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DIFFERENTIAL PERTURBATION OF ERYTHROCYTE MEMBRANE-ASSOCIATED TRANSPORT AND ENZYME ACTIVITIES BY STRUCTURALLY RELATED LIPOPHILIC COMPOUNDS

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Summary

The alteration of two erythrocyte plasma membrane functions, acetylcholine hydrolysis and glucose exchange, by a series of structurally related small lipophilic compounds which exhibit similar antihemolytic behavior was studied. 2-Methyldimethylaminoazobenzene is a more potent inhibitor of acetylcholinesterase than the 3′-methyl analogue, while the unsubstituted compound fails to inhibit. Esterase inhibition by the 2-methyl compound is noncompetitive and dependent on the anion composition of the assay buffer. The temperature dependence of acetylcholinesterase activity in the presence of the 2-methyl compound suggests that interaction with inhibitor is influenced by the state of lipids tightly bound to the enzyme. Glucose exchange is inhibited to the same extent by both methyl derivatives but not by the unsubstituted dye, and the temperature dependence in the presence of inhibitor is not grossly altered. The lack of correlation between inhibition of membrane function and stabilization of erythrocytes against osmotic hemolysis is discussed.

Introduction

Studies of the perturbation of artificial lipid bilayers and biological membranes by small hydrophobic molecules have concentrated mainly on changes induced in the physical state of the lipid bilayer [1—7]. However, the association of a variety of pharmacological effects, such as anesthesia, tranquilization and narcosis, with the presence of many of these small lipophilic molecules in

Abbreviations: DAB, dimethylaminoazobenzene; 2MeDAB, 2-methyl-dimethylaminoazobenzene; 3′MeDAB, 3′-methyl-dimethylaminoazobenzene; DTNB, 2,2′-dinitro-5,5′-dithiodibenzoic acid.

the plasma membrane indicates a concomitant perturbation of membrane function. Generally, the biological potency of a family of compounds can be directly correlated with the change in lipophilic character resulting from structural modifications which alter partitioning between the aqueous phase and membrane [8]. Although this suggests involvement of the lipid bilayer as a site of action, the mechanism by which membrane function is altered by the presence of small lipophilic molecules is poorly understood.

A model system which has been employed extensively in the characterization of small molecule-membrane interactions has involved quantitation of the ability of the compounds to protect erythrocytes from hypotonic lysis (reviewed in Ref. 8). One of the major conclusions from these studies has been the demonstration that the antihemolytic activity of a small molecule is related to its relative hydrophobicity as defined by its octanol/water or membrane/buffer partition coefficient. The mechanism of protection may result from the ability of the compound to expand the membrane up to 10-fold greater than would be predicted from the van der Waal's volume of the perturbant [8—10]. This expansion by added hydrophobic molecules could arise from a change in the fluidity of the membrane lipids and/or conformational changes in membrane protein with attendant alteration of function.

Results of osmotic fragility studies have led to the general observation that the membrane concentration of a family of non-polar compounds is directly related to their partition coefficients with little dependence on structure other than those substitutions which alter partitioning. Because of the observed correlation of antihemolysis with pharmacological potency [8], it is, therefore, not unreasonable to expect that structurally related compounds should alter membrane function in a similar nonspecific fashion. In the course of characterizing a series of structurally related antihemolytic azo dyes of known carcinogenicity as potential perturbants of membrane structure and function, we have encountered what appears to be an exception to this rule. A lack of correlation was found between the ability of the structurally related compounds to confer stabilization against osmotic hemolysis and the effect of the compounds on acetylcholine hydrolysis and glucose exchange across the erythrocyte membrane.

Materials and Methods

Dimethylaminoazobenzene (DAB, Eastman), 2-methyl-dimethylaminoazobenzene (2MeDAB, Aldrich), and 3′-methyl-dimethylaminoazobenzene (3′MeDAB, Aldrich) were recrystallized from ethanol/water and moved as single spots when analyzed by thin-layer chromatography on silica gel developed with benzene or benzene/ethanol (90 : 10, v/v).

Acetylcholinesterase studies. Human erythrocytes, obtained from heparinized blood and washed three times after removal of plasma and buffy coat, were resuspended at a 6% hematocrit in 155 mM NaCl, 2 mM NaH_2PO_4, pH 7.4, and 10 mM glucose. Acetylcholinesterase activity was determined by a modification of the method of Ellman [11] using the assay buffer described by Aloni and Livne [12]. A 20 μl aliquot of the erythrocyte suspension was added to 3 ml of 155 mM sodium phosphate, pH 8, containing either the test compound

dissolved in ethanol or an equivalent amount of ethanol which resulted in a final solvent concentration of 0.33% (v/v). After a 15 min incubation, 1 ml of the mixture and 25 μl of 3.4 mM 2,2'-dinitro-5,5'-dithiobenzoic acid (DTNB, EM Biochemicals) were added to sample and reference cuvettes. After adding 20 μl of buffer to the reference, the reaction was initiated by addition of 20 μl of 25.9 mM acetylthiocholine iodide (Sigma) to the sample cuvette. The increase with time of the absorbance at 412 nm was followed on a Cary 118C recording spectrophotometer equipped with thermostated cuvette holders.

Temperature-activity studies of acetylcholinesterase employed the method described by Livne and Bar-Yaakov [13]. The reaction mixture contained 6 ml of assay buffer, 20 μl of solvent or test compound, 50 μl of a 4.8% hematocrit cell suspension, 50 μl of 10.3 mM DTNB and 20 μl of 0.15 M acetylthiocholine iodide. 1 ml aliquots of the reaction mixture were withdrawn at three time intervals and added to a 20 μl mixture of Triton X-100 (3.3%) and 0.19 mg/ml of eserine salicylate (Sigma), an acetylcholinesterase inhibitor. Reaction rates were determined from the best line fitting these three points.

Incubation of red cells with inhibitor was performed in two ways: (1) the assay buffer plus inhibitor (or solvent control) was equilibrated at the temperature at which the enzyme activity was to be measured prior to the addition of an aliquot of cell suspension. After the addition of cells, the mixture was incubated for 15 min before the reaction was initiated by the addition of substrate; (2) cells were added to room temperature buffer containing either the inhibitor or solvent control and incubated for 10 min. The cells were then incubated for 15 min at the assay temperature before addition of substrate.

In order to determine the effect of the different incubation procedures on binding of 2MeDAB to the erythrocytes, triplicate samples were incubated using the two methods described above (low temperature = 6.8° C) and pelleted at 17 400 × *g* for 10 min (4° C). 1 ml of the resultant supernatant was added to 1 ml of 2.4 N ethanolic HCl and the absorbance at 514 nm was determined [18]. The amount of dye bound to the cells was estimated by comparison with a control containing only 2 MeDAB.

High salt extraction of acetylcholinesterase from washed erythrocyte ghosts was performed as described by Mitchell and Hanahan [14] using 1.2 M NaCl.

Ghosts were prepared by the Dodge method using hypotonic phosphate buffer [15]. Protein was measured according to Lowry et al. [16] using bovine serum albumin as a standard.

Glucose studies. Glucose exchange was measured as described by Hershfield and Richards [17]. Erythrocytes wre preloaded by incubation with 130 mM glucose in 120 mM NaCl; 22 mM sodium phosphate, pH 7.4, for 1 h at 37° C. 5 μl of packed red cells were added to a thermostated sample cuvette containing 2 ml of 6.5 mM glucose in the same buffer plus the test compound or ethanol. At the same time, 5 μl of packed cells were added to the reference cuvette which contained 2 ml of 130 mM glucose containing the same compound as the sample cuvette. The turbidity change caused by cell swelling during the exchange process was followed by continuously recording the absorbance at 700 nm until equilibrium was reached. The exit time was determined by extrapolation of the linear portions of the recording. Control mixtures contained ethanol at a final concentration of 2.4% (v/v). At this con-

centration of ethanol no inhibition of glucose exchange was noted. For temperature studies, the external glucose concentration in the sample cuvette was 4 mM.

Results and Discussion

Previous studies from this laboratory have shown that methyl-substituted derivatives of the azo dye carcinogen, dimethylaminoazobenzene (DAB), protect erythrocytes from osmotic hemolysis in the order: DAB < 2MeDAB = 3'MeDAB [18,19]. The additional stabilization afforded by 2- or 3'-methyl substitution of DAB was related to increased partitioning into the membrane conferred by the methyl group [19]. The studies undertaken in this communication sought to define attendant functional effects of these small lipophilic agents by examining both erythrocyte membrane acetylcholinesterase activity and glucose exchange across the membrane.

Human erythrocyte acetylcholinesterase (EC 3.1.1.7) is located in the external half of the membrane bilayer [20]. Because the activity is extractable from erythrocyte ghosts by high salt, the enzyme has been considered a peripheral membrane protein [21]. It cannot, however, be extracted from intact cells using the same method [14]. Furthermore, the erythrocyte enzyme is sensitive to the lipid fluidity of the membrane [22] and is inhibited by a variety of lipophilic compounds upon interaction with intact erythrocytes [12].

The concentration dependence of acetylcholinesterase inhibition by azo dyes (Fig. 1) indicated that 2MeDAB was a more potent inhibitor than

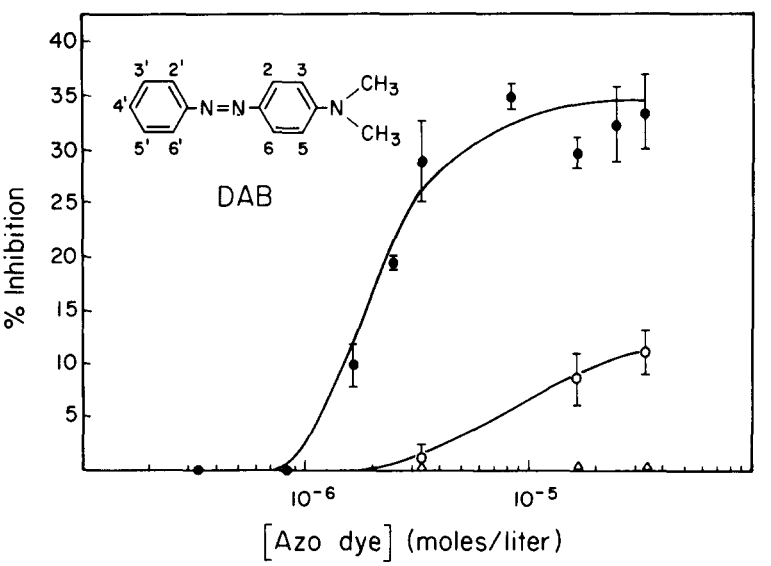

Fig. 1. Concentration dependence of erythrocyte membrane acetylcholinesterase inhibition by azo dyes. The enzyme activity of intact cells was assayed at 21.6° C. Inhibition is expressed as a percentage of the rate of hydrolysis of an ethanol control (final concentration, 0.33%, v/v). Each point represents an average value ± S.E. ●——●, 2MeDAB; ○——○, 3'MeDAB; △——△, DAB.

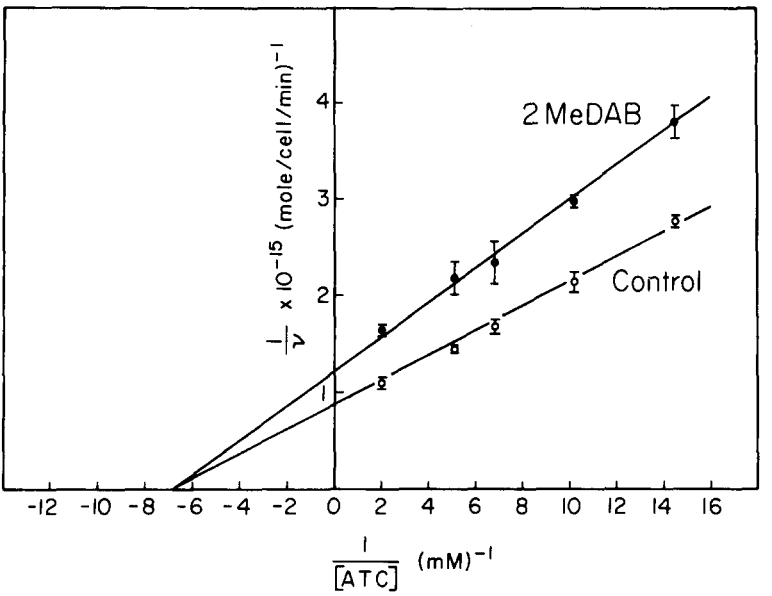

Fig. 2. Kinetics of erythrocyte membrane acetylcholinesterase activity. The enzyme was assayed at 21.6°C. Each point represents the average value of a triplicate determination ± S.D. The control contained ethanol at a final concentration of 0.33% (v/v). 2MeDAB was present at a final concentration of 8.25 μM. ATC, acetylthiocholine.

3'MeDAB at all concentrations studied, while unsubstituted DAB caused no inhibition of the enzyme over the same concentration range. These results clearly differ from osmotic protection experiments in which DAB, 2MeDAB, and 3'MeDAB cause 50% antihemolysis at 8.3 μM, 5.1 μM and 5.1 μM, respectively, with maximum protection from lysis occurring at approximately 20 μM for all three dyes [18,19] (see Fig. 5).

Because of the unexpected lack of correlation between protection from osmotic hemolysis and inhibition of red blood cell membrane acetylcholinesterase, the inhibition by 2MeDAB was studied in greater detail. The compound inhibited the enzyme in a non-competitive manner (Fig. 2), and the kinetic parameters derived from a Lineweaver-Burk analysis of the activity as a function of substrate concentration in the presence (8.2 μM) and absence of inhibitor are K_m = 147 μM; $V = 8.7 \cdot 10^{-16}$ mol/min per cell; K_i = 5.4 μM.

Temperature studies of acetylcholine hydrolysis by the erythrocyte enzyme show a discontinuity in the Arrhenius plot at approx. 20°C [12,23] which has been correlated with the presence of tightly bound lipid in the case of the bovine erythrocyte enzyme [24]. Therefore, the temperature behavior of the enzyme in the presence of 2MeDAB was examined. Arrhenius plots of acetylcholinesterase activity in both intact cells (Fig. 3B) and in a high salt (1.2 M NaCl) solubilized form (Fig. 3A) exhibit biphasic curves with a change in slope at approx. 20°C. Since acetylcholinesterase is isolated by the high salt method as a lipid-protein complex [14], this suggests that the change in the Arrhenius plot is due to a temperature-induced alteration in tightly associated lipid and

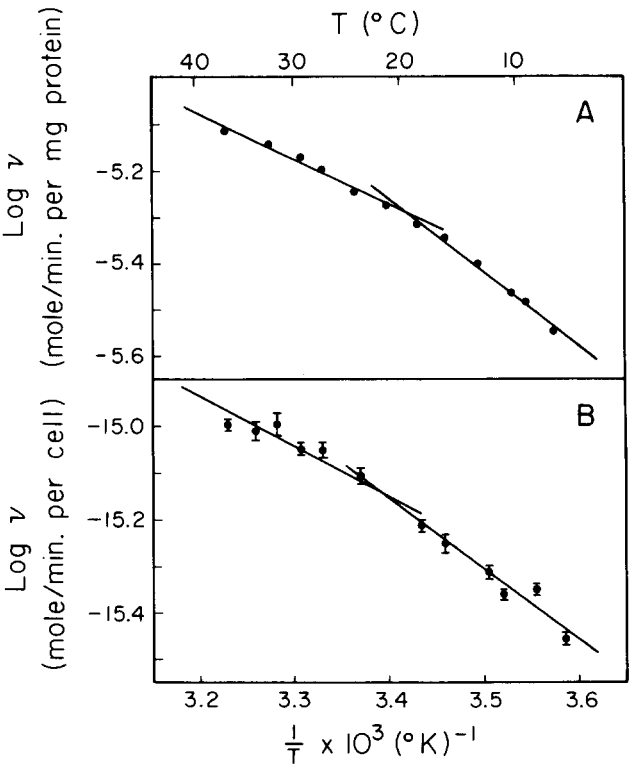

Fig. 3. (A) Temperature dependence of solubilized erythrocyte membrane acetylcholinesterase activity. Acetylcholinesterase solubilized by treatment of washed erythrocyte ghosts with 1.2 M NaCl was assayed by adding an aliquot of solubilized enzyme to buffer preincubated to the assay temperature, as described in Materials and Methods for intact cells. The assay concentration of membrane protein was 0.65 μg/ml. Each point is the average of a triplicate determination. (B) Temperature dependence of intact erythrocyte acetylcholinesterase activity. For this temperature plot, cells were added to a buffer solution containing ethanol (0.33%, v/v) which had been preincubated to the temperature at which the assay was performed. The red cells were incubated at this temperature for 15 min before the reaction was initiated by the addition of substrate. Each point in the Arrhenius plot is the average of a triplicate determination ± S.D. Preincubation at room temperature did not affect the shape of the plot.

not to a general temperature-dependent membrane phenomenon in the case of this enzyme.

In the presence of 2MeDAB, the shape of the Arrhenius plot below 20°C was found to depend on the temperature at which the intact cells were incubated with inhibitor prior to assay. When cells were added to a solution of buffer plus inhibitor precooled to the assay temperature, the plot exhibited a discontinuity in the vicinity of 20°C with decreasing inhibition as the temperature was lowered (Fig. 4A). However, when the cells were exposed to inhibitor at room temperature (approx. 25°C) and then equilibrated to the assay temperature prior to the addition of substrate, the temperature behavior above 20°C appeared to be independent of the change in incubation procedure, while below 20°C the dependence of inhibition on the assay temperature was no longer evident (Fig. 4B). The temperature dependence of inhibition was not due to differential binding of 2MeDAB as a result of the incubation procedure.

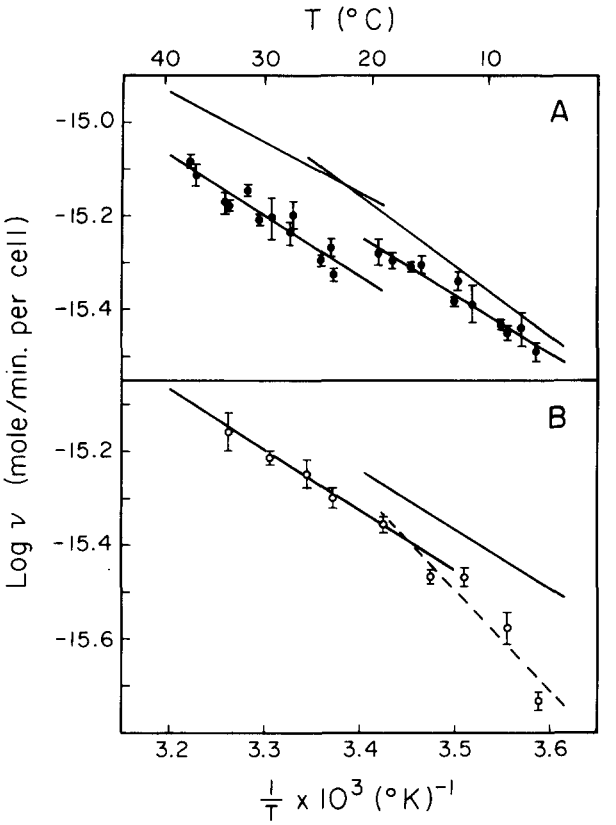

Fig. 4. Effect of 2MeDAB on the temperature dependence of intact erythrocyte acetylcholinesterase activity. (A) Cells were added to a buffer solution containing inhibitor (3.26 μM) which had been preincubated to the assay temperature and the cell suspension was then incubated for 15 min prior to the addition of substrate. The intersecting solid lines represent the temperature behavior of control cells and are redrawn from Fig. 3B. (B) Cells were incubated with 2MeDAB (3.26 μM) for 10 min at room temperature and then at the assay temperature for 15 min prior to the addition of substrate. The solid lines are redrawn from (A) to allow comparison of the effect of the two incubation methods on the inhibition pattern.

When binding as a function of incubation was measured, 54.7 ± 1.3% of the inhibitor remained in the supernatant when cells were added to precooled buffer containing 3.3 μM 2MeDAB and 57.9 ± 1.0% remained in the supernatant when cells were incubated with inhibitor at room temperature prior to cooling.

The results of the temperature studies suggest that the lipids associated with the enzyme must be in a fluid state at the time of incubation with 2MeDAB in order for inhibition to occur. The discontinuity in the Arrhenius plot occurring when the cells were incubated in precooled buffer containing inhibitor (Fig. 4A) may result from an inability of the dye to partition into the site where it acts as a non-competitive inhibitor if the surrounding lipid is in the gel state. Alternatively, below 20°C the enzyme may exist in an altered configuration which is controlled by the fluidity of its tightly bound lipid and which is less reactive with the inhibitor.

TABLE I

INHIBITION OF ACETYLCHOLINESTERASE: DEPENDENCE ON BUFFER COMPOSITION

All assay buffers contained 5 mM sodium phosphate (pH 8). Assay temperature: 21.3°C. Controls contained ethanol at a final concentration of 0.33% (v/v). 2MeDAB was present at a final concentration of 8.25 μM. Values are reported as the average of a triplicate determination ± S.D.

Assay buffer	Control activity (fmol/min per cell)	+2MeDAB (fmol/min per cell)	% inhibition
100 mM $NaPO_4$	1.01 ± 0.02	0.72 ± 0.02	28.7
100 mM Na_2SO_4	0.92 ± 0.03	0.74 ± 0.01	19.6
150 mM NaCl	0.91 ± 0.02	0.88 ± 0.03	—
150 mM $NaHCO_3$	0.97 ± 0.04	0.96 ± 0.04	—

There was no inhibition of acetylcholinesterase activity by 2MeDAB when the activity of washed erythrocyte ghosts prepared by hypotonic lysis was measured. Acetylcholinesterase solubilized by high salt extraction of the ghosts was also uninhibited by 2MeDAB. Furthermore, by changing the assay buffer in which intact cells were incubated with 2MeDAB, it was found that the inhibition was dependent on the type of anion present at the time of exposure of the cells to inhibitor (Table I). When the effect of inhibitor was assayed in buffers containing anions which are rapidly transported across the red cell membrane, viz. HCO_3^- and Cl^-, no inhibition by 2MeDAB was observed. This finding, that acetylcholinesterase sensitivity to inhibition was associated with a transmembrane potential, was first reported by Livne and Bar-Yaakov [13], using linolenoyl sorbitol as an inhibitor. The necessity for the presence of an anion gradient at the time of exposure to inhibitor may explain the lack of inhibition noted when either the extracted enzyme or unsealed ghosts were assayed.

Taken together, the acetylcholinesterase results suggest that: (1) the azo dyes selectively perturb the enzyme; (2) the inhibitor acts indirectly (non-competively) and may interfere with the association of tightly bound lipid; (3) the transmembrane potential determines the state of some membrane component involved, since inhibition is dependent on the anion composition of the incubation buffer.

Examination of the effect of the azo dyes on another membrane function showed that, in contrast to the inhibition pattern for acetylcholinesterase, glucose exchange across the membrane was depressed to the same extent by 2MeDAB and 3'MeDAB, whereas DAB caused no inhibition at any concentration (Fig. 5). These results indicate that inhibition of membrane activity depends on the structure of the azo dye and that this structural dependence varies, to some extent, with the membrane function under study.

An Arrhenius plot of the rate of glucose exchange as a function of temperature is shown in Fig. 6. There is a change in slope of the plot at approx. 23°C seen both in the control and in the presence of 2MeDAB ($1.22 \cdot 10^{-5}$ M). However, there is no evidence that 2MeDAB grossly affects the temperature characteristics of exchange across the membrane. Lacko and Wittke [25] have reported that Arrhenius plots of glucose exchange in the presence of inhibitory steroids also show the same discontinuity as the control plots, and small

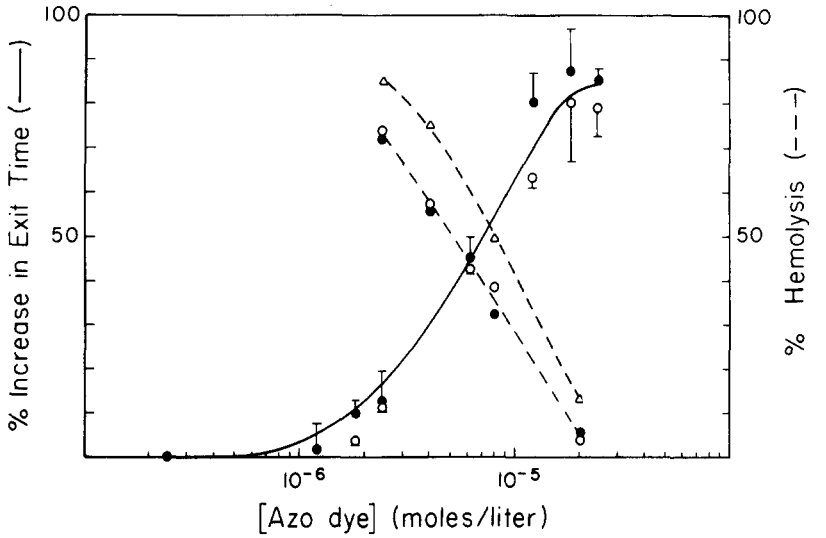

Fig. 5. Concentration dependence of azo dye inhibition of glucose exchange across the erythrocyte membrane. Inhibition is expressed as the percentage increase in the exit time (± S.D.) when measured in the presence of the azo dye compared to an ethanol control (2.4%, v/v). DAB caused no inhibition of glucose exchange when examined at the same concentrations. This figure also illustrates the concentration range over which the azo dyes protect erythrocytes from osmotic hemolysis [18,19]. ●——●, 2MeDAB; ○——○, 3′MeDAB; △——△, DAB.

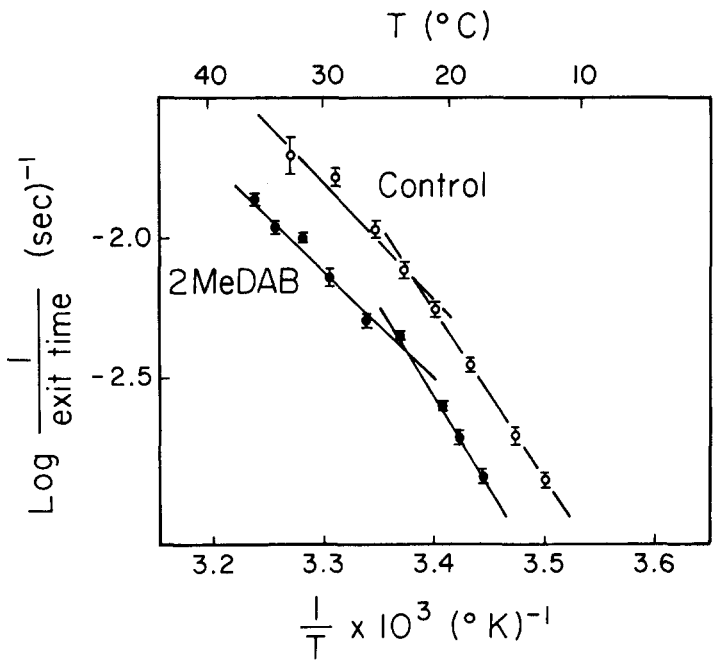

Fig. 6. Effect of 2MeDAB on the temperature dependence of glucose exchange across the erythrocyte membrane. Since the rate of glucose exchange across the membrane is inversely proportional to the exit time, this quantity was used directly for comparison of the rate of exchange in the presence of 2MeDAB (final concentration, 12.2 μM) with the rate of exchange in the presence of ethanol (final concentration, 2.4%, v/v). Each point represents a triplicate determination ± S.D.

changes in activation energy were attributed to fluidity changes induced in the membrane by the steroids.

The interaction of small lipophilic molecules with biomembranes may result in either stimulation or inhibition of membrane enzymes, while transport systems which utilize carriers are inhibited by a variety of hydrophobic molecules [8]. The effect of altered lipid composition on sugar transport in bacteria has led to the observation that the fluidity of membrane lipids influences the transport function [26]. Similarly, it has been found that the lipid composition of the membrane affects the behavior of associated enzymes [27]. In the case of several membrane enzymes, an intimate interaction with specific lipids ('annular lipids' [28,29]) has been postulated, and the state of these lipids necessary for enzyme function may be either fluid [30] or immobilized [31]. Thus, the effect of small hydrophobic molecules on membrane enzymes and transport proteins may be caused by direct interaction with the protein, by disruption of annular lipid surrounding the protein, or by an alteration of the fluidity of the lipid bilayer as a whole.

Using various model systems, evidence has been presented both for [2,4,32] and against [7,33] the induction of lipid fluidity changes by pharmacologically effective concentrations of a variety of small lipophilic compounds. However, it should be noted that the direction of lipid perturbation is influenced by the lipid composition of the model system under study [6,34] and may change as a function of concentration [2,7].

The correlation of erythrocyte stabilization with the membrane concentration of small lipophilic molecules suggested that all three azo dyes tested should have qualitatively similar effects on membrane function but exhibit quantitative differences in the concentration dependence (2MeDAB = 3'MeDAB vs. DAB). The lack of inhibition of two erythrocyte membrane functions by unsubstituted azo dye at any concentration studied was, therefore, unexpected and suggests that there may be a more specific structure-dependent localization or orientation within the membrane than could be predicted from the antihemolytic properties of the compounds. It is interesting, in this regard, that while tetrahydrocannabinol and cannabidiol both protect erythrocytes from osmotic hemolysis [35], the former disorders and the latter orders the lipids of phosphatidylcholine/cholesterol liposomes [36]. Although the molecular basis for correlating inhibition with structure is as yet unknown, the manifestation of differential effects on membrane function has implications concerning the interaction of lipid probes with membrane proteins and points out the danger of generalizations concerning the membrane localization of structurally similar molecules. Work in progress in this laboratory utilizing an unrelated series of compounds extends the observations presented in this paper [37].

Acknowledgments

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