maximal effects were obtained with nickel ion (Fig. 8). This situation was reminiscent of the activation energy-ionic radius profile for TNBS incorporation described earlier (Fig. 2).

The membrane perturbational effects of each divalent cation, therefore, represents some balance between protein and phospholipid structural alterations. It seemed reasonable that ions perturbing membranes by the same type of mechanism would exhibit some constant relationship between phospholipid and protein labelling which would be independent of actual ion potency. We therefore determined the ratio of phospholipid labelling to protein labelling for each of the divalent cations studied, and the results are presented in Table 2. Cations increased phospholipid labelling preferentially, so that in all cases ratios of phospholipid to

Table 2. Influence of cations on relative labelling of phospholipds and proteins by TNBS

Cation[a]	Ratio $\frac{\text{maximal phospholipid labelling}}{\text{Maximal protein labelling}}$
None	1.4 ± 0.2
Mg^{++}	5.6 ± 0.3
Ca^{++}	5.8 ± 0.2
Sr^{++}	5.3 ± 0.3
Ba^{++}	4.8 ± 0.4
Ni^{++}	1.8 ± 0.2
Co^{++}	2.2 ± 0.1
Mn^{++}	3.4 ± 0.1
Na^{+}	3.0 ± 0.2

[a] Divalent cations were tested at a concentration of 1 mM, Na^{+} at a concentration of 10 mM. These results represent the mean ± SEM obtained with two different membrane preparations. Membranes were labelled with TNBS and chromatographed as described for Fig. 7. Maximal phospholipid and protein labelling were estimated from the maximal absorbance at 335 nm of resolved phospholipid and protein peaks for each ion.

protein labelling were greater than 1 (the ratio in the absence of added cations). The alkaline earth cations (Mg^{++}, Ca^{++}, Sr^{++} and Ba^{++}) appeared to form a distinct group with ratios of approximately 5, while other divalent cations, already shown to possess marked protein perturbing properties, formed a heterogeneous group with ratios in the range of 2–3. The results with 10 mM Na^{+} indicate that nonspecific ionic strength effects also favor the labelling of phospholipids over proteins.

Membrane Sulfhydryl Group Reactivity

The protein perturbational effects of divalent cations were examined using an alternative approach involving a study of their influence on the reactivity of membrane sulfhydryl groups towards 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) [10]. Results were expressed as incremental increases (or decreases) in DTNB incorporation in the presence of divalent cations relative to the situation in the absence of added cations (Fig. 10). All divalent cations except nickel and cobalt produced comparable increases in sulfhydryl group labelling, possibly as the result of a rather nonspecific structural effect. Nickel produced a significantly greater enhancement of DTNB incorporation than other ions, while co-

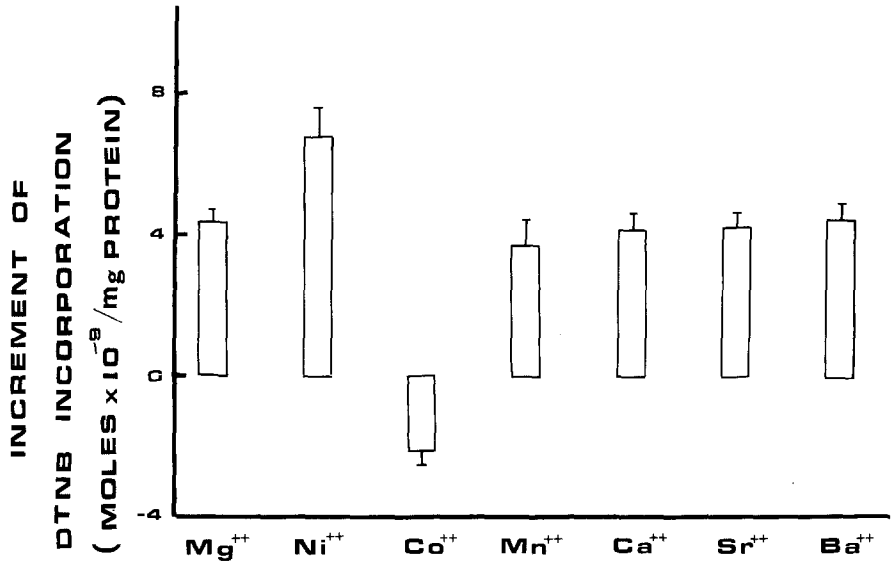

Fig. 10. Modification of membrane sulfhydryl groups by DTNB in the presence of divalent cations. Membranes (0.2 ml) were combined with DTNB (10 mM, final concentration), imidazole buffer (pH 7.4, 50 mM final concentration) and divalent cation (final concentration 1 mM) in a total volume of 3.0 ml. Following incubation for 30 min at 37 ± 0.5 °C, samples were centrifuged and from the absorbance of supernatants at 412 nm the number of moles of membrane sulfhydryl modified was calculated [10]. Results are expressed as incremental increases (or decrease, in the case of Co^{++}) in DTNB incorporation in the presence of divalent cation relative to the control situation in the absence of added cations. Each point represents the mean ± SEM of experiments with three different membrane preparations

balt reduced sulfhydryl group reactivity. This latter effect probably results from complexation of cobalt ion with membrane sulfhydryls. Regardless of underlying mechanisms, however, these experiments demonstrated that divalent nickel and cobalt ions differ from each other in their protein perturbing properties.

Hemolysis of Intact Erythrocytes

A logical extension of the foregoing studies of the membrane perturbational effects of monovalent and divalent cations in the isolated erythrocyte membrane was an investigation of the functional consequences of these structural alterations in intact erythrocytes. The influence of these ions on the hemolysis of erythrocytes exposed to hypotonic stress was examined. The results of these experiments using divalent cations or monovalent cations, at final concentrations of 1 and 10 mM, respectively,

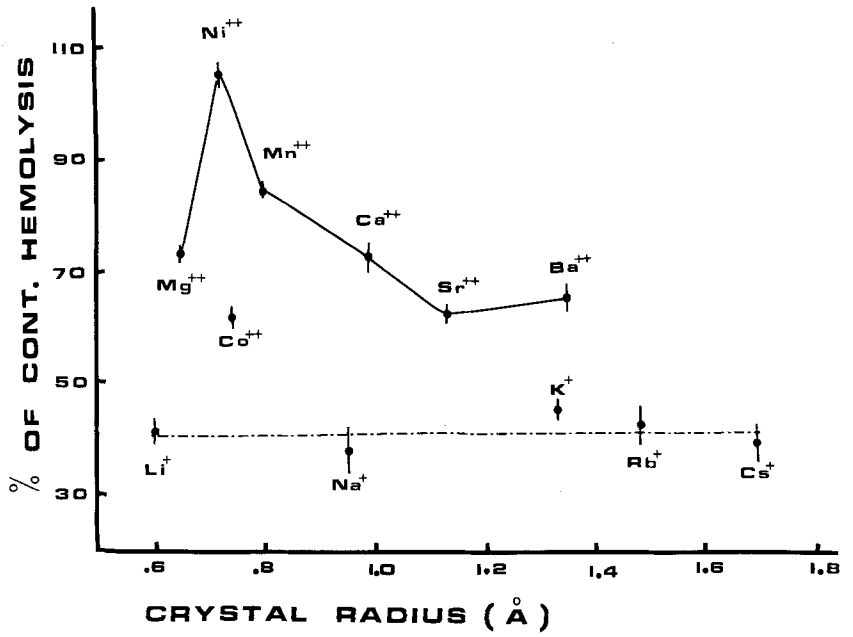

Fig. 11. Effects of monovalent (10 mM final concentration) and divalent (1 mM final concentration) cations on hypotonic hemolysis of intact erythrocytes. The hemolytic procedure, which involved an initial equilibration of cation with the erythrocytes followed by a hypotonic challenge, is described in *Methods*. Results were expressed as per cent control hemolysis (in the absence of added cations) which averaged between 55 and 65% of the total hemolysis (in distilled water). Each point represents the mean ± SEM of triplicate experiments using three different batches of blood

are shown in Fig. 11. The monovalent cations produced a size-independent decrease in hemolysis which was most likely attributable to changes in ionic strength. In contrast, divalent cations exerted effects which were markedly dependent on ionic radius and all ions, with the exception of nickel, decreased hypotonic hemolyis relative to the control (where no divalent cations were present). The antihemolytic-ionic radius profile (Fig. 11) bore a resemblance to the patterns for both activation energy for TNBS incorporation and protein labelling by TNBS. The deviation of cobalt ion from the foregoing bell-shaped relationship characterizing antihemolysis may well result from a stabilization of membrane sulfhydryls (Fig. 10), the integrity of which is known to be an important determinant of hemolysis [44]. For the other divalent cations, a correlation ($r=0.91$) appeared to exist between hemolysis behavior in intact erythrocytes (Fig. 11) and ratio of TNBS incorporation into phospholipids and proteins of isolated erythrocyte membranes (Table 2). The qualitative

(size-independent) effects of monovalent cations in both systems were also in agreement.

Discussion

The influence of inorganic cations on the structural and functional characteristics of biological membranes is likely mediated by some combination of charge neutralization or screening [6] and chelation, on the one hand, and associated configurational alterations on the other. Although a considerable amount of information is available regarding the many diverse functional consequences of membrane-cation interaction in various systems, the lack of fundamental information regarding the structural consequences of membrane-cation interaction has precluded a detailed understanding of these effects at the molecular level. The experiments described here represent an attempt to derive such information using the human erythrocyte as a model membrane system.

Our studies have shown that the interaction of divalent cations with erythrocyte membranes causes extensive perturbation of both protein and protein amino group reactivity indirectly as a result of membrane pendent effects of divalent cations on TNBS incorporation (Fig. 2) contrasted with the weaker, size-independent stimulation produced by monovalent cations (Fig. 3). Since model studies with butylamine showed that cations did not appreciably alter intrinsic amino group reactivity towards TNBS, it was assumed that ions influenced membrane lipid and protein amino group reactivity indicrectly as a result of membrane structural perturbations. Our observation that divalent cations can also influence the maximal level of incorporation of TNBS into membranes suggests that accessibility factors are important in determining this enhanced amino group reactivity. In the case of monovalent cations, stimulation of TNBS incorporation was attributed to nonspecific ionic strength effects, while such an explanation was clearly inadequate to explain the behavior of divalent cations. The structural alterations induced in protein and phospholipid components by divalent cations differed in size dependence, so that Ni^{++} produced maximal incorporation of TNBS into protein, while phospholipid labelling was greatest in the presence of Co^{++} (Fig. 8). The size dependence of divalent cation-stimulated protein labelling tended to parallel the effects of these ions on Arrhenius activation energy for TNBS incorporation, and divalent cation-stimulated phospholipid labelling exhibited a size dependence similar to that observed

for cation-induced increases in rate of TNBS incorporation at 25 °C (Fig. 2). This apparent correlation between structural and kinetic aspects of TNBS incorporation suggested a role for membrane proteins in influencing activation energy and for membrane phospholipids in determining rate of TNBS incorporation.

Activation energy for TNBS incorporation appeared to be largely determined by electrostatic features relating to probe permeation. Thus, the activation energy for the incorporation of picryl chloride, a neutral analogue of the anionic TNBS molecule, was minimally affected by divalent cations (Fig. 5). Experiments utilizing proteolytic or phospholipase enzyme pretreatment of membranes provided evidence that proteins are important in determining the cation-sensitive permeability barrier to TNBS. For most divalent cations, while phospholipase A pretreatment had no significant effect on activation energy, trypsinization markedly decreased the ability of cations to lower activation energy (Fig. 6, *upper*). Membrane sialic acid carboxyl groups, which may be released on trypsinization [20], did not appear to influence the permeation barrier, since extensive depletion of membrane sialic acid with neuraminidase did not alter basal or cation-stimulated TNBS incorporation (Fig. 4). The insensitivity of Ni^{++} and Co^{++} to the effects of trypsinization (Fig. 6, *upper*) and the patterns of membrane sulfhydryl group reactivity towards DTNB in their presence (Fig.10) served to distinguish the membrane actions of these two ions from those of the other divalent cations. The increased activation energy for PC incorporation in the presence of Ni^{++} (Fig. 5), another manifestation of its unique properties, suggested that this cation was able to influence permeation in a manner not directly related to the electrostatic characteristics of the probe.

Cation-dependent increases in the rate of TNBS modification were critically dependent on the integrity of membrane phospholipids. Phospholipase A treatment, which by hydrolyzing long-chain fatty acids of phospholipids drastically alters the hydrophobic characteristics of the membrane [17], results in a substantial decrease in cation-stimulated rates for all cations, with particularly large decreases being observed with Mg^{++}, Ca^{++}, Sr^{++} and Ba^{++} (Fig. 6, *lower*). Trypsinization, on the other hand, had no detectable effect on the increased rate of incorporation produced by these four cations. Despite the threefold greater rate of incorporation of PC as compared with TNBS (attributed, on the basis of our butylamine model studies, to greater permeation by PC rather than to a greater intrinsic reactivity), the dependence of rate on ionic radius was very similar for both probes (Fig. 5). The low rate

observed for Ni^{++} was presumably the result of the high activation energy term discussed previously. Perturbations in the labelling of PE paralleled divalent cation-induced increases in the rate of TNBS modification in whole membranes (Fig. 9). Although the decreased reactivity of PS towards amino group modifying reagents has been noted previously [16], the results of studies using artificial phospholipid systems demonstrating effective divalent cation binding by acidic phospholipids such as PS [21, 39], but not neutral phospholipids such as PE [19, 32], would perhaps have predicted that PS labelling would be influenced to a greater extent by divalent cations than labelling of PE. These findings, which illustrate the problems in attempting to extrapolate from effects of divalent cations in pure lipid systems to the situation in an intact membrane, may well be indicative of a modulatory influence of membrane structural components on the ability of membrane phospholipid anionic groups to interact with divalent cations and/or on the nucleophilic properties of phospholipid amino groups.

The perturbational effects of divalent cations on protein and lipid components probably involve some combination of direct effects and modulatory influences involving mutual lipid-protein interactions. In connection with the former, preliminary experiments using bovine serum albumin as a model protein have shown that certain similarities exist between stimulatory effects of divalent cations on the incorporation of TNBS into bovine serum albumin and into the protein components of erythrocyte membranes. These results are suggestive of an important role of direct protein-divalent cation interactions in determining the labelling of erythrocyte membrane proteins by TNBS. The role of alterations in lipid-protein interaction in mediating the membrane perturbational effects of divalent cations is currently being examined in our laboratory using bifunctional cross-linking agents. Regardless, divalent cations differ in their ability to perturb both lipids and proteins in the membrane and it would be expected that the functional consequences of membrane-ion interaction would depend in some manner on the balance between these two properties. On the basis of the ratio between phospholipid labelling and protein labelling, divalent cations fall into two groups, with the alkaline earths (Mg^{++}, Ca^{++}, Sr^{++} and Ba^{++}) forming one homogeneous group and Ni^{++}, Co^{++}, and Mn^{++} forming a second heterogeneous group (Table 1). Similar grouping of these cations may be made on the basis of their functional properties in excitable tissues. Thus, the slow inward current in mammalian ventricular myocardium can be carried not only by Ca^{++}, but also by Mg^{++}, Sr^{++} and Ba^{++} [23],

while Ni^{++}, Co^{++} and Mn^{++} inhibit this slow inward current [24]. Ca^{++} has been shown to exert little effect on K^+ conductance changes in squid axons, while Co^{++} and Ni^{++} slowed K^+ channel kinetics, an effect tentatively attributed to interaction with protein components of the membrane [2]. In studies of the influence of various divalent cations on presynaptic release of acetylcholine, the effects of Mg^{++}, Sr^{++} and Ba^{++}, which appeared to involve changes in charge density due to screening, could be distinguished from the more complex actions of Mn^{++} and Ni^{++}, which were believed to result from binding of these ions to motor axons and terminals [31]. The well-known ability of divalent cations to alter the conductance-voltage characteristics of nerve axons [12] has been attributed in some systems to a combination of screening and specific binding [6, 29], while in the crayfish axon, the identical effects of Mg^{++}, Ca^{++}, Sr^{++}, Ba^{++}, Ni^{++} and Co^{++} on the threshold for spike initiation suggest that screening alone is involved. Finally, in the present studies we have shown that divalent cations modify the hemolytic behavior of intact erythrocytes in a rather complex size-dependent fashion. In attempting to draw a parallel between the interaction of cations with intact erythrocytes and with isolated erythrocyte membranes, one is faced with a twofold problem. First, in intact cells, only the outer membrane surface can participate in cation binding, while in leaky ghosts, both inner and outer surfaces are accessible to divalent cations. Second, structural alterations produced during the course of membrane isolation may alter the consequences of membrane-cation interaction and/or lead to the exposure of additional cation binding sites. Despite these complexities, the similar antihemolytic properties and comparable ratios of phospholipid to protein labelling of alkaline earth cations (Fig. 11; Table 2) are consistent with a common site and/or mechanism of interaction of these ions with the membrane, possibly involving screening. The contrasting properties of Ni^{++}, Co^{++} and Mn^{++} most likely are governed by more specific interactions at the level of membrane proteins (Figs. 8, 10 and 11; Table 2). The similarities between the effects of cations in intact erythrocytes and in isolated erythrocyte membranes suggests that the external surface of the membrane may figure prominently in the observed membrane structural alterations. Experiments using resealed ghosts with normal or inverted orientation could provide more information on this point.

In conclusion, it would appear that some parallel may exist between the perturbational effects of divalent cations in erythrocyte membranes and in the membranes of electrically excitable tissues, as has been

suggested to be the case with lipid-soluble anesthetics [37, 40]. The qualitative and quantitative differences between the behavior of monovalent and divalent cations are interesting in the light of the proposal that membrane depolarization may involve configurational changes in axonal membranes resulting from alterations in the ratio of univalent to divalent cations at the external surface [18]. We are currently involved in the application of the approaches described here to the study of ion-induced changes in excitable membranes.

This work was supported by The Medical Research Council of Canada, Grant #68-4356. Maureen Garnett is the holder of a Medical Research Council Studentship.

References

1. Bartlett, G.R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**:466
2. Begenisich, T., Lynch, C. 1974. Effects of internal divalent cations on voltage-clamped squid axons. *J. Gen. Physiol.* **63**:675
3. Burger, S.P., Fujii, T., Hanahan, D.J. 1968. Stability of the bovine erythrocyte membrane. Release of enzymes and lipid components. *Biochemistry* **7**:3682
4. Burt, D.H., Green, J.W. 1971. The sodium permeability of butanol-treated erythrocytes. The role of calcium. *Biochim. Biophys. Acta* **225**:46
5. Cook, G.M.W., Eylar, E.H. 1965. Separation of the M and N blood group antigens of the human erythrocyte. *Biochim. Biophys. Acta* **101**:57
6. D'Arrigo, J.S. 1973. Possible screening of surface charges on crayfish axons by polyvalent metal ions. *J. Physiol. London* **231**:117
7. Dunn, M.J. 1974. Red blood cell calcium and magnesium: Effects upon sodium and potassium transport and cellular morphology. *Biochim. Biophys. Acta* **352**:97
8. Edwards, W., Phillips, J.H., Morris, S.J. 1974. Structural changes in chromaffin granules induced by divalent cations. *Biochim. Biophys. Acta* **356**:164
9. Ehrström, M., Eriksson, L.E.Göran, Israelachvili, J., Ehrenberg, A. 1973. The effects of some cations and anions on spin-labelled cytoplasmic membranes of *B. subtilis*. *Biochem. Biophys. Res. Commun.* **55**:396
10. Ellman, G.L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**:70
11. Flanagan, M.T., Hesketh, T.R. 1973. Electrostatic interactions in the binding of fluorescent probes to lipid membranes. *Biochim. Biophys. Acta* **298**:535
12. Frankenhaeuser, B., Hodgkin, A.L. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol. London* **137**:218
13. Godin, D.V., Schrier, S.L. 1970. Mechanism of inactivation of erythrocyte membrane adenosine triphosphatase by carbodiimides. *Biochemistry* **9**:4068
14. Godin, D.V., Wan Ng, T. 1972. Trinitrobenzenesulfonic acid: A possible chemical probe to investigate lipid-protein interactions in biological membranes. *Mol. Pharmacol.* **8**:426
15. Godin, D.V., Wan Ng, T. 1973. Studies on the membrane-perturbational effects of drugs and divalent cations utilizing trinitrobenzenesulfonic acid. *Mol. Pharmacol.* **9**:802

16. Gordesky, S.E., Marinetti, G.V., Segel, G.B. 1973. The interaction of 1-fluoro-2,4-dinitrobenzene with amino-phospholipids in membranes of intact erythrocytes, modified erythrocytes, and erythrocyte ghosts. *J. Membrane Biol.* **14**:229
17. Gul, S., Smith, A.D. 1972. Hemolysis of washed human red cells by the combined action of Naja naja phospholipase A_2 and albumin. *Biochim. Biophys. Acta* **288**:237
18. Inoue, I., Kobatake, Y., Tasaki, I. 1973. Excitability, instability and phase transitions in squid axon membrane under internal perfusion with dilute salt solutions. *Biochim. Biophys. Acta* **307**:471
19. Ito, T., Ohnishi, S.-I. 1974. Ca^{2+}-induced lateral phase separations in phosphatidic acid-phosphatidylcholine membranes. *Biochim. Biophys. Acta* **352**:29
20. Jackson, L.J., Seaman, G.V.F. 1972. Physicochemical properties of some glycopeptides released from human erythrocyte membranes by trypsin. *Biochemistry* **11**:44
21. Jacobson, K., Papahadjopoulos, D. 1975. Phase transitions and phase separations in phospholipid membranes induced by changes in temperature, pH, and concentration of bivalent cations. *Biochemistry* **14**:152
22. Jan, K.-M., Chien, S. 1973. Influence of the ionic composition of fluid medium on red cell aggregation. *J. Gen. Physiol.* **61**:655
23. Kohlhardt, M., Haastert, H.P., Krause, H. 1973. Evidence of non-specificity of the Ca channel in mammalian myocardial fiber membranes. *Pfluegers. Arch.* **342**:125
24. Kohlhardt, M., Herdey, A., Kübler, M. 1973. Interchangeability of Ca ions and Sr ions as charge carriers of the slow inward current in mammalian myocardial fibers. *Pfluegers Arch.* **344**:149
25. Lenard, J. 1970. The thiobarbituric acid assay of sialic acids. *Biochemistry* **9**:1129
26. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265
27. Manery, J.F. 1966. Effects of Ca ions on membranes. *Fed. Proc.* **25**:1804
28. Meiri, V., Rahamimoff, R. 1971. Activation of transmitter release by strontium and calcium ions at the neuromuscular junction. *J. Physiol. London* **215**:709
29. McLaughlin, S.G.A., Szabo, G., Eisenman, G. 1971. Divalent ions and the surface potential of charged phospholipid membranes. *J. Gen. Physiol.* **58**:667
30. Miledi, R. 1973. Transmitter release induced by injection of calcium ions into nerve terminals. *Proc. R. Soc. London* **183**:421
31. Muller, R.V., Finkelstein, A. 1974. The electrostatic basis of Mg inhibition of transmitter release. *Proc. Nat. Acad. Sci. USA* **71**:923
32. Ohnishi, S., Ito, T. 1974. Calcium-induced phase separations in phosphatidylserine-phosphatidylcholine membranes. *Biochemistry* **13**:881
33. Papahadjopoulos, D., Poste, G., Schoeffer, B.E., Vail, W.J. 1974. Membrane fusion and molecular segregation in phospholipid vesicles. *Biochim. Biophys. Acta* **352**:10
34. Pinto da Silva, P. 1972. Translational mobility of the membrane intercalated particles of human erythrocyte ghosts (pH-dependent, reversible aggregation). *J. Cell Biol.* **53**:777
35. Poste, G., Allison, A.C. 1973. Membrane fusion. *Biochim. Biophys. Acta* **300**:421
36. Razin, S. 1972. The reconstitution of biological membranes. *Biochim. Biophys. Acta* **265**:241
37. Roth, S., Seeman, P. 1971. All lipid-soluble anesthetics protect red cells. *Nature, New Biol.* **231**:284
38. Rubalcava, B., Martinez de Muñoz, D., Gitler, C. 1969. Interaction of fluorescent probes with membranes. I. Effect of ions on erythrocyte membranes. *Biochemistry* **8**:2742
39. Schnepel, G.H., Hegner, D., Schummer, U. 1974. The influence of calcium on the molecular mobility of fatty acid spin labels in phosphatidylserine and phosphatidyl-inositol structures. *Biochim. Biophys. Acta* **367**:67

40. Seeman, P. 1972. The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24**:583
41. Spero, L., Siemens, A.J., Toulis, G.A. 1973. Tetrodotoxin inhibition of Na^+-induced conformational change in excitable membranes. *Mol. Pharmacol.* **9**:330
42. Steck, T.L., Fairbanks, G., Wallach, D.F.H. 1971. Disposition of the major proteins in the isolated erythrocyte membrane. Proteolytic dissection. *Biochemistry* **10**:2617
43. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234**:1971
44. Weed, R.I., Reed, C.F. 1966. Membrane alterations leading to red cell destruction. *Am. J. Med.* **41**:681