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THE SODIUM PERMEABILITY OF BUTANOL-TREATED ERYTHROCYTES THE ROLE OF CALCIUM

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SUMMARY

1. The effects of mono- and bivalent cations, local anesthetics, treatment temperature and phospholipase C treatment on the sodium permeability of *n*-butanol-treated human red cells were investigated. The sodium content and per cent hemolysis of the erythrocytes were measured during and subsequent to treatment with 0.4 M butanol (final concn.) in otherwise isosmotic media.

2. It was found, with the use of ^{22}Na and under conditions precluding a change in cellular Na^+ concentration, that 0.4 M butanol effected a 20- to 40-fold increase of Na^+ influx at 15°.

3. The bivalent cations, Ca^{2+}, Sr^{2+} and Ba^{2+}, partially reversed the subsequent increase of Na^+ permeability of butanol-treated cells. This protective effect was in the order $Ca^{2+} > Sr^{2+} > Ba^{2+}$. Mg^{2+} had no effect. The monovalent cations, Li^+, Na^+, K^+, NH_4^+, Rb^+ and Cs^+, showed no meaningful effects.

4. The local anesthetics, tetracaine and procaine, partially reversed the effects of butanol in a manner similar to Ca^{2+}.

5. Ca^{2+} partially reversed the Na^+ hyperpermeability of butanol-treated erythrocytes which were also treated with phospholipase C. However, the bivalent cation did not return the Na^+ permeability to the non-phospholipase-treated control level.

6. It was shown that the Na^+ permeability and the ability of Ca^{2+} to reverse the effects of butanol were dependent on the incubation temperature during butanol treatment.

7. The results were discussed in terms of the mechanism of action of *n*-butanol and the role of calcium in preserving the integrity of the erythrocyte membrane.

INTRODUCTION

The exposure of human or rabbit erythrocytes to 0.4 M *n*-butanol in an otherwise isosmotic salt solution causes these cells to become hyperpermeable to Na^+ and K^+ (refs. 1–3). Although PARPART AND GREEN[2] suggested that the primary effect of the alcohol is to alter surface components of the erythrocyte membrane such as the lipoprotein complexes, few experiments have since been published which were designed

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to elucidate the exact nature of the butanol effect. SLOVITER AND TANAKA[4] treated human red cells with approx. 0.5 M *n*-butanol (final concn.) at 20° but could detect no decrease in the amount of cholesterol or total phospholipid in the membranes. However, no conclusion regarding rearrangement of membrane components can be made from such studies.

This study was undertaken to investigate the influence of Ca^{2+} upon the Na^+ permeability of *n*-butanol-treated red cells and the role Ca^{2+} may play in the structuring of the membrane. It will be shown that exogenous Ca^{2+} partially reverses the butanol-induced hyperpermeability to Na^+ and that this effect is localized at the cell membrane.

During the initial phase of this investigation, the authors proposed that the entrance of butanol into the microstructure of the membrane induced the red cell membrane to imbibe water. Since the effective dielectric constant within the membrane is quite low relative to that of aqueous solutions[5], the entrance of water molecules with their high dipole moments would increase the dielectric constant within the membrane. Therefore, the forces of attraction between oppositely charged groups would decrease (*i.e.* between Ca^{2+} and membrane negative groups, possibly phospholipid moieties).

Recent physicochemical studies of artificial phospholipid membranes[6–8] and of phospholipid–water dispersions[9–11] indicated that the relative amounts of Ca^{2+} and water in the artificial membranes controlled their permeability and the temperature and amount of water determined the liquid-crystalline phase of the phospholipid dispersions, respectively. In view of these investigations, studies were undertaken, here, to determine the effect of the bivalent cations and temperature on the butanol-induced hyperpermeability of human erythrocytes to Na^+.

METHODS

Collection and storage of erythrocytes

Venous blood was collected from healthy, male graduate students into acid–citrate–dextrose medium. The erythrocytes were washed three to four times with a cold, sterile salt solution: 133 mM KCl, 12 mM NaCl, 11 mM potassium phosphates (pH 7.4 at 7°). If the red cells were to be used more than 5 h after collection, they were stored at 4–5° as a 20 % (v/v) cell suspension in a sterile medium consisting of: 50 mM KCl, 12 mM NaCl, 45 mM K_2HPO_4, 10 mM KH_2PO_4, 10 mM $MgCl_2$, 11.1 mM glucose, and 1.12 mM adenosine (pH 7.4 at 4°). Streptomycin and penicillin were added at final concentrations of 50 μg/ml and 50 units/ml, respectively.

Butanol treatment

The method of treating the cells was similar to that of RINEHART AND GREEN[3] with the following exception. 1 vol. of 50 % cell suspension was treated with 4 vol. of 0.4825 M *n*-butanol treatment medium: 12 mM NaCl, 138 mM KCl, 0.4825 M *n*-butanol, 22.5 ideal milliosmolar Tris–phosphate (pH 7.4 at the temperature of treatment). In experiments determining the effect of calcium during butanol treatment, the Tris–phosphate was replaced with 12 mM Tris. The final concentration of butanol in the suspension was calculated using published values for the osmotically active

water in human erythrocytes[12,13] since butanol is known to distribute itself rapidly and equally across the human red cell membrane[14].

Butanol treatment was terminated by the addition of 1 vol. of ice-cold, butanol-free wash solution similar in electrolyte composition to that of the treatment medium. The cells were centrifuged in the cold and washed several times until the concentration of butanol had been reduced more than 200 times.

In each experiment, except those shown in Figs. 1 and 2a, all the red cells were treated together in the same flask. The washed cells were then split into separate vessels such that, for each experimental and control condition, there were quadruplicate incubation flasks.

The day to day variations in the effect of *n*-butanol on red cell permeability could not be satisfactorily explained nor controlled to a low level. However, in separate butanol treatments using the same batch of erythrocytes and performed at the same time, this variability was found to be minimal: mean $\pm$ 4.7 % of the mean. Therefore, in order to compare experiments performed at different times, control incubations were routinely carried out.

Incubation

The appropriate volume of loosely packed, washed red cells was transferred to an incubation medium at 37.5° in siliconized pyrex bottles. The final hematocrit did not exceed 5 %. The incubation medium had a high Na^+ concentration in contrast to the treatment and wash media: 135 mM NaCl, 5 mM KCl, 11.1 mM glucose, and 1.12 mM adenosine. The buffer was either Tris (15 mM) or Tris–phosphate (27.8 ideal milliosmolar) (pH 7.4 at 37.5°). Bivalent cations (as the chlorides) were added from isotonic stock solutions; the appropriate volume of isosmotic choline chloride was added to control media. The local anesthetics, tetracaine–HCl and procaine–HCl, were added from freshly prepared stock solutions; both were recrystallized several times from water before use.

Analytical procedures

After the addition of butanol-treated erythrocytes to the incubation medium, sampling for hemolysis and Na^+ determinations was done over a period from 0 to 5 h. For cellular sodium determinations, the red cells were rapidly washed in ice-cold, isosmotic choline chloride, lysed in 4 mM Tris (pH 9.4), and analyzed with a Na^+-sensitive electrode (Corning No. 476210). No interference from cellular substances was found. Immediately after the sodium analysis, an aliquot of the hemolysate was diluted with Drabkin's solution for the determination of hemoglobin[15].

When the uptake of radioactive $^{22}Na^+$ was determined during butanol treatment, the radioactivity of cells and media was measured in a NaI(Th) crystal well-counter using a Baird-Atomic spectrometre.

The phospholipase C preparation used in this study was judged to be free of contaminating protease activity according to the method of McDONALD AND CHEN[16]. No trichloroacetic acid soluble peptides were detectable in phospholipase C treated red cell ghosts.

The significance of differences between experimental means was evaluated according to the "Student's *t*" test[17]. All differences reported are significant to, at least, the 5 % level.

RESULTS

The uptake of $^{22}Na^+$ during butanol treatment

Incubation of erythrocytes in the presence of 0.4 M butanol and $^{22}Na^+$ results in an increased influx of Na^+ (Fig. 1). Between time zero and 20 min, the influx was approx. 20 times greater than in the control cells. A further increase was observed between 20 and 80 min, when the influx was about 40 times that of the control. Under the conditions of treatment, cellular Na^+ concentration did not change throughout the experiment.

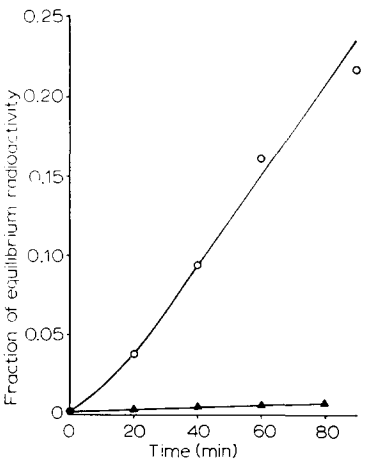

Fig. 1. The relative rate of entry of Na^+ into erythrocytes in the presence and absence of 0.4 M *n*-butanol. Fresh erythrocytes were suspended in radioactive media (labeled with $^{22}Na^+$) containing 12 mequiv Na^+, 150 mequiv K^+, and 55 mmoles phosphate per l either in the presence of 0.4 M *n*-butanol or in its absence. Samples were removed at the times indicated, washed, and centrifuged at 30000 × *g* for 15 min at 4°. 0.1 ml of cells and whole suspension were separately assayed for radioactivity. ○, 0.4 M butanol; ▲, no butanol. The fraction of equilibrium radioactivity = counts/min in cells/total counts/min.

The effects of bivalent cations on hemolysis and Na^+ uptake

When erythrocytes were treated with butanol in the presence of 4 mM $CaCl_2$ and then incubated with or without 4 mM $CaCl_2$, the cells which were exposed to Ca^{2+} throughout gained less Na^+ (Fig. 2a). Red cells treated and incubated in Ca^{2+}-free media gained the most Na^+, while those which were exposed to Ca^{2+} only during one of the procedures were intermediate in the uptake of Na^+.

Other bivalent cations tested, with the exception of Mg^{2+}, partially reversed the disruption of the erythrocyte membrane caused by prior treatment with *n*-butanol (Figs. 2b and 2c). The order of effectiveness with regard to inhibition of hemolysis was $Ca^{2+} > Sr^{2+} > Ba^{2+}$. As the concentration of the bivalent cation increased, the amount of hemolysis decreased. This effect tended towards saturation between 4 and 10 mM for Ca^{2+} and Ba^{2+} and at about 10 mM for Sr^{2+}. The point for 10 mM Ca^{2+} was omitted because 100 % hemolysis was consistently obtained at that concentration. The bivalent cation concentration dependence of Na^+ uptake (Fig. 2c) showed the same qualitative effects as were seen upon hemolysis. At 2, 4 and 6 mM, the differences between the effects of calcium, barium and magnesium were significant (0.05). There

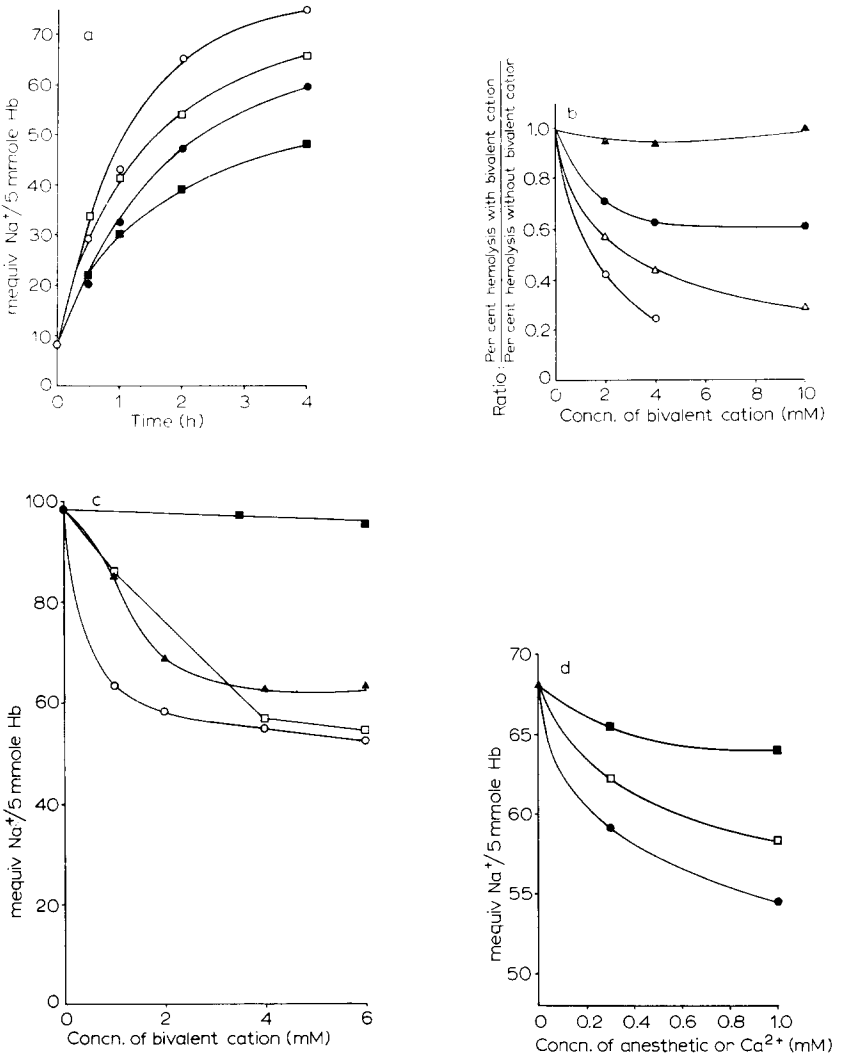

Fig. 2. (a) The effect of Ca^{2+} during butanol treatment and during incubation at 37° on the uptake of Na^+ by red cells at 37°. Erythrocytes were treated in "low Na^+" butanol treatment media at 15° for 30 min with and without 4.0 mM Ca^{2+}. After the treatment was stopped each population of butanol-treated cells was incubated at 37° in a medium containing 130 mequiv Na^+ per l and either 4.0 mM or no $CaCl_2$. Cellular sodium was measured at the indicated times during incubation at 37°. Conditions (butanol treatment, incubation, respectively): ○, Ca^{2+}-free, Ca^{2+}-free; □, Ca^{2+}-free, 4 mM Ca^{2+}; ●, 4 mM Ca^{2+}, Ca^{2+}-free; ■, 4 mM Ca^{2+}, 4 mM Ca^{2+}. (b) Bivalent cation inhibition of hemolysis of erythrocytes treated with butanol. The treatment conditions and incubation conditions are those described in METHODS. Samples were taken at 4.5 h. The ratios of % hemolysis were determined from the 4.5-h samples. ▲, Mg^{2+}; ●, Ba^{2+}; △, Sr^{2+}; ○, Ca^{2+}. (c) Bivalent cation inhibition of Na^+ uptake by butanol-treated red cells. The treatment and incubation conditions were the same as for (a). The Na^+ concentrations of the red cells were determined after 3 h of incubation. ■, Mg^{2+}; ▲, Ba^{2+}; □, Sr^{2+}; ○, Ca^{2+}. (d) The effect of local anesthetics and calcium on sodium uptake by butanol-treated red cells. After butanol treatment, erythrocytes were incubated at 37° in media containing several concentrations of local anesthetic or calcium. The erythrocyte sodium concentration was measured after 3 h at 37°. ■, Procaine; ●, tetracaine; □, Ca^{2+}; ▲, no addition.

was no significant difference between the effects of Ca^{2+} and Sr^{2+} at 4 and 6 mM.

In an attempt to determine the role of Ca^{2+} in erythrocyte membranes, the phospholipids of butanol-treated red cells were altered in two ways: reaction with local anesthetics and treatment with phospholipase C. There is good evidence that

TABLE I

THE EFFECTS OF PHOSPHOLIPASE C ON Na^+ PERMEABILITY AND MEMBRANE PHOSPHOLIPID CONTENT

Red cells were treated with 0.4 M *n*-butanol at 15° for 30 min. After termination of butanol treatment, the cells were divided and treated at 15° in one of the following manners: 10 μg per ml of phospholipase C (Worthington Biochemical Corp., Freehold, N.J.) *plus* 85 units/ml α-antitoxin (control); 10 μg/ml phospholipase alone (phospholipase C). Enzyme treatment was stopped by washing the cells 4 times with ice-cold isotonic buffer containing 2000 times as much α-antitoxin as enzyme. The cells were then incubated at 37.5° in the high Na^+ medium in the presence or absence of Ca^{2+}. At 1.5 h cellular sodium (Na^+_c) (mequiv/5 mmole hemoglobin) was measured, and at 2.25 h membrane phospholipid phosphate was determined by the method of DODGE AND PHILLIPS[20] (P). The values given in the table are the mean ± S.D. The values for the phospholipid phosphate are given in mmoles/5 mmole of hemoglobin.

Treatment	*Parameter measured*	*After treatment with:*		*Ca^{2+} concn. (mM)*	*After incubation in high Na^+ medium*
		Butanol	*Lipase*		
Control	Na^+_c	7.4 ± 0.1	7.4 ± 0.1	0	63.0 ± 0.9
				4	49.0 ± 0.6
	P	3.6 ± 0.1	3.5 ± 0.2	0	3.5 ± 0.2
				4	3.5 ± 0.3
Phospholipase C	Na^+_c	7.4 ± 0.1	7.4 ± 0.1	0	88.6 ± 0.7
				4	56.2 ± 1.1
	P	3.6 ± 0.1	2.7 ± 0.2	0	2.5 ± 0.4
				4	2.5 ± 0.2

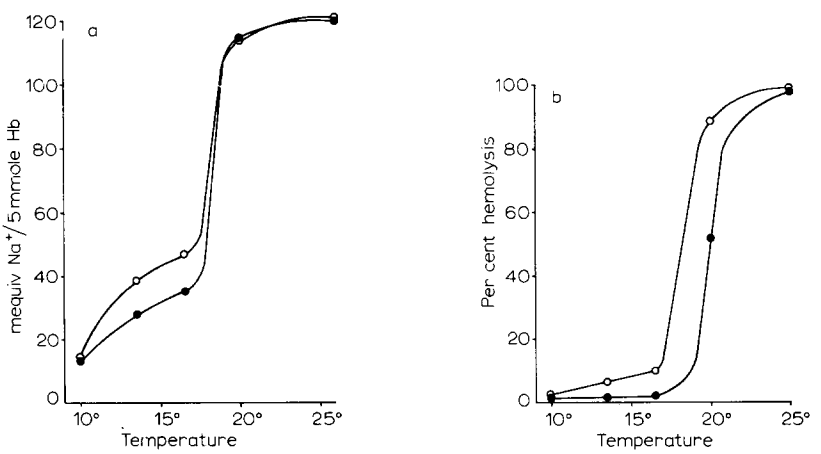

Fig. 3. (a) The effect of butanol treatment temperatures on the Ca^{2+}-inhibited Na^+ permeability of erythrocytes. Erythrocytes were treated at various temperatures for 30 min, at which time treatment was stopped and the cells were incubated at 37° for 2 h in a medium containing 130 mequiv Na^+ per l and either 0 or 4.0 mM Ca^{2+}. The butanol treatment medium was 12 mM NaCl, 138 mM KCl, 22.5 ideal milliosmolar Tris–phosphate, and 0.4 M (final concn.) *n*-butanol (pH 7.4 at treatment temperature). ●, 4 mM Ca^{2+}; ○, 0 mM Ca^{2+}. (b) The effect of butanol treatment temperature on the Ca^{2+}-inhibited hemolysis of erythrocytes. The conditions were the same as for (a).

both local anesthetics and Ca^{2+} chelate phospholipid molecules in a similar manner[18,19]. Therefore, the effects of procaine–HCl and tetracaine–HCl were studied and compared to those of Ca^{+} (Fig. 2d). The efficiency of reducing the Na^{+} uptake was tetracaine $> Ca^{2+} >$ procaine, which is the same order of binding to phospholipids[18].

Treatment of butanol-treated red cells with phospholipase C (phosphatidylcholine, cholinephosphohydrolase, EC 3.1.4.3) (an enzyme which hydrolyzes phospholipids to diglyceride and the phosphorylated nitrogenous base) resulted in a 24 % loss of membrane phospholipid (Table I). No changes occurred with respect to cellular Na^{+} concentration during butanol or enzyme treatment because the treatments were carried out in a medium containing 12 mequiv Na^{+}. Although the absolute decrease brought about with 4 mM Ca^{2+}, was greater in the phospholipase-treated cells, Ca^{2+} was not able to bring the Na^{+} permeability back to control levels.

The effects of butanol treatment temperature on subsequent Na^{+} uptake

Ca^{2+} was able to inhibit Na^{+} uptake into those red cells treated at temperatures below 17.5° (Fig. 3a). Ca^{2+} shifted the curve to the right; *i.e.* the temperature required to achieve the same amount of membrane damage was increased in the presence of 4 mM Ca^{2+}. Similar results were obtained when hemolysis was measured (Fig. 3b), except that the Ca^{2+} effect here was also seen at 20°. The difference between the two figures may well reflect the differing sizes of the permeating substances, the Na^{+} and the hemoglobin molecule.

TABLE II

THE EFFECTS OF ALKALI METAL CATIONS ON Na^{+} PERMEABILITY

All erythrocytes were treated together in 0.4 M *n*-butanol at 15° for 30 min. After treatment, the cells were preincubated at 15° for 1 h in media containing 0, 3, or 60 mM alkali metal chlorides. (The cells which were preincubated in the absence of all of the above cations gained Na^{+} to the level of 47.0 ± 0.5 mequiv/5 mmole of hemoglobin.) The cells were then incubated at 37.5° for 2.25 h at which time the sodium content of the erythrocytes was determined.

Cation	*Cellular Na^{+} (mequiv/5 mmole of hemoglobin)*	
Concn. of alkali metal cation:	*3 mM*	*60 mM*
Li^{+}	49.6 ± 1.3	47.3 ± 0.5
Na^{+}	47.6 ± 0.5	48.0 ± 0.5
K^{+}	45.4 ± 0.5	45.8 ± 0.6
NH_4^{+}	47.3 ± 0.3	39.5 ± 0.5
Rb^{+}	45.1 ± 0.9	44.4 ± 0.5
Cs^{+}	45.8 ± 0.9	45.4 ± 0.3

The effect of preincubation of butanol-treated erythrocytes in media containing the alkali metal cations on subsequent Na^{+} uptake

KAVANAU[21,22] suggested that the quantity and/or quality of water in biological membranes plays a decisive role in the overall structure and function of the membrane. Therefore, the effects of the monovalent cations, which are known to alter the structure of water, were studied (Table II). There were no significant differences between the effects of the monovalent cations except that NH_4^{+} at 60 mM caused significantly less uptake of sodium than the others (1 % level of significance).

DISCUSSION

The fact that the influx of Na^+ into human erythrocytes in the presence of 0.4 M *n*-butanol is approx. 40 times that of the controls (Fig. 1) and that immediately after washing the cells free of butanol the influx decreased to 3 to 4 times control values[23] indicates that the effect of butanol is partially reversible under the conditions of these experiments. A large portion of the increased permeability is due to the presence of the alcohol, but a membrane lesion does remain after the removal of butanol. It is not possible to conclude whether butanol causes two different types of membrane lesions, one of which is partially repaired by the removal of butanol and the other which is repaired by exogenous calcium or one type of damage which is partially reversed by butanol removal and further reduced by exogenous Ca^{2+}.

Ca^{2+} and the other bivalent cations, Sr^{2+} and Ba^{2+}, reduced the Na^+ permeability of butanol-treated red cells. The possibility that these cations are exerting their effects on the erythrocyte membrane is supported by two experimental findings. First, Ca^{2+}, Sr^{2+} and Ba^{2+} also inhibit, in an exactly analogous manner, the butanol-induced hemolysis of red cells. Second, the effects of the local anesthetics parallel those of the divalent cations. It has been recently shown by KWANT AND SEEMAN[24] that the local anesthetic chlorpromazine interacts with the membranes of human erythrocytes. Chlorpromazine, a tertiary amine like the local anesthetics mentioned above, inhibited hypotonic hemolysis. It was also shown that chlorpromazine displaces Ca^{2+} from the red cell membrane[25].

In addition, the permeability of many excitable cells to the monovalent cations, Na^+ and K^+, are known to be reduced in the presence of Ca^{2+} (refs. 26, 28). That bivalent cations do bind to the erythrocyte membrane was shown in studies of hemoglobin-free red cell ghosts. Ca^{2+} bound to the stroma such that the membrane was 90 % saturated at 4–5 mM bivalent cation[29]. The binding of bivalent cations to the ghost was in the order $Ca^{2+} > Sr^{2+} > Mg^{2+}$ (ref. 30). In both studies significant amounts of Mg^{2+} were found to bind.

Studies by LEITCH AND TOBIAS[31] on Ca^{2+} in phospholipid membranes led them to suggest that Ca^{2+} cross-links adjacent phospholipid molecules at their polar head groups causing shrinkage of the membrane and extrusion of water. The possibility that Ca^{2+} reacts with the erythrocyte membrane in a similar manner is suggested by the action of the local anesthetics on butanol-treated red cells. Like Ca^{2+}, the local anesthetics, tetracaine, butacaine, and procaine, form 1:2 complexes with phospholipids in water dispersion[18]. Moreover, the sequence of decreasing ease of saturation of the phospholipids (tetracaine > butacaine > Ca^{2+} > procaine) is the same as the sequence for reversing the butanol damage of whole red cells (tetracaine > Ca^{2+} > procaine). It is thought that Ca^{2+} and the local anesthetics compete for the same membrane sites in nerve and muscle[27,32,33].

In order to confirm the role of phospholipid phosphate as the group which binds membrane Ca^{2+}, the effects of phospholipase C were studied. Two effects on Na^+ permeability were observed. First, in the lipase-treated cells, when placed in a Ca^{2+}-free medium, the Na^+ permeability was much higher than in the controls with or without Ca^{2+}. Second, the addition of Ca^{2+} returned the Na^+ permeability of lipase-treated cells only to the level found for control cells without Ca^{2+}. The fact that Ca^{2+} produced an effect with lipase-treated cells does not lead to a straight-forward con-

clusion. If phospholipase had removed only that small fraction of phospholipid phosphate from which butanol effects the removal of calcium and had not exposed additional phospholipid to the aqueous medium, then the addition of Ca^{2+} would have had no effect on the cells. The analysis of human erythrocytes for membrane lipid phosphate gave a value of 3.90 μmoles/ml of packed cells[20]. The most recent analysis of the calcium content of human red cell membranes gave a value of only 0.016 μgatom/ml packed cells[34]. Therefore, only about 1 % of the phospholipids could be cross-linked by Ca^{2+} at one time in normal red cells if two molecules of phospholipid are chelated for each Ca^{2-}. It is possible that, in butanol-treated cells, more phospholipid molecules become available to Ca^{2+} and additional treatment with the enzyme further increases the fraction of total phospholipids remaining that can bind Ca^{2+} or that the effects of *n*-butanol and phospholipase C are mediated through modifications of the forces involved in Ca^{2+}-dependent lipid–protein, protein–protein, or lipid-dependent protein–protein binding (see ref. 35 for review). The present data do not allow meaningful speculation about the relative importance of each of the above binding-types in mediation of the effects of butanol.

CHAPMAN *et al.*[9] found that, upon addition of water to phospholipids, the transition temperature between the solid crystalline phase and the liquid-crystalline state decreased and additional mesomorphic phases appeared between the liquid-crystalline phase and the liquid-melt phase. Further addition of water (up to 20 % water) decreased the transition temperature to near biological temperature (41°). Therefore, the effect of the butanol treatment temperature upon subsequent Na^+ permeability and hemolysis was investigated in the present study. There was a sharp increase in the degree of membrane disruption as treatment temperature was increased. It may be that *n*-butanol, having penetrated the membrane, caused the membrane to absorb water molecules, priming it for a phase transition of the phospholipids upon transfer to butanol-free incubation media at 37°. The shifting of the membrane "melting" curve to higher temperatures by Ca^{2+} may indicate that such phase changes are strongly dependent on the degree of complexing of adjacent membrane molecules by Ca^{2+}.

CHAPMAN[36] has suggested that membrane-bound water may vary "either in its properties or in its total amount". . . which "in turn may alter transport and diffusion properties across the membrane". Therefore, the effects of incubating butanol-treated erythrocytes in media containing alkali metal cations upon subsequent Na^+ permeability were studied. These cations are known to alter the structure of water in a sequential manner[21]. However, under the conditions of these experiments, no meaningful differences between the effects of the different monovalent cations were observed.

Since Ca^{2+} decreases the permeability of many cells for which transitions within the membrane are known to occur (excitable tissues), it is proposed that Ca^{2+} may have a similar role in erythrocytes which have undergone a transition as a result of treatment with *n*-butanol.

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