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Mechanisms of Local Anesthetic Action on the Permeability of Erythrocytes, Leukocytes, and Liposomes Containing the Erythrocyte Anion Channel Protein

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SUMMARY

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The local anesthetics tetracaine and dibucaine produce complex effects on the permeability of human erythrocytes and rabbit polymorphonuclear leukocytes. Red cell permeability to small hydrophilic nonelectrolytes (i.e., urea), which probably cross the membrane through water-filled transmembrane protein channels, is unaffected or slightly inhibited by dibucaine (0.5 mM). However, larger, more lipophilic, and less permeable nonelectrolytes (i.e., isopropylurea, butanetriol, triethylene glycol, and tetraethylene glycol) are rendered more permeable by dibucaine (and tetracaine). These effects are probably related to the anesthetic-induced increase in membrane lipid fluidity. Leukocytes exhibit much lower water and urea permeability than red cells, indicating a paucity of hydrophilic channels in the membrane. Dibucaine strongly inhibits urea and methylurea permeability in leukocytes. Thiourea, which is more permeable and lipid-soluble, and which probably diffuses through the membrane lipid domain, was unaffected by dibucaine. Anion transport in erythrocytes procedes by way of a specific membrane-spanning protein, band 3 protein. Dibucaine and tetracaine inhibit $SO_4^=$ transport noncompetitively over the same concentration range (0.05-1.0 mM) at which they protect red cells against hypotonic lysis. The inhibition of $SO_4^=$ transport cannot be attributed to a generalized increase in membrane lipid fluidity. Band 3 protein-mediated $SO_4^=$ transport was also demonstrated in liposomes in which the protein was incorporated into the lipid membrane. Dibucaine and tetracaine increased $SO_4^=$ transport somewhat in purely lipid membranes, but strongly inhibited band 3 protein-mediated transport. These results demonstrate that anesthetics can block the function of a protein known to be a specific membrane ion channel. It remains to be determined whether the anesthetics interact directly with the protein itself or affect it by altering those lipids in immediate association with the $SO_4^=$ channels.

INTRODUCTION

Current theories (1-5) dealing with the molecular mechanism(s) of local anestheia fall into three general categories, which attribute anesthetic effects variously to (a) perturbations of the fluidity, surface area, surface potential, and Ca^{++} binding of the lipids of cellular membranes, (b) direct in-

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teractions with proteins that constitute transmembrane ion channels, or (c) conformational changes in the proteins forming the ion channels, which occur secondarily as a result of anesthetic-induced alterations in the physical state of the surrounding lipid milieu.

One theory of anesthesia considers that phospholipid bilayer membranes are composed of highly ordered gel-phase phospholipids in equilibrium with disordered fluid-phase phospholipids. Lateral phase separations occur at the boundaries of the two phases. Such phase separations are proposed to facilitate necessary conformational changes in globular channel proteins which allow sodium ion flux during nerve excitation, i.e., expansion to form an open pore configuration (4), or aggregation of monomers into oligomers (6) via lateral diffusion so as to form the functional Na^+ channels. In either case, it has been proposed that anesthetics prevent the essential protein conformational changes through their disordering effect on the surrounding lipid structure. Lee (7) has proposed that the Na^+ channels are surrounded by specific lipids in the gel state, which, when transformed to the liquid crystalline state by the insertion of anesthetic molecules, result in relaxation of the Na^+ channel to an inactive configuration. These mechanisms need not be specific (i.e., Na^+-selective) but would presumably affect the transport of any substances mediated by similar transmembrane protein channels. Furthermore, the lipid-disordering effect (i.e., melting of the lipid gel state) should increase the permeability of those lipophilic substances which permeate the membrane predominantly by way of the lipid phase of the membrane. The antagonistic effects of pressure on lateral phase separations, phase transition temperature, and fluidity (8, 9) have tended to support lipids as the primary site of anesthetic-membrane interaction. However, other theories predict a similar result but assume proteins to be the site of action.

The most explicit theory dealing with direct anesthetic-protein interactions is that proposed by Eyring *et al.* (10) and Ueda *et al.* (3). On the basis of a thermodynamic analysis of the inhibition of luciferase by anesthetics, it was concluded that hydrophobic interactions between drug and protein led to conformational change and volume expansion of the protein, associated with release of structured water molecules bound to hydrophilic sites of the enzyme.[1] It was proposed that such effects would result in dehydration of the Na^+ channels of nerve membranes and suppression of Na^+ conductance. The ability of pressure to reverse anesthesia is attributed to an antagonism of the specific volume expansion of the protein channels. This theory is also nonspecific, in that it involves anesthetic interactions with hydrophobic regions likely to be common to most intrinsic membrane proteins.

The very nonspecificity of the pharmacological actions of local and general anesthetics, as compared with agents such as tetrodotoxin and saxitoxin, is in harmony with the rather general theories described above. Moreover, this makes evident the reasons for the difficulty in designing decisive experiments to elucidate the fundamental molecular basis for anesthetic action.

In view of the nature of the effects of anesthetics on membrane lipids, their influence on the permeability of relatively hydrophobic solutes might be expected to differ from that on ions or nonelectrolytes, which traverse the membrane via water-filled protein channels. We have therefore studied the influence of several local anesthetics on nonelectrolyte permeability in two types of cells, human erythrocytes and rabbit leukocytes, which differ widely in the relative density of their water channels (12, 13). In addition, we have made comparable studies of anesthetic effects on anion (sulfate) transport in erythrocytes. Sulfate transport was studied because many anesthetics are known to affect anion transport in red cells (14, 15), and there is strong recent evidence which supports

[1] Seeman (11) directly measured membrane expansion due to ethanol and found that synaptosome membranes expanded 50 times more than cholesterol-phosphatidylcholine vesicles. This finding is consistent with the view that conformation changes in membrane protein are responsible for membrane expansion.

the role of a specific intrinsic membrane protein (i.e., band 3 protein) as the anion channel in erythrocytes (16–18). Furthermore, this protein can be extracted from red cell membranes and reconstituted into phospholipid liposomes, thereby conferring increased sulfate permeability to the membrane (19). We therefore compared the effects of local anesthetics on $SO_4^=$ transport in intact erythrocytes and in liposomes containing band 3 protein. The results demonstrate for the first time that anesthetics can inhibit a specific protein-mediated transport system, both in intact cells and in a simple reconstituted membrane system.

MATERIALS AND METHODS

Nonelectrolyte and sulfate permeability of erythrocytes and polymorphonuclear leukocytes. The permeability of human red cell membranes to water and nonelectrolytes was measured by a modified hemolysis and stop-flow technique as described previously (20, 21). The time course of the change in light transmission at 540 nm was measured using a Zeiss spectrophotometer connected to a Grass d.c. amplifier and a paper recorder. Permeability coefficients were calculated using the equations derived initially by Jacobs (20) and later summarized by Stein (22).

For sulfate measurement human red cells were obtained from healthy male or female donors. Heparin (1000 units/ml, 4 ml/liter of blood) was used to prevent clotting. The blood was immediately centrifuged for 15 min at 1500 × g, and the plasma and buffy coat were carefully removed. The packed red cells were then washed three times in 4 volumes of incubation medium, which had the following composition: NaCl, 150 mM; KCl, 5.0 mM; $MgCl_2$, 1.0 mM; $CaCl_2$, 0.25 mM; Na_2HPO_4, 5.0 mM; NaH_2PO_4, 1.0 mM; Na_2SO_4, 10 mM; and glucose, 10 mM (pH 7.4). The washed red cells were resuspended in the incubation medium at 10% hematocrit and were incubated for 15 min in a water bath shaker at 37° before tracer was added. Fluxes were measured using a fast, reproducible technique described previously (23). At a designated time 0.8 ml of a cell suspension was layered on top of a 0.5-ml layer of silicone oil (Versilube F50; density, 1.05 g/ml; Harwick Chemical Corporation, Cambridge, Ma.) in a 1.5-ml microcentrifuge tube. The cells were separated from the suspending medium by a single centrifugation (0.5 min) in an Eppendorf microcentrifuge (maximum speed of 8000 rpm reached in less than 10 sec). The packed red cells were then hemolyzed. A known volume of this hemolysate was taken for hemoglobin measurement, and another for isotope counting. Samples were prepared for counting and the hemoglobin measurements as described previously (24). This general procedure was followed unless otherwise specified. Rate constants and fluxes were calculated using the standard two-compartment analysis (25, 26).

Polymorphonuclear leukocytes were obtained from white albino rabbits (5–7 lb) which had been injected intraperitoneally with 300–500 ml of isotonic sterile solution containing glycogen (0.5 g/liter). The peritoneal exudate was collected 16 hr later in a heparinized flask. The leukocyte-rich exudate was strained through four layers of cheesecloth to remove large clumps of debris. The suspension was gently centrifuged at 500 × g, and the supernatant was removed and replaced by an equal volume of isotonic buffered NH_4Cl. The packed cells were resuspended with a Pasteur pipette and kept at room temperature for 5 min. This procedure was necessary to hemolyze red cells (27). The suspension was centrifuged for 5 min at 500 × g, and the cells were immediately washed twice with a buffered isotonic solution and then resuspended (20 × 10^6 cells/ml). The permeability coefficients of rabbit polymorphonuclear leukocyte membranes to urea, methylurea, and thiourea were measured employing ^{14}C-labeled solutes as described previously (13). A known volume (0.3 ml) of the suspension was layered on top of a silicone oil layer (F50; density, 1.05 g/ml) in 1.5-ml microcentrifuge tubes. Radioactive buffered solution (0.2 ml) was injected into the suspension, and the sample was centrifuged after a set time had elapsed. Mixing was achieved by injecting

the large volume of the buffered solution containing the radioactivity. The cells were separated from the suspending medium by a single centrifugation (0.5 min) in an Eppendorf microcentrifuge. The cells were separated in less than 10 sec. The supernatant and the oil layer were removed by suction, and the pellets were solubilized in 0.1 M NaOH in 2% Na_2CO_3 at 50°. A sample was removed for protein determination, and another sample for counting. It was found that practically no radioactivity could be found in the oil layer (1% of the total counts in the pellet of the first sample). The trapped extracellular space, as determined independently using [^{14}C]inulin, was less than 5% of the total counts in the pellet of the first sample.

Extraction of band 3 protein from erythrocytes. Erythrocyte ghosts were extracted sequentially at 0° with 0.1 N NaOH (28), a series of phosphate buffers, and finally 36 mM phosphate buffer (pH 7.5) containing 0.5% Triton X-100 (29). Variable but usually very small amounts of bands 1, 2, 4.2, and 6 were found in the band 3-enriched Triton X-100 extracts. Band 3 protein usually accounted for more than 95% of the Coomassie blue-stainable protein in Triton X-100 extracts. Triton X-100 also extracted other membrane glycoproteins, such as glycophorin. Before incorporation into liposomes, Triton X-100 was removed (residual concentration, less than 0.1%) from the band 3 protein solutions by adsorption to Bio-Beads SM2 (Bio-Rad) or by extraction with toluene containing egg lecithin (19).

Polyacrylamide gel electrophoresis of erythrocyte membrane proteins was carried out in disc or slab gels containing 5.6% acrylamide, 0.21% bisacrylamide, and 0.2% sodium dodecyl sulfate. Red cell ghosts and proteins extracted from ghosts were dissolved in 1% sodium dodecyl sulfate plus 40 mM dithiothreitol and heated for several minutes in a boiling water bath. The solutions were made 5% with glucose, and either Pyronine Y or bromphenol blue was added as tracking dye. Gels were stained with Coomassie blue for proteins and with periodic acid–Schiff reagent for glycoproteins (30).

Preparation of liposomes containing band 3 protein. Chloroform solutions (2.0 ml) containing 96 mg of egg lecithin plus 4 mg of sodium phosphatidate were evaporated under nitrogen in a rotating flask. Then 50 μl of radioactive sulfate (about 10 μCi), 7.5 ml of buffer solution (150 mM NaCl, 5 mM KCl, 5 mM sodium phosphate, 1 mM $MgCl_2$, 0.25 mM $CaCl_2$, and 10 mM sodium sulfate), 0.5 ml of solution containing band 3 protein (Triton X-100 removed) and 0.15 M NaCl, and several glass beads were added to the flask. The final protein concentration was about 0.37 mg/ml. This solution was mixed by hand in the flask until the thin coat of lipid on the inside of the flask was removed. The resulting lipid suspension was then sonicated (200 W, Branson Sonifier bath) for 10 min. In order to remove sulfate not contained within the liposomes, the lipid suspension was passed through a Sephadex G-50 column and the liposome fraction was collected. To measure $^{35}SO_4^=$ efflux, 2.0-ml aliquots of the liposome dispersion were placed in dialysis bags within screw-capped tubes containing 10 ml of phosphate buffer (as above). The tubes were rotated gently on a rotary mixer. Samples of the external medium (0.25 ml) were taken periodically and replaced with an equal volume of fresh buffer. Samples (0.25 ml) of the liposome dispersion were taken at the beginning and end of the experiment to determine the amount of $^{35}SO_4^=$ remaining within the liposomes.

RESULTS

Nonelectrolyte permeability in red cells and leukocytes. Red cell permeability to a series of nonelectrolytes was studied in the presence of three local anesthetics: dibucaine, procaine, and benzocaine. Benzocaine differs from the other two in that it is not a tertiary amine. The results are summarized in Table 1. The effects of benzocaine and procaine at 0.5 mM were negligible except for some increase in the permeability of 1,2,4-butanetriol in the presence of benzocaine. At this concentration they had much less effect on membrane fluidity than either tetracaine or dibucaine. More striking effects were obtained with dibucaine, which increased the permeability of

TABLE 1

Red cell permeability to nonelectrolytes in the presence of local anesthetics

Compound	Partition coefficient (K_{ether})	Permeability	Change in permeability		
			Dibucaine	Benzocaine	Procaine
		cm/sec × 10^5	%	%	%
Water		915	0	0	0
Urea	0.00047	24	−10		0
Ethylene glycol	0.0053	3.4	−5	0	0
Propylurea		0.62	0	−3	−3
Isopropylurea		0.40	+12	0	−1
1,2,4-Butanetriol		0.24	+56	+16	−4
Triethylene glycol	0.0031	0.10	+80	0	0
Tetraethylene glycol	0.0024	0.07	+19	0	0

four of the low-permeability solutes. It is interesting that the permeability coefficients of human red cell membranes to water and hydrophilic solutes (e.g., urea, ethylene glycol, and propylurea), all of which most likely permeate through hydrophilic transmembrane protein channels, were either not affected or slightly decreased (i.e., urea, ethylene glycol) in the presence of 0.5 mM dibucaine. On the other hand, the permeability coefficients of human red cell membranes to solutes which most likely permeate through the membrane lipid matrix (e.g., isopropylurea, 1,2,4-butanetriol, triethylene glycol, and tetraethylene glycol) were significantly increased by the same concentration of dibucaine. With the exception of tetraethylene glycol, the permeability of these solutes was increased by dibucaine to an extent inversely proportional to their initial permeability coefficients. Expressed in another way, those solutes which permeate the membrane poorly, and probably entirely through the lipid regions of the membrane, were most strongly affected by the local anesthetic. Furthermore, when the permeability coefficient of human red cell membranes to 1,2,4-butanetriol was measured as a function of local anesthetic concentration, it was found to be increased 6-fold by dibucaine over the concentration range 0.3–1.0 mM (Fig. 1) but was unaffected by procaine and benzocaine up to 3 mM.

In comparison with erythrocyte membranes, it is generally found that the permeability of rabbit polymorphonuclear leukocyte membranes to water is very low

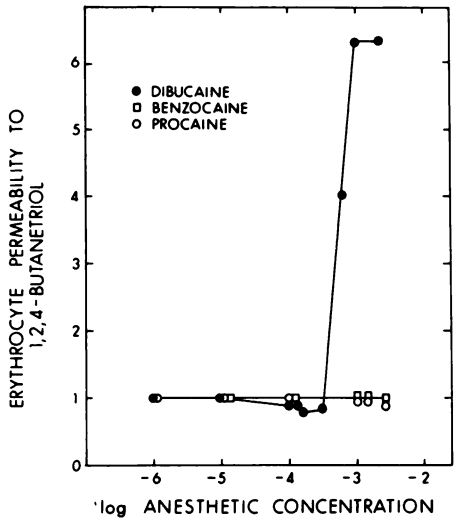

FIG. 1. *Effects of local anesthetics on permeability of human erythrocytes to 1,2,4-butanetriol*

Permeability as a function of anesthetic concentration is expressed relative to the control permeability, taken as 1.0. Control permeability for 1,2,4-butanetriol was 0.24 cm/sec × 10^5.

(12). Based on these and other results, it was concluded that rabbit leukocyte membranes are characterized by a very low density of regions which are available for the permeation of hydrophilic solutes (13). Accordingly, this system should provide a good model with which to study the apparent dual effects of local anesthetics on the movements of hydrophilic and hydrophobic nonelectrolytes. The permeability coefficients for the urea series in the leukocyte are in the order urea < methylurea < thiourea (Table 2). This is in striking contrast to the red cell, where the permeabil-

ity coefficients ($\times 10^{-5}$ cm/sec) vary as follows: urea, 23.9; methylurea, 1.83; thiourea, 0.07 (31). The low permeability of urea in the leukocyte correlates well with the paucity of aqueous transmembrane channels in these cells.

The effects of 0.5 mM dibucaine on the permeability coefficients of rabbit leukocyte membranes to these three solutes—urea, methylurea, and thiourea—are summarized in Table 2. The permeability of urea and methylurea was significantly decreased (55–56%) by dibucaine. Thiourea, however, with a greater lipid solubility, was much more permeable than urea and methylurea and was unaffected by dibucaine. These results and those previously described in red cells demonstrate unequivocally the dual effect of local anesthetics on nonelectrolyte movements across biological membranes.

Sulfate permeability in red cells. The rate of $SO_4^=$ uptake in erythrocytes was inhibited significantly in the presence of certain local anesthetics (Fig. 2), such as tetracaine, quinacrine, and dibucaine. The concentration ranges over which the anesthetics were effective (i.e., 0.05–1.0 mM for dibucaine and 0.05–3.0 mM for tetracaine) correspond to those found previously by Seeman to protect red cells against hypotonic lysis (32). Procaine at concentrations up to 10 mM had no effect on $SO_4^=$ transport. Reducing the temperature from 37° to 21° reduced $SO_4^=$ permeability by 70% (Table 3). Over this temperature range the viscosity of the red cell membrane was found to increase 2-fold (33). The magnitude of the inhibitory effect of dibucaine (1 mM) on $SO_4^=$ permeability was equivalent to that caused by the 16° reduction in temperature (Table 3). At the lower temperatures dibucaine produced only a small further reduction in $SO_4^=$ permeability. Any effect of the anesthetic on $SO_4^=$ permeation through the protein channels at 21° may have been offset by increased anion movement through lipid regions as a result of the considerable fluidizing effect of the anesthetic at that temperature (33). At dibucaine concentrations of 2 mM and above, hemolysis commenced and $SO_4^=$ permeability increased (Table 3).

TABLE 2

Effect of dibucaine on permeability of rabbit neutrophils to certain nonelectrolytes

Compound	K_{ether}	Permeability coefficient	
		Control	0.5 mM dibucaine
		cm/sec $\times 10^5$	
Urea	0.00047	0.40 ± 0.05	0.18 ± 0.02
Methylurea	0.0012	0.48 ± 0.02	0.21 ± 0.01
Thiourea	0.0063	1.30 ± 0.1	1.20 ± 0.08

The antagonism of sulfate permeability by dibucaine was not competitive with respect to sulfate concentration. This is shown in Fig. 3, where the rate constant for sulfate uptake is plotted as a function of sulfate concentration. The ratio of the control rate constant to that in the presence of dibucaine is essentially constant at a value of 2.9 ± 0.48 (SE) over the entire sulfate concentration range of 0.1–15 mM.

Sulfate permeability of liposomes containing band 3 protein extracted from erythrocytes. Anion transport across human red cell membranes is mediated by a specific membrane protein of about 95,000 mol wt, commonly referred to as band 3 protein (16–18). The ability of anesthetics to inhibit $SO_4^=$ transport in intact cells indicated that they affect the anion transport channel. We therefore studied $SO_4^=$ transport in liposomes which contained band 3 protein incorporated into the membrane structure. Rothstein *et al.* (19) demonstrated that a reconstituted membrane containing band 3 protein and lecithin facilitated $SO_4^=$ transport. In addition, the function of the extracted protein as an anion channel was abolished by prior treatment with a specific inhibitor of anion transport, 4,4′-diisothiocyano-2,2′-stilbenedisulfonate. In our experiments, band 3-enriched extracts of erythrocyte membrane (Fig. 4) incorporated into liposomes composed of egg lecithin (96%) and the sodium salt of phosphatidic acid (4%) significantly enhanced $SO_4^=$ efflux at both 21° and 37° (Figs. 5 and 6). It is interesting that over this temperature range the viscosity of lecithin membranes varies from 115 to 40 centipoise below that of red cell

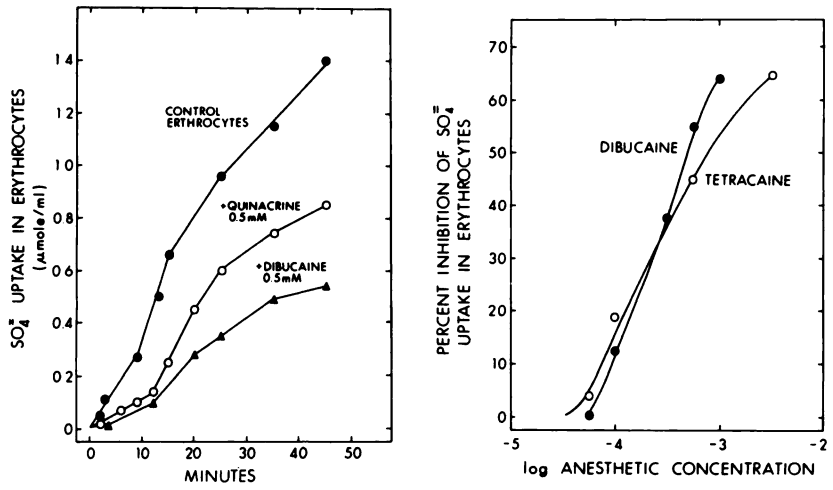

FIG. 2. *Inhibition of $SO_4^=$ uptake in erythrocytes*

Left: Inhibition by quinacrine and dibucaine as a function of time. $SO_4^=$ uptake was measured as described in MATERIALS AND METHODS. Right: Inhibition of $SO_4^=$ uptake as a function of dibucaine or tetracaine concentration.

TABLE 3

$^{35}SO_4^=$ permeability in red cells

Dibucaine	Permeability		
	37°	24°	21°
mM		*μmol/ml/min*	
0	0.066	0.033	0.021
0.01		0.037	0.024
0.1	0.056	0.035	0.019
0.5		0.031	0.019
1.0	0.020	0.028	0.019
2.0		0.038	0.025

membranes (33); thus lowering the temperature does not reduce $SO_4^=$ permeability in liposomes as much as it does in the red cell. Local anesthetics added to purely lipid-containing liposomes enhanced $SO_4^=$ efflux as a function of anesthetic concentration (Figs. 5 and 6). However, when added to band 3-containing liposomes, both tetracaine and dibucaine almost completely arrested the protein-facilitated $SO_4^=$ efflux (Figs. 5 and 6) at either 21° or 37°. The effectiveness of the local anesthetic in liposomes at 21° is in contrast to that in red cells. This result may be related to the significantly lower viscosity of the liposome lipids compared with the red cell membrane lipids at 21°.

Although Triton X-100 was substantially removed from band 3 protein preparations, we considered the possibility that remaining traces of detergent were responsible for enhanced $SO_4^=$ efflux. However, this was clearly not the case. Sulfate

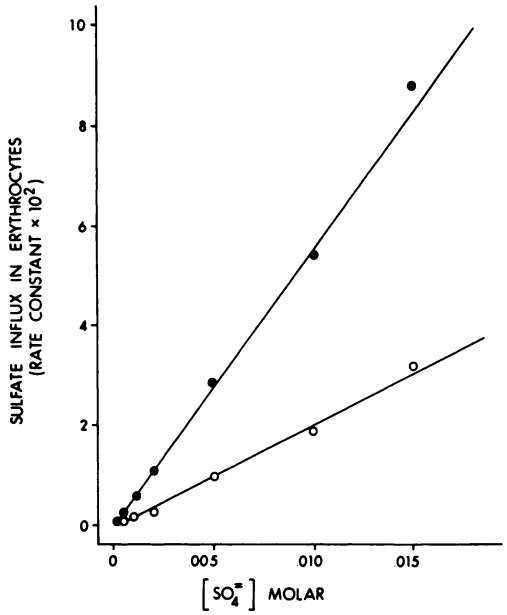

FIG. 3. *Inhibition of rate of $SO_4^=$ influx in erythrocytes by dibucaine as a function of extracellular $SO_4^=$ concentration*

●, control, ○, 0.5 mM dibucaine.

efflux from liposomes lacking band 3 protein was increased by addition of Triton X-100 at concentrations of 0.1% and above, but by direct measurement (34) less than 0.1% Triton X-100 was present in our experiments. Furthermore, $SO_4^=$ efflux induced by the detergent was not inhibited by local anesthetics (Fig. 7), whereas that attributable to the presence of band 3 protein in the membrane was strongly suppressed.

DISCUSSION

Recent theories put forth to interpret the effects of anesthetics on membrane permeability have emphasized either anesthetic-induced changes in lipid fluidity indirectly affecting protein structure, or direct effects on ion channel protein structure. One of the problems with interpretation of fluidity data is that the viscosity of the lipid phase of membranes is not likely to be uniform in the plane of the membrane; lateral phase transitions (4) and quasicrystalline clusters of lipids (35, 36) have been described. Furthermore, the physical state of lipids in immediate association with membrane proteins may differ greatly from that of lipids in the bulk phase (37). It is quite difficult technically, in intact cells, to test the hypothesis that anesthetics cause unique alterations of the physical state of lipids in the immediate environment of ion channels. Neither the ion channels of nerve membranes nor their associated lipids have been identified. However, experiments with isolated band 3 protein from erythrocytes, in well-defined liposomes, offers a system amenable to investigation of some of the pertinent questions concerning the molecular mechanism of anesthetic action. Most significantly, the effects of anesthetics can be assessed on a well-characterized membrane transport channel protein. It would be naive, however, to assume that whatever we learn from this model will necessarily apply strictly to ion channels in nerve membranes.

The involvement of band 3 protein in the regulation of anion transport has been well established (16–18). Furthermore, the identification of band 3 protein in the con-

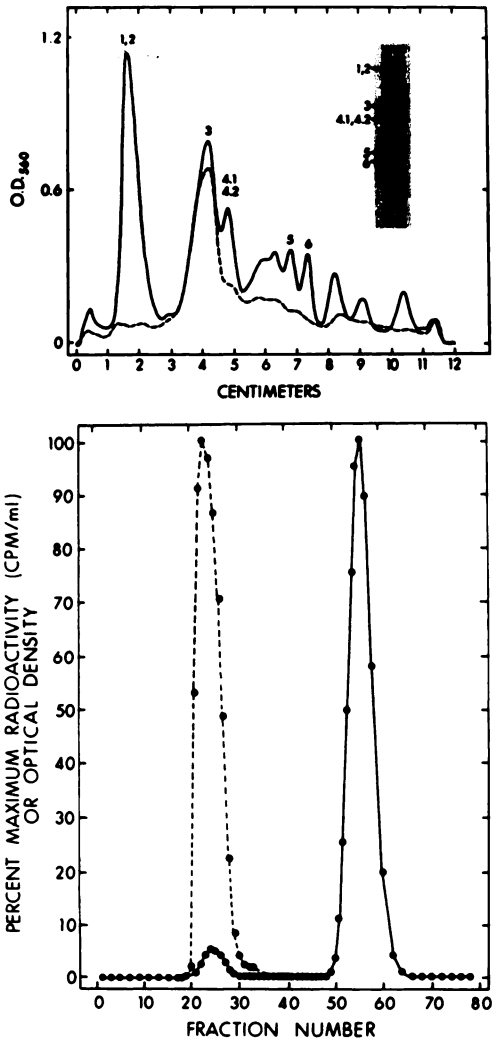

FIG. 4. *Isolation of band 3 protein (upper) and liposomes containing band 3 protein (lower)*

The inset shows polypeptide chains of intact red cell membranes extracted with 1% sodium dodecyl sulfate and separated by polyacrylamide gel electrophoresis (left), and a Triton X-100 extract of red cell membranes after stripping to remove proteins other than band 3 (right). The gels were stained with Coomassie blue. Upper: Densitometric scans of the stained gels, showing the pattern for the total membrane proteins (——) and the Triton X-100 extract of stripped membranes (- - -). Lower: Liposomes prepared with $^{35}SO_4^=$ as described in MATERIALS AND METHODS were passed through a Sephadex G-50 column. The optical density at 450 nm (- - -) measures the presence of liposomes in the eluate. The radioactivity (——) shows the clear separation of free $^{35}SO_4^=$ from that associated with the liposomes. The liposomes contained 20 mg of lipid and 0.13 mg of protein per milliliter.

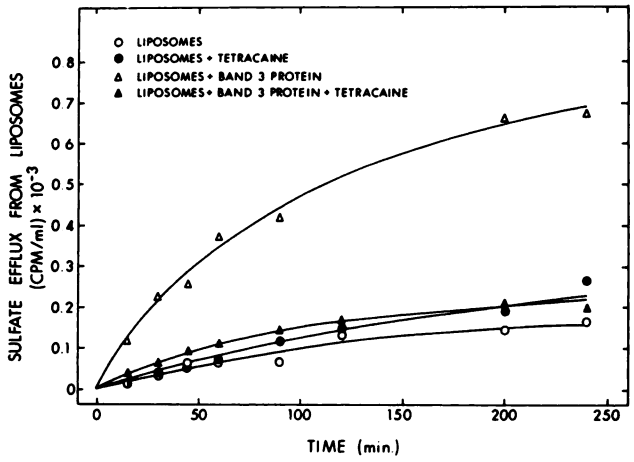

FIG. 5. *Inhibition of band 3 protein-facilitated $SO_4^=$ efflux from liposomes at 21° by tetracaine*
Liposomes were prepared and $SO_4^=$ efflux was measured as described in MATERIALS AND METHODS and Fig. 4. The tetracaine concentration was 0.5 mM.

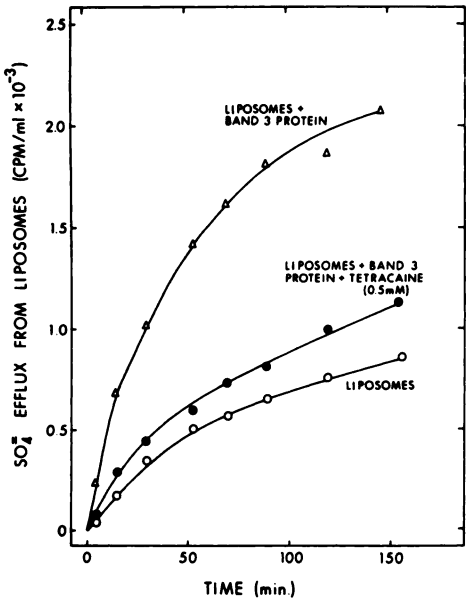

FIG. 6. *Inhibition of band 3 protein-facilitated $SO_4^=$ efflux from liposomes at 37° by tetracaine*

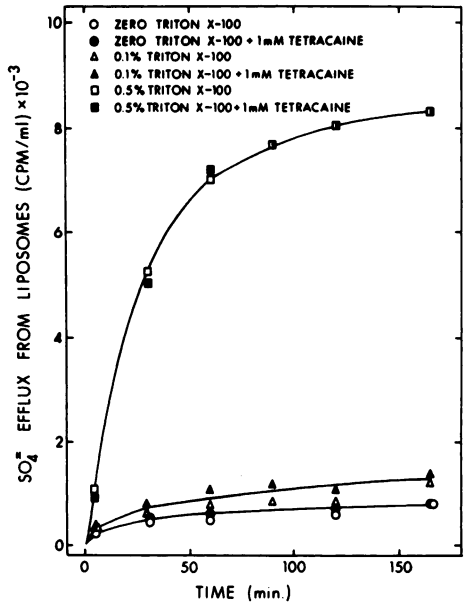

FIG. 7. *Effects of Triton X-100 and tetracaine on $SO_4^=$ efflux from liposomes prepared without band 3 protein*

trol of osmotic water flow (38, 39) greatly strengthens the view that this protein provides the water-filled channels required for the movement of anions and certain small nonelectrolytes across the membrane. Water and chloride permeability is very high in erythrocytes, necessitating that a significant fraction of membrane protein be devoted to these processes. Band 3 protein meets this criterion, since it completely spans the membrane and is the major protein constituent, making up 30–35% of the total protein content (approximately 1×10^6 copies/cell).

Incorporation of erythrocyte membrane extracts highly enriched in band 3 protein into liposomes results in the appearance of intramembrane particles of about 85-A diameter, as revealed by electron micro-

graphs of freeze-fractured, reconstituted vesicles (40). These particles closely resemble those seen in intact erythrocyte membranes. The extraction procedures for band 3 protein are not entirely selective, so that incorporation of small amounts of additional proteins into liposomes, especially glycophorin, the other minor glycoproteins, and spectrin, is entirely likely. With respect to anion and water transport in the intact cell, however, glycophorin is unlikely to be directly involved (41, 42).

The most important finding from these experiments is that local anesthetics inhibit $SO_4^=$ transport both in intact erythrocytes and in reconstituted liposome membranes containing band 3 protein. This effect of the anesthetics on liposomes was observed only when they contained incorporated band 3 protein. In purely lipid membranes anesthetics increased $SO_4^=$ permeability. The latter effect is likely due to the increased lipid fluidity and positive membrane surface potential imparted to the membranes by the local anesthetics. From the observation that anesthetics inhibit $SO_4^=$ transport in liposomes containing band 3 protein, as well as in intact erythrocytes, we may conclude with reasonable certainty that the local anesthetics cause some derangement of the protein constituting the anion channels. It remains to be determined whether or not the anesthetics affect the protein directly in the manner proposed by Eyring *et al.* (10).

Both lowered temperature and local anesthetics inhibit $SO_4^=$ permeability, but their effects on over-all membrane fluidity are quite the opposite. The temperature coefficient for $SO_4^=$ transport is quite high, i.e., Q_{10} = 4.5–5.2 (14), and significantly greater than that for monovalent anions. A reduction of temperature from 37° to 21° increases the viscosity of the red cell membrane by about 125 centipoise, an increase of 100% (33). It is interesting that the fluidity of red cell membranes decreases between 35° and 21° about 6-fold faster (centipoise per degree) than over the range from 20° to 0°. Tetracaine, on the other hand, produced only a 10-centipoise fall in red cell membrane microviscosity at 37° (33). Inhibition of $SO_4^=$ transport in red cells by local anesthetics was very pronounced at 37°, but not apparent at 22°. Yet the fluidity of the membrane is increased by local anesthetics to a much greater degree (about 10-fold) at 22° than at 37° (33). It is clear, therefore, that an over-all increase in membrane fluidity cannot satisfactorily account for the inhibition of $SO_4^=$ transport by local anesthetics. However, we cannot rule out the possibility that anesthetics induced greater changes in fluidity highly localized to the lipids in immediate association with anion channels. Moreover, it is conceivable that channel protein departs from its correct configuration if the viscosity of the surrounding lipid is either higher or lower than some optimum level. Also, a direct effect of temperature on protein conformation cannot be excluded.

A good correlation exists between anesthetic effectiveness and protection of erythrocytes against hypotonic lysis (1, 32). Similarly, the inhibition of $SO_4^=$ transport by dibucaine and tetracaine occurs at concentrations which stabilize the erythrocyte membrane. The lack of effect of procaine (up to 3 mM) with respect to $SO_4^=$ permeability is also consistent with Seeman's (32) observation that procaine increased osmotic stability of the erythrocyte only above 10 mM concentration. The stabilization of erythrocyte membranes by tertiary amine local anesthetics is quite pH-dependent (32), but we have not yet studied the pH dependence with respect to inhibition of $SO_4^=$ permeability. This is a complex problem, because anion permeability itself is highly sensitive to pH (14).[2] Many of the agents which protect against hemolysis and decrease anion permeability in erythrocytes are anionic or cationic substances which also produce crenation or cupping of the erythrocyte membrane (1, 14). Because of the asymmetry of membrane lipid distribution and surface charges, the charged drugs are believed to bind differentially to the two membrane surfaces, thereby inducing asymmetrical

[2] Gunn and Cooper (43) concluded that both charged and un-ionized forms of tetracaine inhibit Cl^- self-exchange at 0°, but that the latter is more potent.

expansion of the membrane (1, 44). Both types of agents, anionic and cationic, are similar in that they protect against hypotonic lysis, inhibit ionic permeability at certain concentrations, and may lyse erythrocytes at higher concentrations (1). Asymmetrical expansion of the lipid bilayer may disrupt the required structural organization of proteins constituting the ion channels of the membrane. One of the difficulties with this explanation is that there are no good quantitative data which compare the degree of membrane shape change with permeability effects. A more fundamental stumbling block is that many agents produce crenation in erythrocytes without affecting anion transport (45).

Local anesthetics exhibit a dual effect on permeability of nonelectrolytes in erythrocytes and leukocytes. In erythrocytes the permeability of small, moderately lipophilic solutes (e.g., isopropylurea, 1,2,4-butanetriol, triethylene glycol, and tetraethylene glycol) was enhanced, an effect which may be related to the ability of anesthetics to lower erythrocyte membrane viscosity. This increased permeability induced by dibucaine and tetracaine was not the result of a general increase in cell permeability or hemolysis for the following reasons. First, lysis did not occur at 1 mM tetracaine or dibucaine, in agreement with the results of Seeman (32). Second, the permeability increases were quite selective; that for some nonelectrolytes was unaffected, whereas $SO_4^=$ transport was strongly inhibited at the same concentrations. At higher concentrations of anesthetics (e.g., dibucaine $\geqq$ 2 mM) some hemolysis (5–20%) occurred and $SO_4^=$ influx increased. Increased nonelectrolyte permeability induced by the anesthetics occurred approximately in the concentration range known to induce the prolytic stage (32). The prolytic stage is associated with cellular K^+ loss, but occurs prior to hemoglobin release. The same underlying effect on the membrane may account for the increases in both K^+ and nonelectrolyte permeability.

It is well known that the effects of anesthetics on cell permeability, both quantitative and qualitative, differ significantly from one cell type to another, and even within the same cell types from different species. For example, Hunter *et al.* (46, 47) concluded that butanol increased erythrocyte permeability to nonelectrolytes which penetrate by simple diffusion, but inhibited what was assumed to be carrier-mediated permeation. Among the cellular factors we can identify as important in influencing these effects are (a) the presence of different specific pathways (e.g., protein channels vs. lipid domains) for membrane permeation by various solutes, (b) the relative concentration of transmembrane protein channels for solute transfer, and (c) the regional chemical composition and fluidity of the membrane lipids. The first two points are well illustrated by our experiments dealing with permeability of solutes of the urea series in red cells and leukocytes. It is apparent that the density of transport channels in the surface membrane of the two types of cells influences the response to anesthetics quite vividly. Urea permeability, which is quite likely to occur via transmembrane water-filled channels, is 50 times greater in the red cell than in the leukocyte. Dibucaine reduced urea permeation only weakly in the erythrocyte, but inhibited it strongly in the leukocyte. The much stronger effect of local anesthetics on urea and methylurea permeability in the leukocyte is clearly associated with the limited availability of pathways for hydrophilic solute permeation in that cell. Dependence of the effect of anesthetics on the mode of solute permeation is further emphasized by their effects on the permeability of other nonelectrolytes in red cells. The poorly permeable solutes 1,2,4-butanetriol, triethylene glycol, and tetraethylene glycol are larger and more lipophilic than urea and presumably must penetrate the cell through the lipid regions of the membrane. Their permeability is strikingly enhanced by appropriate concentrations of dibucaine and tetracaine, because of the reduction in membrane lipid viscosity. In the case of a solute of greater permeability and lipid solubility (e.g., thiourea in leukocytes), increased lipid fluidity was apparently not an important factor influencing permeation. In general, we may conclude that anesthetics will tend to increase

the permeability of solutes which penetrate the membrane via the lipid regions. On the other hand, highly permeable lipid-soluble solutes are not as significantly aided by fluidity changes of the magnitude produced by anesthetics. However, because fluidity is highly temperature-dependent, the effects of anesthetics on the permeability of lipophilic solutes will be expected to vary significantly with this parameter.

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