Biochimica et Biophysica Acta, 512 (1978) 579—591

BBA 78152

THE EFFECTS OF ANION TRANSPORT INHIBITORS ON STRUCTURAL TRANSITIONS IN ERYTHROCYTE MEMBRANES

JULIAN W. SNOW, JOHN F. BRANDTS and PHILIP S. LOW *

Department of Chemistry, University of Massachusetts, Amherst, Mass. 01003 (U.S.A.)

(Received December 2rd, 1977)

Summary

Red blood cell membranes have been labeled with several covalent and non-covalent inhibitors of anion transport and their heat capacity profiles determined as a function of temperature. Covalent inhibitors include the amino reactive agents 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid, pyridoxal phosphate and 1-fluoro-2,4-dinitro benzene. The non-covalent inhibitors include several well known local anesthetics. The study was undertaken in order to identify regions of the membrane involved in anion transport. Covalent modification in all cases resulted in a large upward shift of the C transition, which is believed to involve a localized phospholipid region. Evidence is presented which indicates that Band III protein and this phospholipid region are in close physical proximity on the membrane. Addition of non-covalent inhibitors affects the membrane in either or both of two ways. In some cases, a lowering and broadening of the C transition occurs; in others the B_1 and B_2 transitions are altered. These latter transitions are believed to involve both phospholipid and protein, including Band III. These results may indicate that the non-covalent inhibitors produce their inhibitory effect on anion transport at least in part by interacting with membrane phospholipid.

Introduction

The ability of the red blood cell to transport monovalent inorganic anions through the membrane enables it to transfer oxygen and carbon dioxide between tissue and lungs, which is its primary function. It is quite likely that a common transport system exists for these monovalent anions and for divalent

* Present address: Department of Chemistry, Purdue University, West Lafayette, Ind. 47907, U.S.A.
Abbreviations: DADS, 4,4′-diamino-2,2′-dihydrostilbene disulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DNFB, 1-fluoro-2,4-dinitrobenzene; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid.

anions such as sulfate [1,2]. Several investigations have been conducted in recent years in order to elucidate the nature of this transport system [3]. The results of these investigations, although not always in agreement with each other, indicate that the process is complicated, requiring both protein and lipid, and consisting of at least two steps [4—6].

Kinetic studies of anion transport inhibition have revealed a large number of reversible, non-competitive inhibitors, most of which are amphipathic in nature [6—8]. Among these compounds are local anesthetics such as tetracaine and phenol. Other potent non-covalent inhibitors include dipyridamole and phenylbutazone. Although the exact mode of action for these inhibitors is not known, the non-competitive nature of the inhibition implies that the molecules react reversibly with a site separate from the anion binding site.

The use of specific inhibitors which act by covalent bond formation has been successful in identifying membrane components which participate in anion transport. Labeling experiments with several amino-active reagents have demonstrated that the 100 000 molecular weight protein, Band III, is involved in the process [9,10].

The usefulness of scanning microcalorimetry in learning about local membrane structure has been discussed previously [11,12]. Evidence has been obtained in this laboratory which indicates the involvement of protein, including Band III, and phospholipid in the calorimetric transitions of the red cell membrane. Accordingly, we felt it would be of interest to determine the effects of several non-covalent and covalent inhibitors of anion transport on the calorimetry of erythrocyte ghosts. In this paper, we report that the regions of the membrane responsible for the two transitions mentioned above are affected by these inhibitors at concentrations in the inhibitory range. It is speculated that these two regions may be associated spatially in the membrane and that the integrity of each is required for anion transport.

Materials and Methods

The chemicals used in this study were obtained from the following sources: 4,4′-diaminostilbene-2,2′-disulfonic acid (DADS), octylamine hydrochloride, benzyl alcohol and salicylate from Eastman Chemical Company; phenylbutazone, pyrodoxal phosphate and tetracaine from Sigma Chemical Company; phloretin from K and K Labs; 1-fluoro-2,4-dinitrobenzene (DNFB) from Pierce Chemical Company; 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) from the British Drug House through Gallard-Schlesinger Chemical Company; phenol from Mallinckrodt Chemical Company. All these chemicals were used without further purification except DADS, which was recrystallized once from water. A sample of dipyridamole was a gift from Boehringer Ingelheim. Ltd. 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) was synthesized from DADS according to the method of Cabantchik and Rothstein [10].

Membranes were prepared from freshly drawn human blood by the method of Dodge et al. [13]. The appropriate concentrations of non-covalent inhibitors were dissolved in 310 ideal milliosmolar sodium phosphate buffer, at pH 7.40 unless otherwise indicated, and introduced into the membrane by two or more

washings. At the relatively low concentrations employed in this study, the inhibitors did not alter the pH of the buffered solutions.

Covalent inhibitors were reacted with whole cells in 310 imosM sodium phosphate buffer, usually at pH 7.40, under various conditions. Thus, different concentrations of DIDS were reacted with whole cells for 30 min at 37°C. (10% hematocrit). This was followed by three washes in cold, isotonic Tris buffer, pH 7.40, and three more with Tris buffer containing 0.5% albumin before lysis. DNFB labeling was accomplished in a manner similar to that of Poensgen and Passow [14] by reacting whole cells for 30 min at 37°C (10% hematocrit).

Pyridoxal phosphate labeling, followed by reduction of the resulting Schiff's base with sodium borohydride, was performed by the method of Cabantchik et al. [15]. Erythrocytes were exposed to pyridoxal phosphate at 37°C, pH 8.0, for 10 min and then for 10 min at 0°C before centrifugation and removal of the supernatant. The cells were then reduced with sodium borohydride at 0°C for 10 min using a concentration 10 times in excess of the pyridoxal phosphate. Finally the cells were washed repeatedly in ice-cold sodium phosphate buffer prior to lysis. SITS labeling was accomplished according to Cabantchik and Rothstein [10]. Cells were reacted with 0.1 mM SITS in isotonic sodium phosphate for 10 min at 5°C (20% hematocrit), then washed 3 times with buffer alone. This procedure was repeated twice before lysis. In all cases, ghosts were prepared by the method of Dodge et al. [13].

Permeability measurements were conducted as follows. Recently outdated blood was washed three times in wash buffer (5 mM sodium phosphate monobasic/5 mM Na_2SO_4/145 mM NaCl at pH 7.40). The cells were then incubated at 10% hematocrit in the wash buffer for 90 min at 37°C in a shaker bath with tracer amounts of $^{35}SO_4^{2-}$ to load the cells. They were then labeled with DIDS as described previously. The cells were centrifuged, washed twice in cold Tris-bovine serum albumin solution (25 nM Tris/5 mM Na_2SO_4/120 mM NaCl/0.5% bovine serum albumin, pH 7.40 at 22°C) and then once in cold Tris solution. Efflux measurements were performed at 37°C by following the appearance of $^{35}SO_4^{2-}$ in the extracellular medium.

The methods [12,18] and instrumentation [16,17] used in conducting heat capacity measurements have been described previously.

Results

Calorimetric investigation of inhibition by covalent inhibitors. Fig. 1 shows a typical heat capacity profile for erythrocyte ghosts in isotonic sodium phosphate, pH 7.4. Of the five transitions labeled in the diagram, the A transition has been shown conclusively to be due to partial unfolding of spectrin [11]. The B_1 and B_2 transitions have been suggested to involve both lipid and protein, with protein components consisting of Bands IV.1 and IV.2 in the B_1 transition and Band III in B_2 (ref. 19 and Brandts, J.F., Carlson, R., Taverna, R.D., Snow, T. and Lysko, K., unpublished). Several lines of evidence show that the C transition is primarily due to phospholipid, and may involve the melting of an ordered phospholipid region which involves only a small fraction of the total membrane lipid [12]. The D transition leads to extensive protein unfolding, based on results of CD studies [11].

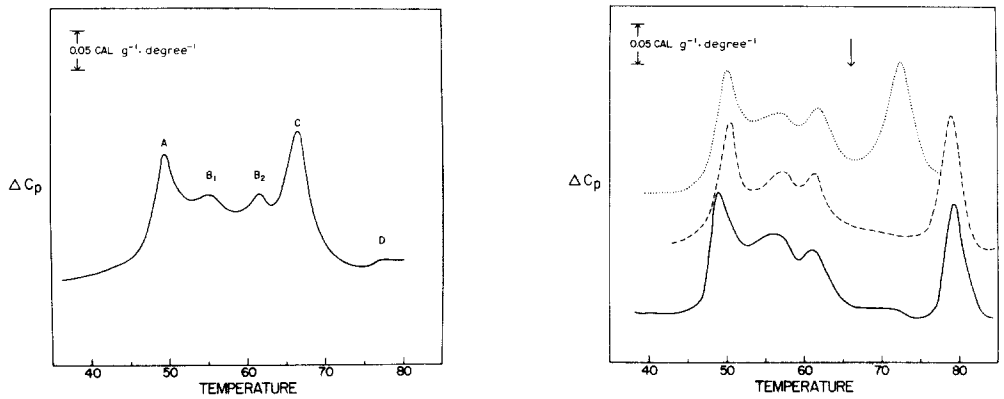

Fig. 1. The heat capacity profile for red cell ghosts in 310 imosM sodium phosphate buffer, pH 7.4. The calorimetric transitions are labeled A, B_1, B_2, C and D. Unless otherwise specified all the calorimetric scans in this study were obtained using identical buffer solutions.

Fig. 2. The effects of 0.1 mM SITS (———), 1 μM DIDS (- - - - -), and 1 mM pyridoxal phosphate (. . . . .) on the calorimetric transitions of the red cell membrane. The arrow at 66°C indicates the position of the C transition in unmodified membranes. Whole cells were labeled with the inhibitors and membranes were prepared as described in Materials and Methods.

In order to assign possible functional roles in anion transport to any of the structural regions giving rise to these thermal transitions, we have determined the calorimetry of erythrocyte ghosts labeled with several chemical modifiers of anion permeability (Fig. 2). The stilbene derivatives DIDS and SITS have been shown to be effective, non-penetrating inhibitors of sulfate and chloride transport and bind quite specifically to Band III [10,14,25]. Under conditions employed in labeling these cells, for example, DIDS is known to label protein with very high specificity, with binding to glycoprotein occurring to a smaller extent. The concentrations of DIDS (1 μM) and SITS (100 μM) used in labeling the cells whose endotherms appear in Fig. 2 are in the inhibitory range. The only major modification for both DIDS and SITS treated membranes occurs in the lipid-related C transition, which is shifted up by 12°C from 66 to 78°C. The small transition at 71°C for SITS-labeled ghosts is probably due to tightly bound, unreacted SITS which is only partially released by washing, since repeated exposure to SITS followed by several washes with buffer results in an increase in size of the 78°C peak and a corresponding decrease in size of the 71°C peak. The tendency for SITS to bind tightly, but non-covalently, to red cells has been noted previously [10].

It is of interest to establish whether the DIDS-membrane interaction is susceptible to modification by extensive bilateral cleavage of membrane proteins. For this purpose, whole cells were labeled with sufficient DIDS to produce 100% inhibition of sulfate transport, and then were subjected to lengthy bilateral proteolysis (0.1 mg/ml papain for 48 h) in a lysing buffer. After this treatment, examination by SDS polyacrylamide gel electrophoresis showed that all major protein bands are gone, leaving only a few, low molecular weight polypeptide cleavage products.

As can be seen from the calorimetric scan in Fig. 3, these DIDS-labeled, proteolysed membranes show only a single transition centered near 75°C. That the

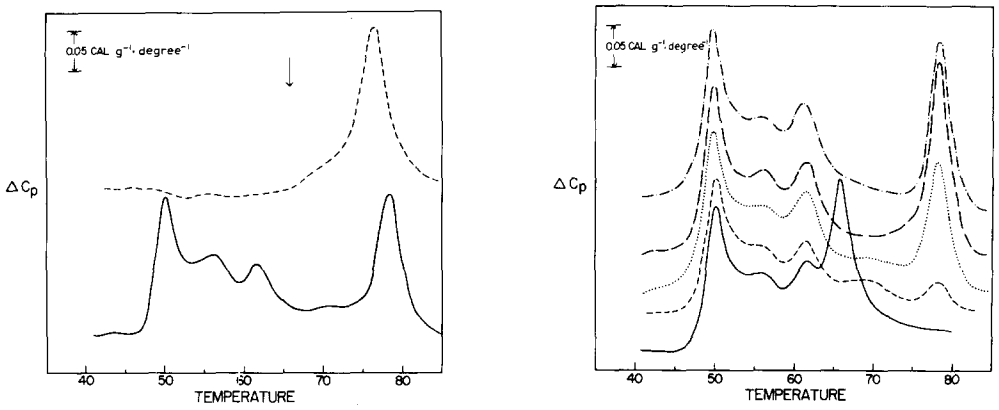

Fig. 3. The effect of exposing DIDS-treated membranes in 20 imosM sodium phosphate, pH 7.4, to papain (0.1 mg/ml) for 48 h at 5°C. DIDS labeling was accomplished using whole cells. ———, DIDS-treated membranes ; - - - - - -, DIDS-treated membranes following proteolysis.

Fig. 4. The effects of different concentrations of DIDS on red cell membranes. The percent inhibition of sulfate flux produced by these concentrations relative to untreated cells is also shown in parentheses. ——— (0%), untreated cells; - - - - - - (73%), 1 μM; · · · · · · (89%), 1.5 μM; — — — (99%), 2 μM; - · - · - · (100%), 3.9 μM.

protease-resistant transition is the C transition has been established from time-dependent proteolysis studies of unlabeled membrane, where it has been shown that all other transitions progressively decrease with incubation while the C transition remains virtually unaffected [12]. In the case of the DIDS-treated membrane, there is a small shift to lower temperature (approx. 3°C), but otherwise extensive bilateral proteolysis has no effect on the C transition.

Similarly, external proteolysis of SITS-treated cells had no effect on the modified C transition nor on any other transition except for a reduction in size of B_1 (not shown).

A series of calorimetric scans for several DIDS concentrations were obtained to determine whether or not the effect on the C transition could be correlated with a loss in transport capabilities. Permeability measurements and calorimetry were determined for identically prepared samples. The results, shown in Fig. 4, demonstrate that there is indeed a qualitative, if not quantitative correlation. The endotherm corresponding to 73% inhibition shows that considerably more than half of the enthalpy change during the C transition now occurs at 78°C. At 99% inhibition virtually all the C transition is shifted to the higher temperature. Thus, calorimetric modification occurs over the same range of DIDS concentrations as inhibition.

Another inhibitor of sulfate transport, pyridoxal phosphate, forms a reversible Schiff's base with membrane amino groups which can be irreversibly fixed by reduction with sodium borohydride. A short exposure time (10 min, 37°C, pH 8.0) minimizes penetration into the cell [15]. Under these conditions, the inhibitor is known to react with lysine residues of Band III and to a lesser extent with glycoproteins. Labeling of lipids under these conditions is negligible. The calorimetric results for cells labeled in this manner (1.0 mM) are similar to the results obtained from DIDS- and SITS-treated cells (Fig. 2). Only the

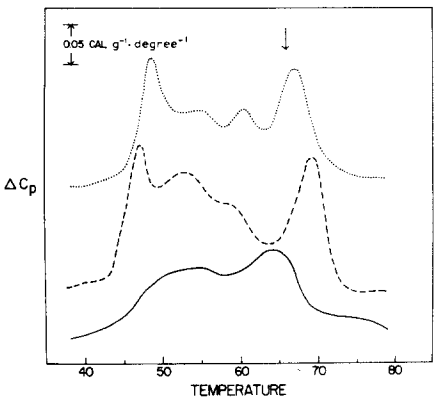

Fig. 5. The effects of labeling whole cells with different conentrations of DNFB. Membranes were prepared subsequent to DNFB treatment: · · · · · · 0.25 mM; - - - - - - 1 mM; ——— 3 mM.

C transition is significantly affected, and this shows an upwards shift of 7°C, from 66° to 73°C, relative to the control. With essentially no labeling of lipid in this case [15], reaction with protein is also apparently responsible for the modulation of the C transition.

Fig. 5 shows the effects of adding three different concentrations of DNFB, a rather non-specific inhibitor of anion transport which is known to penetrate the membrane and label phospholipids (predominantly phosphatidylethanolamine) as well as membrane proteins [14]. Reaction with amino groups is believed to produce the inhibitory effect. The three curves represent, from top to bottom, increasing concentrations of DNFB in the inhibitory range. Even at the intermediate concentration all the transitions are modified. This is undoubtedly due to the non-specific nature of the probe and its ability to penetrate the membrane. It is of interest to notice the behavior of the C transition produced by this inhibitor. The two lower concentrations produce an upward shift of 1 and 3 degrees, whereas the highest concentration results in a downward shift of several degrees. It is quite likely that the pattern of labeling with DNFB is similar to that for trinitrobenzene sulfonate, which has been studied quite extensively in this laboratory (Taverna, R.D. and Brandts, J.F., unpublished). Labeling cells with low concentrations of trinitrobenzene sulfonate results in an upward shift of the C transition, and binding occurs largely to protein rather than lipid. At higher concentrations the C transition is shifted down and binding to lipid occurs to a larger extent.

Calorimetric investigation of inhibition anesthetics and other non-covalent inhibitors. Heat capacity profiles of red cell membranes treated with solutions of several different noncovalent inhibitors of anion transport have been obtained and are presented below. In view of the similar non-competitive nature of inhibition by these compounds, it is important to learn whether their modification of the membrane structure, as is indicated by their effects on the calorimetry, are also similar. The concentrations employed were in the inhibitory range, generally at I_{50} values (concentrations of inhibitor producing 50% inhibition) taken from the literature [6,8].

The results, shown in Fig. 6—8, indicate that the effects produced by the dif-

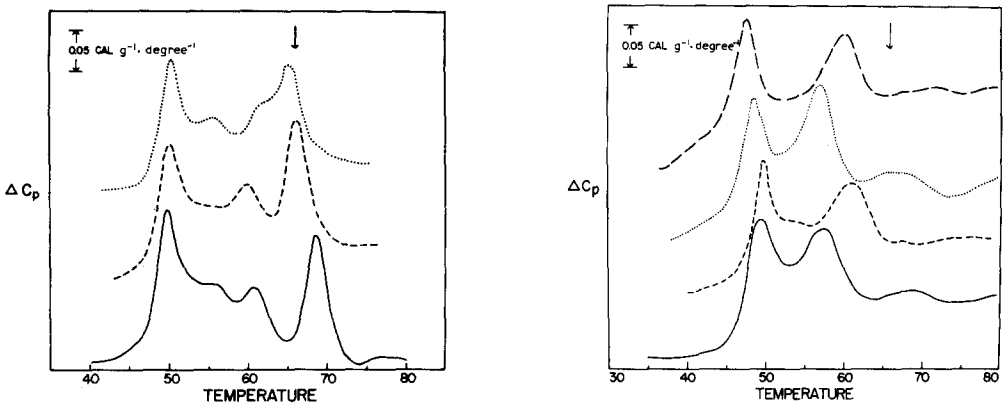

Fig. 6. The effects of exposing red cell membranes to solutions containing various reversible, non-competitive inhibitors of anion flux. The concentrations used are listed in parentheses and have been shown to produce 50% inhibition of sulfate flux in whole cells (I_{50} concentrations): ——— (0.05 mM), dipyridamole; - - - - - - (0.25 mM), phloretin; · · · · · · (0.21 mM), phenyl butazone.

Fig. 7. The effects of exposing red cell membranes to solutions containing other reversible, noncompetitive inhibitors present at I_{50} concentrations: ——— (60 mM), benzyl alcohol; - - - - - - (14.9 mM), phenol; · · · · · · (100 mM), butanol; — — — (6.9 mM), octylamine.

ferent inhibitors are complex and do not follow a single pattern. Phloretin (Fig. 6) causes the disappearance of the B_1 transition and lowers the B_2 transition temperature (T_m) from 62° to 59°C. Phenylbutazone, on the other hand, modifies only the C transition by lowering the T_m slightly (Fig. 6). At higher phenylbutazone concentrations (not shown) the C transition is lowered further and begins to merge on the temperature axis with the B_2 transition. Dipyridamole (Fig. 6), the most potent noncovalent inhibitor studied (I_{50} = 5 M), modifies only the C transition by shifting the T_m slightly upward. Increasing the concentration of dipyridamole by an order of magnitude resulted in an upward shift of the C transition by an additional 3°C (not shown). The enthalpy change (i.e. integrated area) for the B_1 transition was observed to change for several samples

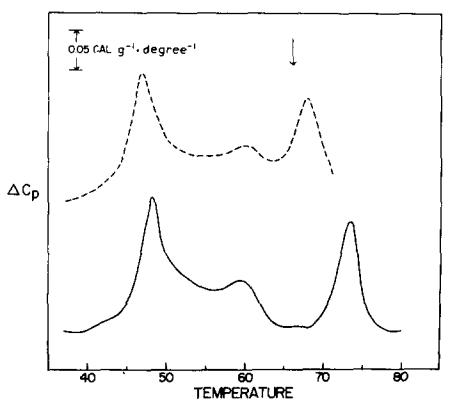

Fig. 8. The effects of exposing DIDS-labeled membranes to solutions of two reversible, non-competitive inhibitors at I_{50} concentrations. Whole cells were labeled with DIDS prior to lysis: ———, DIDS + phenol; - - - - - -, DIDS + octylamine.

treated with dipyridamole, but this aspect of the results was not completely reproducible.

The calorimetric results for membranes treated with solutions of three other local anesthetics and an aliphatic amine are shown in Fig. 7. At approximately I_{50} concentrations phenol, butanol, benzyl alcohol and octylamine all lower the T_m of the C transition and broaden it slightly. The B_1 transition has also disappeared in all cases. Since the modified C transition obscures B_2, an indication of the effects of these inhibitors on the B_2 transition was determined by labeling whole cells with DIDS prior to the preparation of ghosts. The effects of both phenol and octylamine on the B_2 transition were then determined using the DIDS-treated membranes (Fig. 8). Two observations are of interest. First, both phenol and octylamine lower the T_m of the B_2 transition at their respective I_{50} concentrations. The effect for phenol is somewhat greater than for octylamine. In both cases, the B_1 transition is diminished in size. Second, the effects of DIDS and the two local anesthetics are approximately additive and hence seemingly independent of each other; e.g., phenol reduces the T_m of the C transition by 5°C both in the presence and absence of DIDS labeling. This suggests that phenol and DIDS act at different sites in effecting their modifications of the regions of the membrane giving rise to the C transition. For DIDS-labeled membranes suspended in a solution of dilute octylamine the lowering of the C transition is actually somewhat greater than that caused by octylamine in membranes with no prior DIDS treatment, also suggesting separate sites but with some interaction between sites.

In two instances, reversibility of the calorimetric effect of inhibitors was examined. Membranes were treated with either octylamine or phenol at the concentrations given in Fig. 7. The inhibitors were subsequently washed out and the calorimetric scans determined in isotonic phosphate buffer, pH 7.4. In the case of membranes penetreated with octylamine, the scan was identical to that expected for a membrane which had never been exposed to the inhibitor. In the case of pretreatment with phenol, all transitions were of normal size and temperature, except for the B_1 transition which was missing. Since the effects

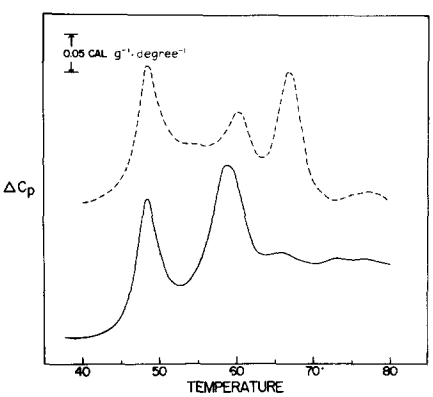

Fig. 9. The effects of exposing red cell membranes to solutions of two reversible, non-competitive inhibitors at concentrations approximately 10-fold greater than I_{50} concentrations: ——— (10 mM), tetracaine; - - - - - - (20 mM), salicylate.

of these inhibitors on anion transport are reversible, this may suggest that the integrity of the 'B_1 domain' is not required for anion transport.

Finally, salicylate, tetracaine and DADS exert only minor influences on the endothermic transitions at I_{50} concentrations (not shown). Tetracaine produces a very slight downard shift of the B_2 transition. A 10-fold increase in tetracaine concentration shifts both the C and B_2 transitions down to form a single observable peak at 59°C (Fig. 9). The B_1 transition is not present in this scan. Increasing salicylate concentration to 20 mM results in a lowering of the B_2 transition to 60°C and disappearance of B_1. The C transition is not affected.

These results show that different non-covalent inhibitors have different effects on the membrane transitions, although the largest alterations are always restricted to the B_1, B_2 and C transitions. It is interesting that those inhibitors which effect the B_2 transition by shifting it down in temperature also, without exception, cause reduction in area or elimination of the B_1 transition. This suggests that the two alterations are coupled, and we will refer to such an inhibitor as a 'B effector'. Likewise, an inhibitor which decreases T_m for the C transition is a 'C effector', and this effect can occur in the presence (e.g., octylamine) or absence (e.g., phenyl butazone) of any effect on the B transitions.

Discussion

Current theories of the anion transport system postulate the existence of either a titratable 'carrier' or fixed, positive charges as the main feature of the system. In the former model, the anion is thought to combine chemically with a specific protein site, either a mobile carrier or a site associated with a gate-like mechanism [2,21]. Detailed characterization of the molecular structure of the carrier is largely speculative. The transport process is thought to consist of at least two steps [22] which necessitate lipid involvement, as well as protein. Recent studies employing amino reagents have led to the development of a model containing Band III as the principal protein participant [4]. The model appears to have features compatible with both theories mentioned above.

The calorimetric results obtained in this study provide certain new evidence which is consistent with the above model and which should be of further use in elucidating the transport mechanism. The results obtained using covalent inhibitors will be discussed first, since the specific and permanent nature of their interactions lead to a more secure interpretation.

All of the specific covalent inhibitors studied (DIDS, SITS, and pyridoxyl phosphate) produce the same single effect on the membrane transitions, i.e., the C transition shifts higher by some 8—12°C when the inhibitor · membrane complex is formed. Since all three molecules produce the same physiological response (transport inhibition) and the same structural response (C shifted higher), it seems likely that there is a direct relationship. Therefore, we speculate that the C 'region' of the membrane (i.e., the local region of the membrane involved in the C transition) is either directly or indirectly associated with anion transport. That this is true was further confirmed by the studies showing close correlation between transport inhibition and shift of the C transition at various levels of DIDS binding (Fig. 4).

The specific membrane site whose attachment to covalent inhibitors causes the shift of the C transition is, all likelihood, on Band III. To support this contention, we first of all cite studies [10,23,24] showing the high specificity of DIDS for Band III. Although there is some quantitative disagreement between laboratories, estimates put the binding to Band III in the range of 80 - 95% of the total binding. The principal sites of binding other than Band III are the sialoglycoproteins. Cabantchik and Rothstein [10] have shown that external proteolysis of DIDS-labeled membranes causes the release of much of the DIDS bound to sialoglycoproteins, but causes little or no release of the DIDS bound to Band III. Our studies of DIDS-treated membranes subsequently subjected to bilateral papain proteolysis showed that the shift of the C transition caused by DIDS binding is not reversed by proteolysis. Therefore, it seems fairly certain that it is not the binding of DIDS to glycoproteins which is responsible for the effect on the C transition. Binding of DIDS to phospholipids at the concentrations of DIDS used here has never been demonstrated to occur. If it does occur, the data of Cabantchik and Rothstein indicated that it must involve less than 1% of the total reacted DIDS. This means that a maximum of only approx. 0.01% of the lipid molecules of the membrane could be DIDS-labeled. This would seem to be far too small an amount to be responsible for the upward shift of the C transition by some 10°C. Finally, it has been shown before [10,24] that the binding of DIDS to Band III is strongly correlated with anion transport inhibition and we have shown that a correlation exists between transport inhibition and the shift of the C transition. It follows then, that the shift of the C transition is correlated with binding of DIDS to Band III. All of these facts then allow us to conclude with some confidence that the effect on the C transition must be caused by the binding of DIDS to Band III.

The C region of the membrane thereby appears to be involved in anion transport and somehow regulated by interaction of Band III with covalent inhibitors. Further interpretation of our data beyond this point is speculative. However, a fairly convincing case can now be made to support the idea that the C transition involves strong phospholipid participation and that it might be due to the melting of a small lipid region which is highly ordered at physiological temperature [12]. The evidence in favor of this idea includes: (1) the lack of CD change (223 nm) in the C transition argues against protein unfolding as the primary structural change; (2) the C transition shows a high sensitivity to many amphipathic additives which are known to shift lipid melting transitions; (3) it is the only one of the erythrocyte transitions which is insensitive to bilateral proteolysis; (4) the C transition disappears progressively as a function of phospholipid cleavage using either phospholipase C or A_2 and (5) the C transition is the only transition which can be 'extracted' from the membrane by brief exposure to lipid-extracting solvents such as *n*-butanol (unpublished results). If this idea does prove to be true, then the binding of DIDS to Band III must somehow exert strong regulation over a lipid region in the erythrocyte membrane. This probably means that Band III is located in close proximity to the lipid region and suggests the further possibility that the anion transport site may be critically dependent upon local lipid architecture as well as on the specificity present in Band III for anion recognition.

A discussion of the effects of anesthetics and other non-covalent inhibitors should be prefaced with the observation that binding to the membrane is reversible and therefore a function of temperature during the calorimetric scan. With temperature-induced structural changes occurring in the membrane, it is possible that both the affinity and number of binding sites vary. Also, many of these types of inhibitors are known to be general effectors of phospholipid transitions [28—30] at the concentrations necessary to inhibit anion transport, so that there is no guarantee modifications seen in the calorimetric scans are necessarily related to transport inhibition. For example, butanol [29] and tetracaine [27] both lower the transition temperature and broaden the melting peak for phosphatidylcholine and phosphatidylserine liposomes, respectively. The ability of local anesthetics such as tetracaine to inhibit the uptake of Ca^{2+} implies a specific interaction with the head group of acidic phospholipids [30] and it has been suggested that these anesthetics exert their effect by preferentially interacting with domains of acidic phospholipids in biological membranes [17]. In general, the properties of local anesthetics have been attributed to their ability to penetrate the lipid bilayer [31].

Several patterns were evident in the calorimetric results on non-covalent inhibitors and these could be of some relevance to the mode of action as transport inhibitors. Modification of the heat capacity profile of ghosts by these inhibitors were confined in the B_1, B_2 and C transitions. No large effects on the A and D transitions were seen. These latter two transitions are the only ones associated with large CD changes (223 nm) and probably are protein-unfolding reactions [11]. There is evidence of lipid involvement in the B_1 and B_2 transitions [12] as well as the C transition, discussed earlier. Although there is as yet no evidence suggesting protein involvement in the C transition, it has been demonstrated by SDS polyacrylamide gel electrophoresis that Bands IV.1 and IV.2 undergo changes in disulfide crosslinking patterns in the B_1 transition and that Band III does so in the B_2 transition (ref. 19 and Brandts, J.F., Carlson, R., Taverna, R.D., Snow, J. and Lysko, K., unpublished). Thus, the B transitions may involve some type of change in both protein and lipid structure, even though the lack of CD change argues against extensive protein unfolding. At any rate, the inhibitors do appear to interact most strongly with those three erythrocyte transitions which had been thought to involve phospholipid participation on the basis of independent evidence.

Of all the noncovalent inhibitors studied, only one was found to produce a modification of membrane transitions similar to that seen for each of the covalent inhibitors. Dipyridamole causes a large upward shift in the T_m for the C transition with no change in any of the other transitions. This was the most potent non-covalent inhibitor examined in this study, and it seems likely that it inhibits in much the same way as the covalent inhibitors. Although many of the other noncovalent inhibitors interact with the C transition, all of them lower the T_m. It was shown that at least two of these (phenol and octylamine) exert this effect both in the presence and absence of DIDS saturation, so that they must bind at different sites in the C region than do the more specific covalent inhibitors.

All of the remaining non-covalent inhibitors besides dipyridamole can be categorized as either B effectors (lower T_m for the B_2 transition, while simulta-

neously causing B_1 to diminish in size) or as C effectors (lower T_m for the C transition), or as both. Two (phloretin and salicylate) were found to the B effectors, one (phenyl butazone) a C effector, while most (octylamine, phenol, butanol, benzyl alcohol, and tetracaine) acted as both B and C effectors. Although all of these inhibitors exerted some effect at their respective I_{50} concentrations, some were more effective than others so that the correlation between inhibitor potency and calorimetric modifications is only an approximate one.

In summary, these results suggest that two structural regions of the membrane may be part of the anion transport system. The involvement of the C region is suggested by its high sensitivity to all three covalent inhibitors and by its sensitivity to most non-covalent inhibitors. Although the B_2 region shows no sensitivity to covalent inhibitors, its reversible interaction with many non-covalent inhibitors and its known involvement with Band III suggest that this region could also be part of the anion transport system. If so, then these two regions might be spatially close to one another on the membrane. This is also suggested from studies (to be published) on ghosts isolated from sheep and cows, where the B_2 and C transitions are coupled into a single transition in isotonic phosphate buffer, pH 7.4. It is only at lower salt that these two transitions separate and give the pattern characteristic of human erythrocyte ghosts. The ability of these two transitions to couple under certain conditions might also be an indication of close proximity of the B_2 and C regions.

It was previously suggested [32] that certain ion channels are 'surrounded by an annulus of lipid, which is in the crystalline, or gel, state'; and that this lipid 'channel' is proximally located to a protein which confers transport specificity. Local anesthetics, it is argued, inhibit transport by interacting with the crystalline lipid phase, thereby closing the channel. Although this suggestion was made specifically for sodium transport, certain features of the model seems attractive for explaining our results on anion transport. In addition to the known involvement of Band III, covalent inhibitors and certain non-covalent inhibitors drastically modify the structural stability of the C region. Existing evidence suggests that the C transition has strong lipid involvement and that it may be a lipid melting transition. Thus, the C region, at physiological temperature, could be a highly ordered, localized region of crystalline lipid in close contact with Band III. Further studies are necessary in order to provide discriminating evidence for or against this picture of the anion transport site.

Acknowledgements

This work was supported by grants from the National Institutes of Health (GM-11071 and HL-17878) and from the National Science Foundation (PCM-74-21665 AOZ).

References

1 Schnell, K.F., Gerhardt, S. and Schöppe-Fredenburg, A. (1977) J. Membrane Biol. 30, 319—350
2 Gunn, R.B. (1973) in Erythrocytes, Thrombocytes, Leucocytes (Deutsch, E. and Wilmanns, W., eds.), p. 77, Georg Thieme Verlag, Stuttgart

3 Sachs, J.R., Knauf, P.A. and Dunham, P.B. (1975) in The Red Blood Cell (Surgenor, D.M., ed.), Vol. II, pp. 613—703, Academic Press, New York
4 Rothstein, A., Cabantchik, Z.I. and Knauf, P. (1976) Fed. Proc. 35, 3
5 Passow, H. (1970) Prog. Biophys. Molec. Biol. 19, 425—467
6 Deuticke, B. (1972) in Biomembranes 3, Passive Permeability of Cell Membranes (Manson, L.A., ed.), p. 381, Plenum Press, New York
7 Gunn, R.B. and Cooper, Jr., J.A. (1975) J. Membrane Biol. 25, 311—326
8 Schnell, K.F. (1972) Biochim. Biophys. Acta 282, 265—276
9 Ho, M.K. and Guidotti, G. (1975) J. Biol. Chem. 250, 675—683
10 Cabantchik, Z.I., and Rothstein, A. (1974) J. Membrane Biol. 15, 207—226
11 Brandts, J.F., Erickson, L., Lysko, K., Schwartz, A.T. and Taverna, R.D. (1977) Biochemistry 16, 3450—3454
12 Brandts, J.F., Taverna, R.D., Sadasivan, E. and Lysko, K. (1978) Biochim. Biophys. Acta 512, 566—578
13 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
14 Poensgen, J. and Passow, H. (1971) J. Membrane Biol. 6, 210—232
15 Cabantchik, Z.I., Balshin, M., Breuer, W. and Rothstein, A. (1975) J. Biol. Chem. 250, 5130—5136
16 Jackson, W. (1970) Ph.D. Thesis, University of Massachusetts
17 Jackson, W. and Brandts, J.F. (1970) Biochemistry 9, 2294—2301
18 Jackson, W., Kostyla, J., Nordin, J. and Brandts, J.F. (1973) Biochemistry 12, 3602
19 Brandts, J.F., Lysko, K., Schwartz, A.T., Erickson, L., Carlson, R., Vincentelli, J. and Taverna, R.D. (1976) Colloques Internationaux du C.N.R.S., No. 246, L'eau et les Systems Biologiques, 169—175
20 Drickamer, L.K. (1976) J. Biol. Chem. 251, 5115—5123
21 Gunn, R.B., Dalmark, M. Dosteson, D.C. and Weth, J.O. (1973) J. Gen. Physiol. 61, 185—206
22 Deuticke, B. (1970) Naturwissenschaften 13, 172
23 Lepke, S., Fasold, H., Prinz, J. and Passow, H. (1976) J. Membrane Biol. 29, 147—177
24 Ship, S., Shanni, Y., Breuer, W. and Rothstein, A. (1977) J. Membrane Biol. 33, 311—323
25 Cabantchik, Z.I. and Rothstein, A. (1972) J. Membrane Biol. 10, 311—330
26 Cabantchik, Z.I. and Rothstein, A. (1974) J. Membrane Biol. 15, 227—248
27 Jain, M.K., Wu, N.Y. and Wray, L.V. (1975) Nature 255, 494—495
28 Ohnishi, S. and Ito, T. (1974) Biochemistry 13, 881—887
29 Seeman, P. (1972) Pharm. Rev. 24, 583—655
30 Papahadjopoulos, D. (1972) Biochim. Biophys. Acta 265, 169—186
31 Papahadjopoulos, D., Jacobson, K., Poste, G. and Sheperd, G. (1975) Biochim. Biophys. Acta 394, 504—519
32 Lee, A.G. (1976) Nature 262, 545—548