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Functional Evidence for Distinct Interaction of Hydrophobic Arylisothiocyanates with the Erythrocyte Anion Transport Protein

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Summary. Human erythrocytes were treated with various hydrophobic arylisothiocyanates under conditions which favor modification of distinct proteinaceous nucleophiles. The morphological appearance of phenylisothiocyanate-treated cells was discoid and membrane-bound hydrolases (human acetylcholinesterase, sheep phospholipase A_2) were fully active following membrane modification. Noncharged hydrophobic arylisothiocyanates, including phenylisothiocyanate, β-naphthylisothiocyanate and heterobifunctional azidoarylisothiocyanates inhibited [^{35}S]-sulfate efflux irreversibly. Protection against modification-induced inhibition of sulfate transport was attained by the simultaneous presence of the specific reversible anion transport inhibitor 4,4'-dinitrostilbene-2,2'-disulfonate. Selective protection of a functionally relevant domain of band 3 is concluded to occur based on the above-derived information.

Key Words erythrocyte membrane · chemical modification · arylisothiocyanates · band 3 protein · anion transport

Introduction

Anion exchange in erythrocytes is mediated by a 95,000-dalton polypeptide, known as band 3 protein. Under physiological conditions the protein effects transmembrane exchange of Cl^- and HCO_3^- anions. The protein-mediated process is inhibited *in vitro* by inorganic and organic anions, structurally related to physiological substrates (for reviews on the subject *see* Deuticke, 1977; Cabantchik, Knauf & Rothstein, 1978; Knauf, 1979). Investigations which included reversibly and irreversibly binding inhibitors have indicated that the minimum requirement for anion transport inhibition is the availability of at least one of differing molecular characteristics. The efficient anion transport inhibitor bears a negative charge (anion), most obviously to interact noncovalently with a positively charged group allocated in the functional domain of the protein (Cabantchik et al., 1978; Knauf, 1979). Furthermore, inhibition is significantly enhanced by the reagent's lipophilicity (Barzilay, Ship & Cabantchik, 1979; Cousin & Motais, 1982) and by the electron-attracting capacity of substituents within the inhibitor molecule (Barzilay et al., 1979; Kitagawa, Terada & Kametami, 1982). Reagents which provide either of the characteristics mentioned in conjunction with a covalently reactive function are appropriate for selective modification of the anion transport protein.

Hydrophobic arylisothiocyanates fulfill the second and the third requirement for selective covalent inhibition of anion transport. Polar negatively charged groups which may direct the inhibitors to the anion binding site of band 3 are absent. The reagents are therefore of particular use for the exploration of otherwise inaccessible hydrophobic domains of band 3. Apolar arylisothiocyanates favorably partition into lipophilic media and are thus capable of covalent membrane protein modification at sites where crucial transmembrane functions may occur. The covalent modification reaction effects thiocarbamylation of reactive nucleophilic groups ($R—S^-$; $R—O^-$: $\rangle N^-$; $R—NH_2$) which, in an apolar environment, is most favorable for lysine ε-amino groups (Sigrist & Zahler, 1982).

In this study the effects of hydrophobic arylisothiocyanates on erythrocyte morphology, membrane-bound hydrolase activities and the process of anion exchange are investigated. With respect to anion transport, the inhibitory potency of various arylisothiocyanates including hetero-bifunctional azidoarylisothiocyanates are compared by analyzing [^{35}S]-sulfate equilibrium exchange in labeled erythrocytes. Distinct interaction of hydrophobic arylisothiocyanates with the erythrocyte membrane is concluded from experiments which document that arylisothiocyanate-induced anion transport in-

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hibition can be protected by specific reversible anion transport inhibitors.

Abbreviations

PITC: phenylisothiocyanate; NITC: β-naphthylisothiocyanate; APITC: *p*-azidophenylisothiocyanate; ITCNA: 5-isothiocyanato-1-naphthalene azide; H_2DIDS: 4,4′-diisothiocyano-2,2′-dihydrostilbene disulfonic acid; DNDS: 4,4′-dinitro-2,2′-stilbene disulfonic acid.

Materials and Methods

Chemicals

Chemicals of highest purity commercially available were obtained from the following sources: phenylisothiocyanate from Fluka, Switzerland; *p*-sulfophenylisothiocyanate, β-naphthylisothiocyanate from Aldrich, Belgium; 4,4′-dinitro-2,2′-stilbene disulfonate disodium salt from ICN (K & K) Chemicals; phloretin from Serva, Germany; bovine serum albumin from Sigma. *p*-Azidophenylisothiocyanate and 5-isothiocyanato-1-naphthalene azide were prepared as described elsewhere (Sigrist et al., 1982). [^{35}S]-Na_2SO_4 was purchased from New England Nuclear.

Erythrocytes and Erythrocyte Membranes

Human blood (citrate anticoagulant) was obtained from the Blood Transfusion Service of the Swiss Red Cross, stored at 4°C and used for experiments within one week after withdrawal. Sheep blood collected into citrate-dextrose solution was supplied by the local slaughterhouse. Erythrocyte membranes were prepared by the procedure of Dodge and collaborators (1963).

Hydrophobic Label Application and Labeling Procedures

Human erythrocytes were washed three times (6,000 × *g*, 4 min, 4°C) by sedimentation in Na/K buffer, pH 7.0 (70 mM NaCl, 50 mM KCl, 5 mM K_2SO_4, 20 mM sodium phosphate, pH 7.0). Hydrophobic arylisothiocyanates dissolved in ethanol were either combined directly with red cells or erythrocyte membranes (PITC, APITC) or added to erythrocytes as sonicated (12 min, bath-type sonicator) suspensions in aqueous buffer (NITC, ITCNA). In either procedure the final concentration of ethanol in the modification media was below 2%.

Erythrocytes (50% cell suspension in Na/K buffer, pH 7.0) and erythrocyte ghost membranes (4 mg/ml) in 10 mM sodium phosphate buffer, pH 7.0, respectively, were modified (1 hr, 37°C) under continuous agitation. Labeled cells were washed three times by sedimentation (6,000 × *g*, 3 min, 4°C) in Na/K buffer. Excess label was removed from modified membranes by repeated (3 times) sedimentation (100,000 × *g*, 20 min, 4°C) before hydrolase activities were assayed.

Morphological Characterization

Suspensions of arylisothiocyanate-labeled erythrocytes (15%) in Na/K buffer, pH 7.0, 0.2% bovine serum albumin were observed in phase contrast under a Dialux 20 EB light microscope (Leitz-Wild). Erythrocytes were morphologically indexed according to Fujii and Tamura (1979). An arbitrarily chosen morphological score of +4 was ascribed to fully crenated cells; a score of −4 represents the state of complete cell invagination.

Enzyme Assays and Analytical Procedures

Phospholipase A_2 activity of sheep erythrocyte membranes was assayed as described by Zahler and Kramer (1981), using egg[^{3}H]-phosphatidylcholine as substrate (prepared according to Stoffel, Lekim & Sang Tschung, 1971). Acetylcholinesterase activity was determined in human erythrocyte ghost membranes according to the procedure of Ellman et al. (1961). Membrane protein content was analyzed in the presence of 0.1% sodium dodecyl sulfate by the Lowry et al. (1951) procedure, bovine serum albumin serving as standard.

Sulfate Exchange Efflux

Inhibition of sulfate exchange by arylisothiocyanates was investigated in human erythrocytes which were equilibrated (1 hr, 37°C) in Na/K buffer, pH 7.0, and loaded (1 hr, 37°C) with trace amounts of [^{35}S]-Na_2SO^4 in 70 mM NaCl, 50 mM KCl, 5 mM K_2SO_4, 20 mM sodium phosphate buffer, pH 7.0. Subsequently, extracellular [^{35}S]-sulfate was removed by washing the cells in cold (4°C) Na/K buffer. Modification by arylisothiocyanates was then carried out as previously described. Sulfate exchange efflux from labeled, washed erythrocytes was measured at 37°C. Packed cells (0.3 ml) were suspended in 6 ml Na/K buffer, pH 7.0. Aliquot samples (0.5 ml) were withdrawn at defined periods of time (cpm(t)) and transferred into 0.1 mM phloretin solution (0.5 ml) at 4°C. Erythrocytes were sedimented in an Eppendorf centrifuge (6,500 × *g*, 2 min, 4°C) and the supernatant containing released [^{35}S]-sulfate was treated with trichloroacetic acid (5% wt/vol final) to remove trace amounts of protein before assessment of radioactivity. Radioactivity at isotopic equilibrium (cpm(∞)) was measured in an aliquot cell suspension which had been deproteinized as above. Rate constants for the exchange of sulfate were calculated according to Barzilay and Cabantchik (1979*a*).

Experiments describing the effect of DNDS on arylisothiocyanate modification entailed the presence of 2 mM DNDS 5 min before and during covalent arylisothiocyanate modification. Following modification labeled membranes were thoroughly washed (7 times) in cold Na/K buffer, pH 7.0, by sedimentation (6,000 × *g*, 3 min, 4°C). Sulfate efflux measurements were carried out as above.

Results and Discussion

Morphology of Arylisothiocyanate-Treated Erythrocytes

It is well established that the morphology of intact erythrocytes may undergo drastic changes due to

the action of reagents which penetrate the membrane. Generally, it has been observed that anionic reagents produce crenation of the red cells while cationic probes produce membrane invagination (Deuticke, 1968; Fujii et al., 1979). Modification studies utilizing hydrophobic reagents demand careful control of the cell morphology, particularly if membrane transport functions are to be investigated. Figure 1 shows human erythrocytes which were modified with the hydrophobic probe PITC (Fig. 1*A*) and, for comparison, cells treated with the anionic reagent *p*-sulfophenylisothiocyanate (Fig. 1*B*). In control experiments only the corresponding solvents were present (Fig. 1*C*). Arylisothiocyanate-labeled cells (≥200 per set of experiment) were morphologically inspected and semiquantitatively indexed according to Fujii and collaborators (1979). The noncharged hydrophobic reagent does not induce detectable changes of the erythrocyte shape. Contrastingly, *p*-sulfophenylisothiocyanate which is known to covalently modify erythrocyte band 3 and aminophospholipids (Ho & Guidotti, 1975; Drickamer, 1977) produces membrane extrusions in a significant number of labeled cells. Morphological changes in the corresponding control cells are insignificant (Table 1).

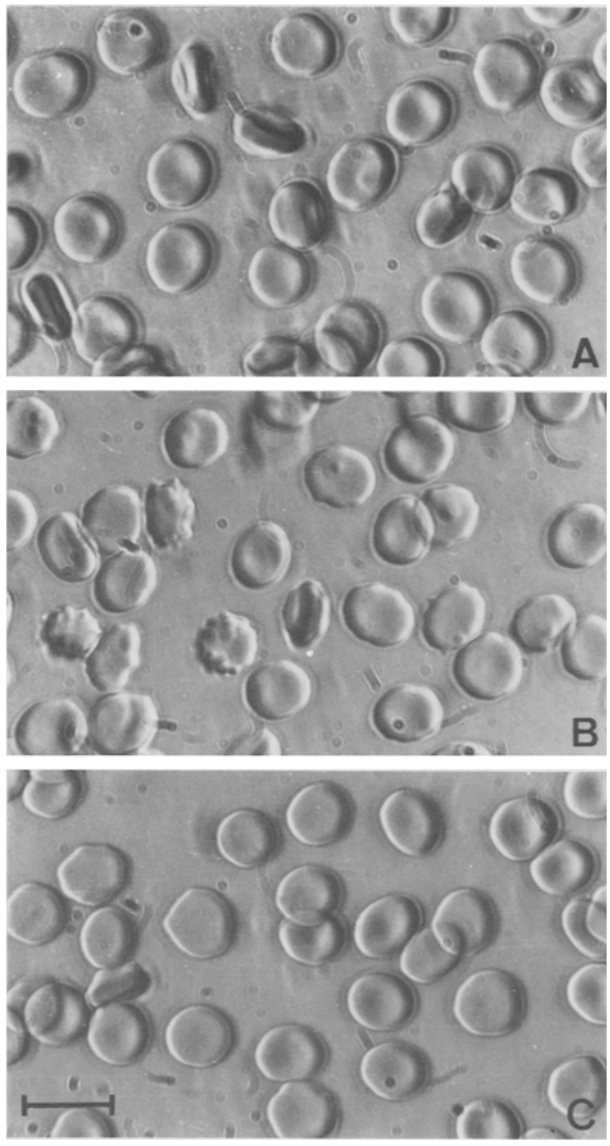


Fig. 1. Morphology of arylthiocarbamylated erythrocytes. Human erythrocytes (50% cell suspension) were modified with the apolar probe phenylisothiocyanate (5 mM, Fig. 1*A*) or the polar reagent *p*-sulfophenylisothiocyanate (5 mM, Fig. 1*B*) for 60 min at 37°C in 70 mM NaCl, 50 mM KCl, 5 mM K_2SO_4, 20 mM sodium phosphate buffer, pH 7.0. After repetitive sedimentation the erythrocyte shape was microscopically examined (Na/K-phosphate buffer, pH 7.0, containing 0.2% bovine serum albumin). Erythrocytes treated with 0.5% ethanol are depicted in Fig. 1*C*. (— 10 μm)

Membrane-Bound Hydrolase Activities

Enzymatic activities of membrane-bound hydrolases were analyzed to investigate the consequences of hydrophobic agents on lipid-protein interactions. Human erythrocyte acetylcholinesterase and sheep erythrocyte phospholipase A_2 have been chosen as "marker" enzymes. Both enzymes require lipids (or amphipathic detergents) for the expression of enzymatic activity for fundamentally different reasons. Unimpaired hydrophobic interactions are essential for full expression of acetylcholinesterase activity (Wiedmer, Di Francesco & Brodbeck, 1979), whose catalytic activity is directed entirely towards the outside of the cell membrane (Steck, 1974). Ca^{2+}-dependent hydrolysis of membrane phosphatidylcholine is attained by a membrane-bound outside-oriented phospholipase A_2 (Frei & Zahler, 1979). The latter enzyme is expected to report exclusively on subtle changes in enzyme-substrate (membrane phosphatidylcholine) interaction.

Arylisothiocyanate modification and subsequent enzyme assays were carried out with erythrocyte membranes. As shown in Fig. 2*A*, acetylcholinesterase remains fully active when up to 10 mM PITC was utilized for membrane modification. Similarly, inhibition of sheep phospholipase was not significant unless ≥5 mM reagent was used (Fig. 2*B*). Neither enzyme activity was inhibited by the polar probe *p*-sulfophenylisothiocyanate (Fig. 2*A* and *B*) and hydrolase assays were not influenced by residual amounts of PITC present within modified bilayer membranes (*data not shown*). Membrane-bound hydrolase activities were not altered below 5

Table 1. Morphological analysis of arylisothiocyanate-modified erythrocytes[a]

Modification	Morphological index
Phenylisothiocyanate, 5 mM	+ 0.01
Control (0.5% ethanol)	+ 0.01
p-Sulfophenylisothiocyanate, 5 mM	+ 0.43
Control (Na/K buffer, pH 7.0)	+ 0.05

[a] The morphological index [Σ(morph. score) × (number of transformed cells/total cell number)] has been applied to semiquantitatively allocate probe-induced shape changes in labeled erythrocytes (Fujii & Tamura, 1979). Morphological index: +4 = fully crenated cells; −4 = complete cell invagination.

mM label concentration although the hydrophobic probe PITC accumulates two- to eightfold within the hydrophobic core of the lipid bilayer (Sigrist & Zahler), 1978). The inherent lipid-protein interactions necessary for enzyme function are therefore not disturbed by the hydrophobic arylisothiocyanate unless the enzymes become covalently modified (phospholipase A_2 at 10 mM PITC).

The hydrophobic arylisothiocyanates favorably partition into the apolar membrane domain according to their physicochemical properties. Disorganization of the erythrocyte membrane does not occur as shown by the preservation of the cell shape (Fig. 1) and membrane hydrolase functions (Fig. 2). Furthermore, irreversible inhibition of anion (sulfate) efflux by nonsulfonated arylisothiocyanates is impaired by the presence of the active site-directed, reversible inhibitor DNDS (Fig. 4). The results thus indicate a direct or indirect, yet distinct interaction of the hydrophobic probes with a domain which is crucially involved in the anion transfer process.

Inhibition of [^{35}S]-Sulfate Exchange by Hydrophobic Arylisothiocyanates

Irreversible inhibition of anion fluxes in erythrocytes has been attained by modification of amino acid side chains which may take part in anion exchange catalysis and/or substrate binding as summarized in Table 2. The target functions include lysine ε-amino groups, arginine guanidino functions and carboxyl groups. At present, participation of histidine imidazole NH in the anion transfer process cannot be excluded. The inorganic anion exchange system is, however, not affected by SH modifying reagents (Deuticke, Richert & Beyer, 1978).

Erythrocyte band 3 modification by the nonsulfonated hydrophobic probe PITC and concomitant

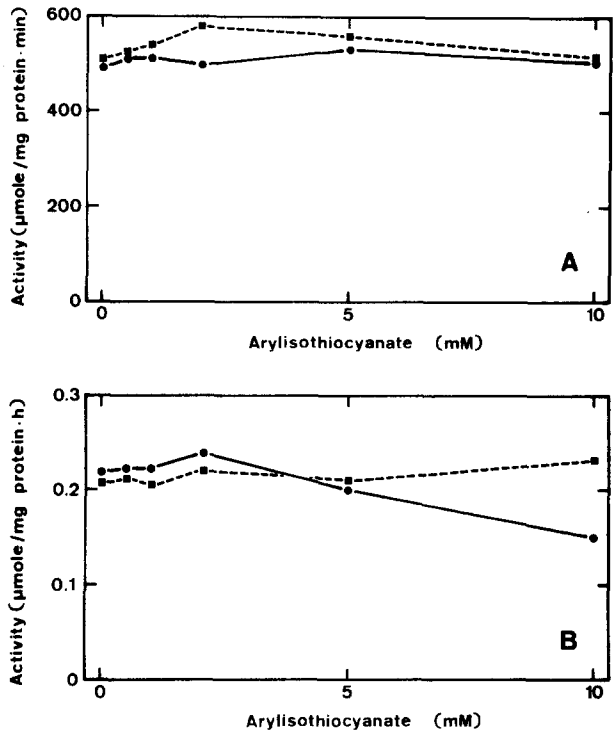


Fig. 2. Hydrolase activities of arylisothiocyanate-labeled erythrocyte membranes. Erythrocyte membranes (4 mg/ml) were incubated with (●—●) phenylisothiocyanate or (■—■) *p*-sulfophenylisothiocyanate for 60 min at 37°C. The sedimented washed membranes were assayed for (*A*) acetylcholinesterase activity (human erythrocytes) or (*B*) phospholipase A_2 activity (sheep erythrocytes)

inhibition of phosphate influx into labeled cells has been reported previously (Sigrist, Kempf & Zahler, 1980). In the present study, the initial observation has been elaborated and extended to sulfate equilibrium exchange which requires a nonchanging internal cell volume unimpaired by the hydrophobic label. Moreover, the media used for sulfate flux measurements contain equal concentrations of Na^+ and K^+ across the erythrocyte membrane. Therefore, changes of the transmembrane ion distribution caused by irreversible inhibition of the erythrocyte Na^+/K^+-ATPase are unlikely.

[^{35}S]-sulfate equilibrium exchange experiments demonstrated that both hydrophobic reagents PITC and NITC inhibit sulfate efflux. The inhibition is concentration-dependent (Fig. 3). The extent of inhibition increases with the duration of the modification step (*data not shown*). Half-maximal transport activity was attained with 4.3 mM PITC (Fig. 3*A*) and 5.4 mM NITC (Fig. 3*B*), respectively. The corresponding crosslinker molecules APITC and ITCNA were comparably effective in blocking an-

Table 2. Exploration of the erythrocyte anion exchange protein by covalent modification

Reagent	Target function	Preferentially labeled segment of band 3[a]	Suggested domain of inhibitor action	References
4,4′-Diisothiocyano-di-hydrostilbene-2,2′-disulfonate	Lys-NH_2	17 K	17 K	Ramjeesingh et al., 1980 Passow et al., 1982
Di-iodosulfophenyl-isothiocyanate	Lys-NH_2	17 K	17 K	Mawby and Findlay, 1982
p-Sulfophenyliso-thiocyanate	Lys-NH_2	17 K	17 K	Ho and Guidotti, 1975
Phenylisothiocyanate	Lys-NH_2	10 K[b]	35 K	Sigrist et al., 1980 Kempf et al., 1981
1-Fluoro-2,4-dinitro-benzene	Lys-NH_2	17 K		Passow et al., 1982
Formaldehyde/$NaBH_4$	Lys-NH_2	60 K; 35 K	35 K	Jennings, 1982
Pyridoxal 5-phosphate/$NaBH_4$	Lys-NH_2	17 K; 35 K	35 K	Cabantchik et al., 1975 Nanri et al., 1983
N-(4-azido-2-nitro-phenyl)-2-aminoethyl-sulfonate: NAP-taurine	Unspecific	17 K	17 K	Knauf and Rothstein, 1980
p-Chloromercuriben-zene sulfonate	Cys-SH		No inhibition	Deuticke et al., 1978
Phenylglyoxal; 1,2-cyclohexanedione	Arg(guanidino group)	9 K[c]	35 K	Bjerrum, 1983; Wieth et al., 1982; Zaki, 1981, 1983
1-Ethyl-3-(4-azonia-4,4′-dimethylpentyl)-carbodiimide/tyrosine ethyl ester	Asp/Glu-β/γ-COOH	35 K	35 K	Bjerrum, 1983

[a] Covalent binding of selective inhibitors is restricted to defined segments of band 3, which are obtained by proteolysis of erythrocytes and erythrocyte membranes. Externally applied chymotrypsin cleaves the band 3 protein into 60 K and 35 K integral fragments (Cabantchik & Rothstein, 1974). Trypsin treatment at the cytoplasmic face of the membrane obtained from chymotrypsinized cells produces 17 K and 35 K membrane-associated fragments and removes a 40 K water-soluble fragment at the cytoplasmic face of the membrane (Steck et al., 1978).

[b] The 10 K membrane-integrated fragment has been isolated from pepsin-digested erythrocyte membranes (Kempf et al., 1981). The peptide is allocated on the C-terminal 35 K segment of band 3 (Tanner et al., 1979).

[c] Following excessive chymotrypsin digestion of phenylglyoxal-labeled membranes a modified 9 K peptide has been isolated, which is a subfragment of the 35 K segment (Bjerrum, 1983).

ion fluxes by thiocarbamylation (Table 3). The inhibitor potencies of the reagents correlate with the molecular dimensions rather than the physicochemical process of label/bilayer interaction: PITC emulgates in aqueous solutions whereas APITC, ITCNA and NITC form amorphous precipitations in polar media. Structural features in conjunction with the inherent chemical reactivity are most probably decisive for individually preferred domains of inhibition.

Protection of [^{35}S]-Sulfate Exchange by DNDS

DNDS is a reversible inhibitor of anion transport. The reagent competes for the anion binding site, which for structural reasons is most probably common to the H_2DIDS recognition domain in Band 3 (Barzilay & Cabantchik, 1979*b*). The reversible probe DNDS is further capable of masking functionally essential proteinaceous groups allocated in the 35 K segment as indicated by the selective modification of band 3 with pyridoxal 5-phosphate/$NaBH_4$ (Nanri, Hamasaki & Minakami, 1983). Comparably, the presence of 2 mM DNDS during covalent arylisothiocyanate modification protects the anion exchange system from irreversible inhibition (Fig. 4*A* and *B*). In accordance with the inhibitor efficiency the degree of protection is more pronounced for PITC (94%) than for NITC (55%).

Although electrostatic interactions with positively charged groups are missing, anion exchange

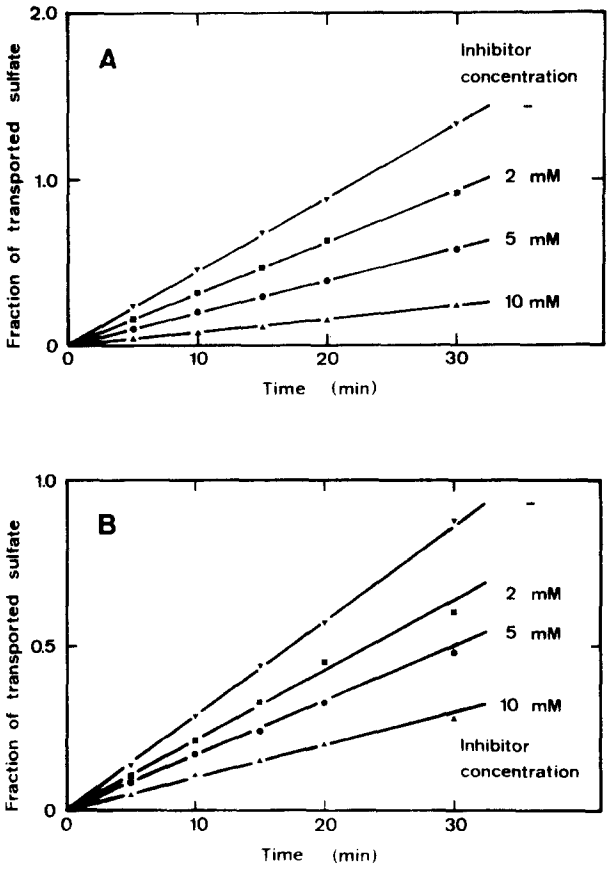
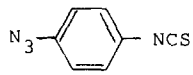
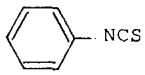
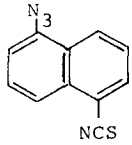
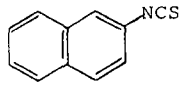


Fig. 3. Inhibition of sulfate exchange by hydrophobic arylisothiocyanates. [^{35}S]-sulfate-loaded human erythrocytes (50% cell suspension) were labeled with *A*) phenylisothiocyanate or *B*) naphthylisothiocyanate by incubation at 37°C for 60 min in Na/K buffer, pH 7.0. After removal of unreacted label [^{35}S]-sulfate efflux was analyzed at 37°C, pH 7.0, as described in Materials and Methods. The fraction of transported sulfate $-\ln\left(\frac{\text{cpm}(\infty) - \text{cpm}(t)}{\text{cpm}(\infty) - \text{cpm}(o)}\right)$ was calculated according to Barzilay and Cabantchik (1979*a*)

Table 3. Inhibition of sulfate exchange by hydrophobic azidoarylisothiocyanates and their respective parent compounds[a]

Inhibitor (5 mM)	Structure formulae	[^{35}S]-sulfate efflux (% of control)
p-Azidophenylisothiocyanate (APITC)		53 ± 2(3)
Phenylisothiocyanate (PITC)		55 ± 5(5)
5-Isothiocyanato-1-naphthalene azide (ITCNA)		31 ± 5(3)
β-Naphthylisothiocyanate (NITC)		47 ± 3(5)

[a] Erythrocytes were equilibrated with [^{35}S]-sulfate and modified (60 min, 37°C, Na/K buffer, pH 7.0) with the indicated inhibitors. Prior to [^{35}S]-sulfate efflux measurements, the erythrocytes were washed (4°C) by sedimentation. All procedures were carried out under subdued light. The number of experiments are indicated in parentheses.

inhibition is attained by hydrophobic arylisothiocyanates. Due to the reagent's apolarity, the domains of inhibition and covalent binding may expectedly differ from the reversible and/or covalent binding sites of aromatic (stilbene-, naphthalene-, and benzene) sulfonic acids (Rakitzis, Gilligan & Hoffmann, 1978; Barzilay & Cabantchik, 1979*a,b*; Ramjeesingh, Gaarn & Rothstein, 1980). Site-directed modification of band 3 by various sulfonated stilbene and benzene isothiocyanates has been allotted to a 17 K transmembrane segment, a subfragment of the N-terminal 60 K chymotryptic segment of band 3 (Ho & Guidotti, 1975; Ship et al., 1977; Ramjeesingh et al., 1980; Mawby & Findlay, 1982; Passow et al., 1982). The 17 K peptide is likewise modified by 1-fluoro-2,4-dinitrobenzene (Passow et al., 1980) and the photolabeled reagent N-(4-azido-2-nitrophenyl)-2-amino-ethyl sulfonate: NAP-taurine (Knauf & Rothstein, 1980). Covalent label binding to the 17 K segment of band 3 has been correlated with transport inhibition effected at the site of covalent interaction.

Recent findings indicate that anion transport inhibition is equally achieved by arginine-specific reagents (Zaki, 1981; 1983; Wieth, Bjerrum & Borders, 1982; Bjerrum, 1983), by carboxyl group labeling (Bjerrum, 1983), reductive methylation (Jennings, 1982) and selective covalent binding of pyridoxal 5-phosphate (Nanri et al., 1983). Evidence has been accumulated from the later studies that partial or exclusive label binding and concomitant anion transport inhibition may occur at the 35 K C-terminal segment of band 3 (Table 2). Moreover, *p*-sulfophenylisothiocyanate suppresses binding of the hydrophobic probe PITC to the peptic peptide P 5 allocated within the 35 K segment of band 3 (Tanner, Williams & Kyle, 1979; Kempf et al., 1981).

Covalent interaction of the apolar arylisothiocyanates with band 3 favorably occurs within the erythrocyte membrane. The ultimate requirement for thiocarbamylation is the reactivity of the interacting nucleophile which is imposed by its local environment. Brock, Tanner and Kempf (1983) have allocated one of the PITC binding sites on the

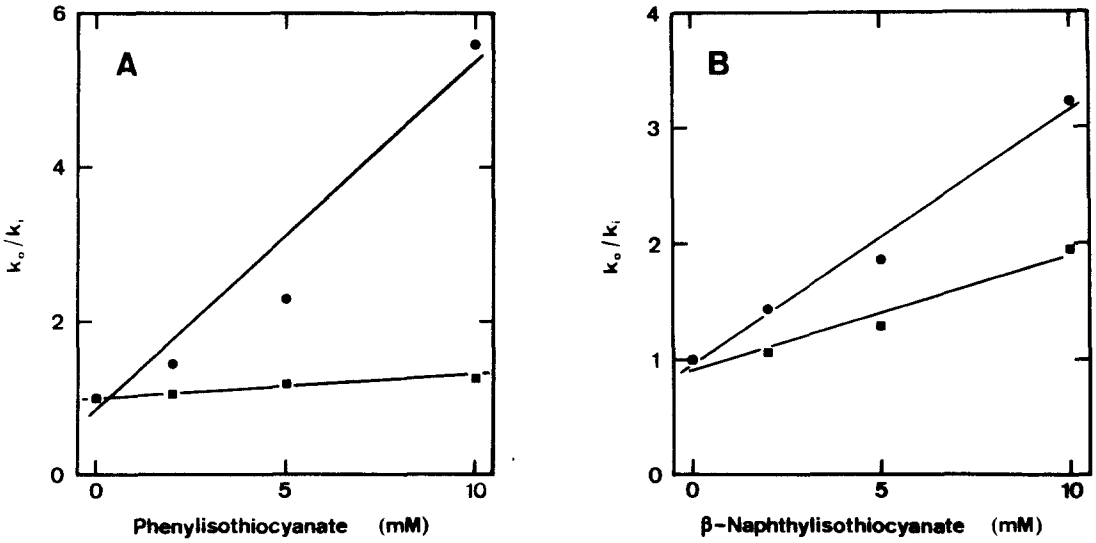


Fig. 4. Protective effect of DNDS on hydrophobic arylisothiocyanate modification (relative Dixon plots). [^{35}S]-sulfate-loaded erythrocytes were modified with irreversible binding arylisothiocyanates in the presence (■) or in the absence (●) of 2 mM DNDS. The rate constants k_o (without inhibitor) and k_i (with inhibitor) were calculated according to Barzilay and Cabantchik (1979*a*). Cells were exposed to phenylisothiocyanate (*A*) or β-naphthylisothiocyanate (*B*) for 60 min, at 37°C, pH 7.0. The erythrocytes were then washed seven times at 0°C with Na/K buffer, pH 7.0 and [^{35}S]-sulfate efflux was measured at 37°C, pH 7.0

peptic peptide P 5. The phenylthiocarbamylated lysine ε-amino group has been topographically alloted within the inner leaflet of the erythrocyte bilayer membrane. In contrast, the positively charged sites of band 3 which preferentially interact with sulfonated arylisothiocyanates are presumably located on the outer leaflet of the membrane. The dual action of sulfonated arylisothiocyanates entails reversible (DNDS) or irreversible (*p*-sulfophenylisothiocyanate) binding to the 17 K peptide and commensurate amino group protection of the C-terminal part of band 3. The protected groups are therefore likely to provide a hydrophobic domain juxtaposed or allosterically related to the anion recognition site. They are, as functionally evidenced, distinctly accessible to hydrophobic arylisothiocyanates. This study thus provides the functional means for site-directed structural exploration of band 3 by differential labeling procedures (Phillips, 1977) in morphologically intact and vital erythrocytes.

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