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Kinetic Analysis of the Inhibition of Sulfate Transport in Human Red Blood Cells by Isothiocyanates

Emmanuel T. Rakitzis*, Paul J. Gilligan, and Joseph F. Hoffman

Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510

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Summary. A kinetic analysis of anion self-exchange in human red blood cells, in the presence of an irreversible inhibitor, is presented and applied to the study of the inactivation of sulfate transport by three isothiocyanates: 3-isothiocyano-1,5-naphthalenedisulfonic acid, disodium salt (INDS), 1-isothiocyano-4-naphthalene sulfonic acid, sodium salt, monohydrate (INS), and 1-isothiocyano-4-benzenesulfonic acid, sodium salt, monohydrate (IBS). The time dependence of the inhibition of sulfate transport by the isothiocyanates used could be described by a single exponential and could be shown to contain a reversible and an irreversible component. In each case a portion of sulfate efflux was found to be resistant to inactivation. The residual portion of the sulfate efflux varied with inhibitor: 4% for INS, 16% for INDS, and 34% for IBS. INS showed the largest reversible inhibitory effect (12% of the flux remaining at 0.2 mM inhibitor concentration), while INDS showed the weakest effect (92% of the flux remaining at 0.3 mM inhibitor concentration). IBS had the highest rate of inactivation while INDS had the lowest. The kinetic analysis further suggests that all three isothiocyanates bind reversibly to an inhibitory site on the membrane before they bind covalently, and therefore irreversibly, to the same site on the membrane. The equilibrium constant for the dissociation of the reversibly-bound complex, K_i, and the rate of irreversible inactivation after all membrane sites are reversibly bound, k_{max}, have been computed for all three inhibitors: INDS ($K_i = 420\ \mu M$, $k_{max} = 5.04\ hr^{-1}$), INS ($K_i = 148\ \mu M$, $k_{max} = 6.48\ hr^{-1}$), and IBS ($K_i = 208\ \mu M$, $k_{max} = 8.11\ hr^{-1}$).

Isothiocyanates have been shown to be potent reversible and irreversible inhibitors of anion transport in human red blood cells (Knauf & Rothstein, 1971; Cabantchik & Rothstein, 1972, 1974; Ho & Guidotti, 1975; Zaki *et al.*, 1975; Ship *et al.*, 1977). The time period required for completion of the inhibition ranges from minutes to hours and, accordingly, is amenable to kinetic analysis. A rate saturation effect with an increase in isothiocyanate concentration has been described (Ho & Guidotti, 1975; Zaki *et al.*, 1975). It therefore appears likely that, as in the case of the irreversible inhibition of enzyme action (Aldridge, 1950;

* *Present address:* Department of Biological Chemistry, University of Athens Medical School, Athens 620, Greece.

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Kitz & Wilson, 1962; Schaeffer, Schwartz & Odin, 1967), an irreversible red cell membrane-inhibitor complex is formed by the covalent attachment of the inhibitor to the binding site of the red cell membrane. A kinetic analysis of the inhibition of sulfate transport in human red cells by three water-soluble isothiocyanates is reported here. An attempt is made to answer two questions concerning the inhibition of anion transport: (i) whether the reversible binding of isothiocyanate on the cell membrane has an effect on anion transport, and (ii) whether the sites on the cell membrane to which isothiocyanate binds irreversibly are uniform and independent, i.e., whether the time dependence of transport inactivation can be described by a single exponential rather than by a summation of exponentials (Ray & Koshland, 1961, 1962; Rakitzis, 1977).

Materials and Methods

Reagents

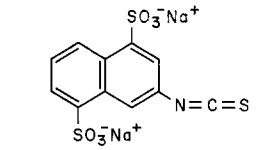
3-Isothiocyanao-1,5-naphthalenedisulfonic acid, disodium salt (INDS) was purchased from Nutritional Biochemicals Corp. 1-Isothiocyano-4-benzenesulfonic acid, sodium salt, monohydrate (IBS), was obtained from Aldrich Chemical Co. Both commercial isothiocyanates were used without further purification since thin layer chromatography (silica gel; ethyl acetate/methanol/water, 15:5:1) revealed no impurities. Their melting points and infrared spectra agreed with those cited in the literature (Braunitzer, Schrank & Ruhfus, 1971; Tietze, 1955).

1-Isothiocyano-4-naphthalenesulfonic acid, sodium salt, monohydrate (INS) was synthesized from thiophosgene and 1-amino-4-naphthalenesulfonic acid, sodium salt (both obtained from Aldrich Chemical Co.). The synthesis followed the method of Tietze (1955) for the preparation of isothiocyanates from amino-arylsulfonates. The parent amine (0.02 moles) was dissolved in 150 ml distilled water, to which 2 ml thiophosgene was added. The reaction mixture was stirred vigorously for 2 hr until fuming stopped; at this time, the pH of the preparation was 1.0. Excess thiophosgene was removed by two 200 ml ether extractions, and active charcoal was added to the aqueous solution. The preparation was filtered through paper, and 25 ml of a 10% NaCl solution (w/v) was added to the filtrate with stirring. Light brown crystals were collected by filtration. The isothiocyanate was recrystallized in 0.4 N HCl by addition of a small volume of the 10% NaCl solution; silver-white crystals were collected by filtration. The overall yield was 70%; thin layer chromatography as performed on the commercial isothiocyanates showed the product to have an $R_f = 0.48$.

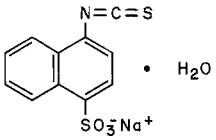
Elemental analysis: $C_{11}H_6O_3NS_2Na \cdot H_2O$
Calculated: C:43.25%, H:2.62%, O:21.00%, N:4.60%, S:21.00%, Na:7.56%
Found: C:43.96%, H:2.93%, O:20.00%, N:4.59%, S:20.54%, Na:8.06%
A trace of NaCl is present (Cl:0.64%)
IR (KBr pellet): 3550 cm^{-1}, 2015 cm^{-1}, 1630 cm^{-1}, 1175 cm^{-1}, 1065 cm^{-1}, 825 cm^{-1}, 760 cm^{-1}.

UV (25 μM aqueous solution): 236 nm ($\varepsilon = 44{,}000$), 318 nm, 333 nm

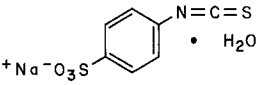
The structures of three isothiocyanates are shown in Fig. 1.



3-Isothiocyano-1,5-naphthalene-disulfonic acid, disodium salt (INDS)



1-Isothiocyano-4-naphthalene-sulfonic acid, sodium salt, monohyrate (INS)



1-Isothiocyano-4-benzenesulfonic acid, sodium salt, monohydrate (IBS)

Fig. 1. Structure of INDS, INS, and IBS

Sulfate Fluxes in the Absence of Inhibitor in the Flux Medium

Sulfate fluxes were measured, in the absence of inhibitor in the flux medium, by the self-exchange method described by Gardos, Hoffman and Passow, (1969). Human blood was freshly drawn from normal adults into a heparinized flask. The blood was centrifuged at 12,100 × *g* and 5 °C for 3 min; the plasma and buffy coat were removed by suction. The red cells were then washed twice with three volumes of a solution whose composition was (in mM): 130 NaCl, 5 KCl, 10 Na_2SO_4, 5 HEPES, and 5 D-glucose (pH adjusted to 7.4 with 0.5 N NaOH). The cells were then incubated in this solution at 37 °C for 2 hr at 6% hematocrit with gentle shaking. The cell suspension was centrifuged at 12,100 × *g* for 5 min, and the supernatant removed by suction. The cells were resuspended at 16% hematocrit in fresh sulfate-chloride medium, to which aqueous $Na_2{}^{35}SO_4$ was added (New England Nuclear, Boston, Mass., 10 mCi/ml. 1 μl/5 ml of cell suspension). This cell suspension was incubated at 37 °C for 1 hr with gentle shaking. The cell suspension was then centrifuged at 12,100 × *g* for 2 min and the supernatant was removed by suction. The cells were resuspended at 37 °C in sulfate-chloride medium at 10% hematocrit. This cell suspension was immediately diluted to 0.5% hematocrit by adding one volume of the cell suspension to 19 volumes of prewarmed buffer to which inhibitor had just been added and dissolved. The cell suspension was incubated at 37 °C with gentle shaking for varying time periods from 10 to 120 min. At the end of this incubation period, the cell suspension was centrifuged at 12,100 × *g* and 5 °C for 5 min. The supernatants were removed by suction and the $^{35}SO_4$-containing cells were washed twice with five volumes of ice-cold $^{35}SO_4$-free solution. This procedure effectively removed excess and reversibly bound inhibitor from the cells. The control experiments were run in the same manner except that inhibitor was omitted from the dilution from 10% hematocrit at 0.5% hematocrit. The rate of efflux of $^{35}SO_4$ was determined by resuspending these washed cells in warm sulfate-chloride medium (37 °C) at 0.5% hematocrit and by measuring the counts appearing at various times, $P(t)$, in the supernatants. The supernatants

were prepared for counting after deproteinizing with 5% trichloroacetic acid solution (w/v), centrifuging at 12,000 × *g* and 5 °C for 3 min. The supernatant counts at isotopic equilibrium $P(\infty)$, were measured from an aliquot of the whole cell suspension, which was deproteinized and centrifuged in the above manner. The rate constant, $k_{SO_4,inh.}$ was determined from a linear least squares fit of the plot of $\ln[(P(\infty)-P(t))/P(\infty)]$ *vs. t.*

Sulfate Fluxes in the Presence of Inhibitor in the Flux Medium

For determination of the sulfate flux when inhibitor is present in the flux medium, the cells were first incubated and loaded with $Na_2{}^{35}SO_4$ as described above. After the 1-hr incubation with $^{35}SO_4$, the cell suspension was centrifuged at 12,100 × *g* for 5 min, the supernatants removed by suction, and the cells were washed twice with five volumes of ice-cold $^{35}SO_4$-free medium. The cells were resuspended at 0.5% hematocrit in medium (37 °C), which contained a known concentration of inhibitor. The values of $P(t)$ and $P(\infty)$ were measured as before. The inhibitor had been dissolved in the solution at 37 °C immediately before the start of the flux determination.

Hemolysis was followed in all experiments by spectrophotometrically measuring the hemoglobin concentrations in the supernatants according to the method of Dacie & Lewis (1968). Because the percentage hemolysis that occurred during the incubation was low whether or not inhibitor was present, the rate constants for sulfate efflux were used uncorrected for hemolysis.

Sulfate fluxes with and without inhibitor in the flux medium were employed in the inhibition studies with INDS. Only sulfate fluxes with inhibitor in the flux medium were used for inhibition studies with the other two isothiocyanates. The effect of INDS on the self-exchange rate of sulfate at two other sulfate concentrations in the buffer was examined using the following solutions whose composition was either 143.5 mM NaCl, 5 mM KCl, 1 mM Na_2SO_4, 5 mM HEPES, 5 mM D-glucose or 2.5 mM K_2SO_4, 90.5 mM Na_2SO_4, 5 mM HEPES, and 5 mM D-glucose. Both solutions were adjusted to pH 7.4 with 0.5 N NaOH.

Kinetic Analysis of Anion Transport in Human Red Cells in the Presence of an Irreversible Inhibitor in the Flux Medium

Sulfate exchange, under low hematocrit conditions where $^{35}SO_4^=$ flow may be assumed to be unidirectional, is described by the equation:

$$-\frac{d[S]}{dt}=k_o[S] \tag{1}$$

where $[S]$ is the intracellular concentration of $^{35}SO_4$ at time t, and k_o is the rate constant for sulfate efflux (Gardos *et al.*, 1969). An irreversible inhibitor may alter the value for k_o through the progressive inactivation of the sulfate transport system, which may be accompanied by reversible inhibition of anion transport. Such reversible inhibition is usually rapid; it may or may not occur at the same site(s) on the membrane as irreversible inhibition. If reversible inhibition occurs rapidly, the rate constant for sulfate efflux assumes another value; call it k_r; k_r will become a time-dependent function with the onset of irreversible modification of the anion transport system, i.e., $k_r(t)$ where the ratio $k_r(t)/k_r$ would equal the fraction of the transport sites not irreversibly bound by inhibitor. If the inhibitor binding sites, at which irreversible modification occurs, are uniform and independent (Ray & Koshland, 1961; Rakitzis, 1977)

$$\frac{k_r(t)}{k_r}=e^{-kt} \tag{2}$$

where k is the inactivation constant. In the most general case, where the inhibitor binding sites may be nonuniform or interacting,

$$k_r(t) = k_r \left(A + \sum_{i=1}^{m} B_i e^{-k_i t} \right). \tag{3}$$

The constants A, B_i, m and k_i arise from the solution of the differential equations for a system of nonuniform or interacting inhibitor binding sites on an enzyme or in a transport system. The solution requires that $A + \sum_{i=1}^{m} B_i = 1$, where m is an empirically derived parameter giving the number of exponential terms in the rate expression. The theory has been derived in detail in a previous paper (Rakitzis, 1977).

Substituting (3) into (1) and integrating from zero time to time t:

$$\ln \frac{[S]_o}{[S]} = k_r A t + k_r \sum_{i=1}^{m} B_i k_i^{-1} (1 - e^{-k_i t}) \tag{4}$$

which, for $m = 1$ reduces to:

$$\ln \frac{[S]_o}{[S]} = k_r A t + \frac{k_r B}{k} (1 - e^{-kt}) \tag{5}$$

where $[S]_o$ is the intracellular concentration of $^{35}SO_4$ at zero time. The constant $k_r A$ is a measure of the inhibitor resistant portion of the sulfate efflux. As time becomes large,

$$\ln \frac{[S]_o}{[S]} = k_r A t + k_r B/k \tag{6}$$

Hence it is readily seen that a plot of $\ln([S]_o/[S])$ *vs.* t gives $k_r A$ as the slope and $k_r B/k$ as the intercept for the limiting case when time becomes large. Equation (5) can be arranged to give:

$$\ln((k_r B/k) + k_r A t - \ln([S]_o/[S])) = -kt \tag{7}$$

using the slope and intercept of Eq. (6). Therefore, $k_r A$, $k_r B$ and k can be determined by graphical methods (Defares & Sneddon, 1960). By definition $A + B = 1$ (Rakitzis, 1977); therefore, $k_r = k_r A + k_r B$, and k_r, A and B can be computed.

The relationship between inhibitor concentration and the inactivation constant, k, can give information on the mechanism of inhibition. When the transport system is inactivated, two mechanisms are most probable: (i) direct, covalent attachment of the inhibitor to the membrane which is independent of any reversible binding, or (ii) initial formation of a reversibly-bound membrane-inhibitor complex, which leads to covalent bonding of the inhibitor to the membrane (Ho & Guidotti, 1975), i.e.,

$$E + I \overset{K_i}{\rightleftharpoons} (EI)_{\text{reversible}} \longrightarrow (EI)_{\text{irreversible}} \tag{8}$$

where E is a free membrane binding site, I is the inhibitor and

$$K_i = [E][I]/[EI]_{\text{reversible}} \tag{9}$$

As in the case of soluble enzymes (Schaeffer *et al.*, 1967), these two mechanisms can be distinguished kinetically. In the first case, the observed rate of irreversible inhibition will be directly proportional to inhibitor concentration. For the second mechanism, k will increase with inhibitor concentration until the reversibly binding membrane sites are saturated with inhibitor molecules. This rate saturation effect requires that

$$\frac{[I]}{k} = \frac{[I]}{k_{\max}} + \frac{K_i}{k_{\max}} \tag{10}$$

where k_{max} is the inactivation constant after all sites are reversibly bound (Aldridge, 1950; Kitz & Wilson, 1962; Schaeffer *et al.*, 1967; Ho & Guidotti, 1975). Hence, a straight line plot of $[I]/k$ *vs.* $[I]$ further confirms the second mechanism (Hanes, 1932), and allows the computation of K_i and k_{max}.

Results and Discussion

Sulfate Efflux in Human Erythrocytes in the Presence of Isothiocyanates in the Flux Medium

The effect of INDS on sulfate efflux in human red cells is shown in Fig. 2. The data are plotted according to Eq. (6). It will be seen that the slope of the line drawn through the points on the graph diminishes with incubation time until it reaches a constant value. The same effect was observed with all concentrations of INDS used. There is no correlation between this inactivation-resistant portion of the flux and inhibitor concentration (Table 1, column *B*). When the results obtained from the slopes and intercepts of Fig. 2 are plotted according to Eq. (7), a straight line relationship is obtained (Fig. 3). This type of relationship was found to hold with all concentrations of the three isothiocyanates used.

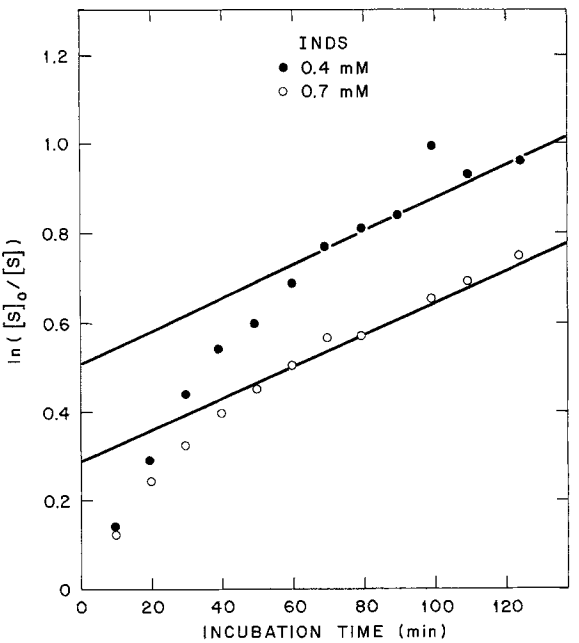


Fig. 2. The time course of sulfate efflux in human red cells in the presence of 0.4 and 0.7 mM INDS in the incubation medium. The results were plotted according to Eq. (6)

Table 1. Kinetic constants of sulfate efflux in human red cells in the presence of INDS in the incubation medium

Inhibitor (mM)	$k_r A$	k	k_r	B	% hemolysis in 60 min.
At 10 mM *sulfate*					
0.3	0.281(±0.07)	2.12(±0.21)	1.57(±0.14)	0.82(±0.08)	2.0
0.4	0.229(±0.01)	2.31(±0.18)	1.54(±0.12)	0.85(±0.07)	2.4
0.5	0.250(±0.09)	2.78(±0.20)	1.68(±0.10)	0.85(±0.06)	1.1
0.6	0.230(±0.01)	2.57(±0.17)	1.31(±0.08)	0.82(±0.04)	4.0
0.7	0.225(±0.02)	3.14(±0.08)	1.29(±0.06)	0.83(±0.05)	3.4
1.0	0.186(±0.02)	5.00(±0.10)	1.09(±0.08)	0.83(±0.06)	2.0
2.5	0.147(±0.03)	5.25(±0.15)	0.985(±0.07)	0.85(±0.10)	4.7
At 1 mM *sulfate*					
0.3	0.317(±0.05)	1.21(±0.10)	1.12(±0.03)	0.76(±0.04)	1.6
At 93 mM *sulfate*					
0.3	0.173(±0.02)	1.98(±0.08)	1.50(±0.05)	0.89(±0.06)	3.4

The values of the kinetic constants are in reciprocal hours. The constants $k_r A$ and k were determined from a linear least squares fit of the data; the parenthetical values are the errors in the regression coefficients. The constants k_r and B are derived as described in the text; their derived errors are in parentheses. The constants are not corrected for hemolysis.

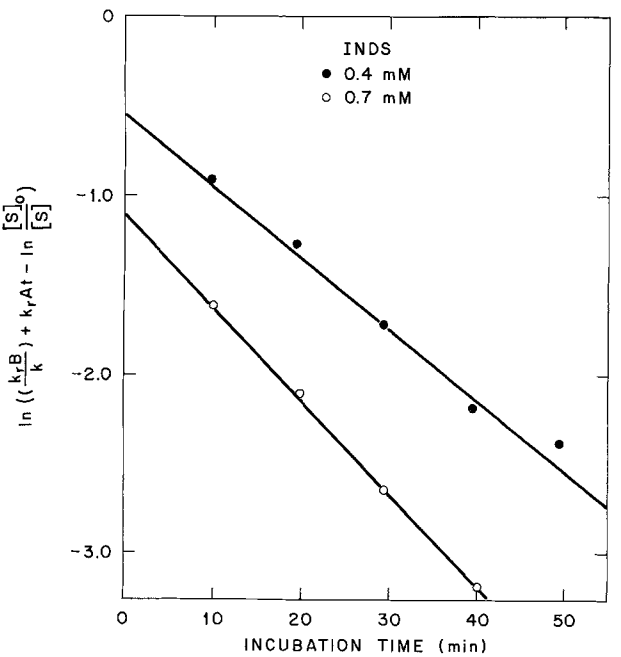


Fig. 3. Determination of the inactivation constant of sulfate efflux by 0.4 and 0.7 mM INDS by plotting the results according to Eq. (7). *See text* for details

These results indicate that the inactivation of sulfate transport by the isothiocyanates used can be described by a single exponential. However, a straight line relationship such as presented in Fig. 3 could also be obtained if the inactivation effect was due to a summation of exponentials; in such a case, the slope of the line drawn through the points of the graph would correspond to the slowest exponential. It is not possible to differentiate between these two possibilities from a graph such as that of Fig. 3, because the basis of this graph cannot be known by first principles. However, it is possible to confirm the finding that the inactivation effect is described by one single exponential by comparing the k value obtained from Eq. (7) with the inactivation effect produced on red cells when the inhibitor is removed by washing before the determination of the flux constant (*see below*).

The average inhibitor-resistant portion of the flux was roughly 16% with INDS (the inhibitor-resistant portion of the flux is computed by dividing the $k_r A$ value, in Tables 1 and 2, by the value for the flux in the absence of an inhibitor, i.e., by 1.4/hr); it was 34% for IBS and 4% for INS (average of the value obtained from the data presented in Tables 1 and 2). The inhibitor-resistant portion of the flux may represent (i) leakage in the red cell membrane (which would include the reversibly inhibited portion), (ii) a subsidiary sulfate carrier system that is totally resistant to inactivation by the inhibitors used, (iii) attachment of the irreversible inhibitor on the active site of the protein functioning unit, but in such a manner that only part of the activity of the unit is

Table 2. Kinetic constants of sulfate efflux in human red cells in the presence of INS and IBS in the incubation medium

Inhibitor (mM)	$k_r A$	k	k_r	B	% hemolysis in 60 min.
INS					
0.10	0.048(±0.01)	2.41(±0.07)	0.323(±0.04)	0.85(±0.04)	2.2
0.15	0.094(±0.03)	3.68(±0.38)	0.278(±0.04)	0.66(±0.05)	1.4
0.20	0.067(±0.02)	3.70(±0.20)	0.310(±0.03)	0.78(±0.02)	1.3
0.25	0.037(±0.01)	3.72(±0.04)	0.179(±0.02)	0.79(±0.03)	2.6
IBS					
0.075	0.137(±0.04)	2.08(±0.02)	0.660(±0.06)	0.79(±0.04)	2.2
0.15	0.638(±0.06)	3.30(±0.10)	1.351(±0.08)	0.53(±0.05)	3.4
0.20	0.670(±0.08)	3.78(±0.15)	1.360(±0.06)	0.51(±0.04)	3.2

Sulfate concentration in the flux medium was 10 mM. The values of the kinetic constants are in reciprocal hours.

The constants and their errors are determined as for Table 1.

destroyed (Ray & Koshland, 1961), or (iv) negative irreversible inhibitor cooperativity, e.g., the covalent attachment of one or more irreversible inhibitor molecules to the protein transport unit, but at a site other than the active site of the unit, so that the modified protein unit is resistant to inhibitor attachment (Levy, Leber & Ryan, 1963; Kitchen & Andrews, 1974; Magee & Ebner, 1974; Schram & Lawson, 1963; Rakitzis, 1977).

Kinetic evidence alone is insufficient to distinguish between all the above possibilities. Leakage may arise from cell lysis or damage to the cell membranes, during repeated washings, without lysis. Hemolysis during the course of inhibition has been found to increase with increased exposure to inhibitor. However, no correlation exists between the extent of hemolysis, which was small, and the noninhibitable portion of sulfate efflux. Tables 1 and 2 reveal no relation between percent hemolysis in 60 min with the values for $k_r A$. The variation of the inhibitor-resistant portion of the flux with inhibitor excludes the possibility that either a sulfate leak or a subsidiary sulfate carrier system is solely responsible for this inhibitor-resistant portion.

The value for the inactivation constant k is dependent on the isothiocyanate concentration (Fig. 2, Tables 1 and 2), and a rate saturation effect was observed. The inactivation constant for INDS was not effected by the sulfate concentration in the medium (Table 1). Assuming a simple equilibrium relationship between the isothiocyanate and the membrane binding site (Aldridge, 1950; Kitz & Wilson, 1962; Schaeffer *et al.*, 1967) the value for K_i (dissociation constant of the reversibly formed membrane-site inhibitor complex, whose subsequent first order reaction leads to irreversible inhibition) and for k_{max} (the inactivation constant when all of the sites are reversibly bound with inhibitor) may be determined by graphical procedures. A plot of $[I]/k$ *vs.* $[I]$, in analogy with the Hanes (1932) plot for enzyme action, is shown in Fig. 4. It will be seen that a straight line relationship is obtained between the $[I]/k$ and $[I]$ values, thus ruling out the possibility of reversible binding cooperatively (Koshland, 1970). All three isothiocyanates used in this study have given essentially similar results with those presented in Figs. 2–4. The values for K_i and k_{max} obtained are given in summary form in Table 3.

The hyperbolic shape of the k *vs.* $[I]$ dependence curve is indicative of the existence of a reversible membrane site-inhibitor complex, in analogy with Michaelis-Menten kinetics of enzyme action. Accordingly, the data presented in Fig. 4 strongly support the assumption that inactivation of the sulfate transport process by the isothiocyanates used in the present study is provided by the mechanism described by Eq. (8). An

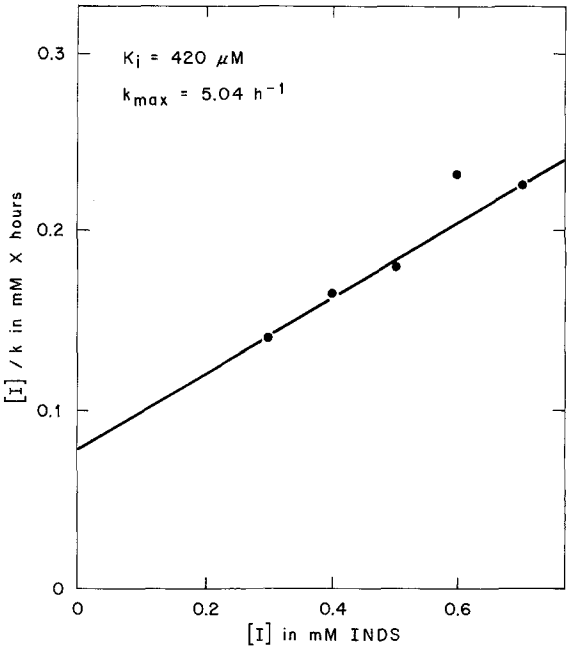


Fig. 4. Determination of the binding constant (K_i) of INDS with the red cell membrane. The results were plotted according to Eq. (10). *See text* for details

Table 3. Kinetic constants of the inactivation of sulfate transport by isothiocyanic acid esters

Compound	K_i (μM)	k_{max} (hr^{-1})
INDS	420(±23)	5.04(±0.26)
INS	148(±50)	6.48(±1.60)
IBS	208(±21)	8.11(±0.40)

The values pertain to a 10 mM sulfate concentration in the incubation medium.

The constants K_i and k_{max} have been determined from a linear least squares fit. The parenthetical values are the errors derived from the errors in the regression coefficients.

alternative mechanism for the inactivation process would be the covalent attachment of the inhibitor on the membrane sites, without the prior formation of a reversible membrane site-inhibitor complex, that is, by a simple bimolecular mechanism (Baker, 1967; Rakitzis, 1974). In such a case the dependence of k on $[I]$ would be a straight line passing through the origin. A similar type curve could also be obtained if inactivation was carried out according to Eq. (8), with K_i being much larger than $[I]$;

Eq. (10) then becomes

$$\frac{[I]}{k} = \frac{K_i}{k_{max}}. \tag{11}$$

In such a case a plot of $[I]/k$ *vs.* $[I]$ would give a straight line parallel to the $[I]$ axis, and with an intercept of K_i/k_{max} on the $[I]/k$ axis.

The large standard errors in the values for K_i and k_{max} for INS and the independence of k of inhibitor concentration may be explained by the observation that INS penetrates the erythrocyte membrane (P.J. Gilligan and J.H. Kaplan, *unpublished results*). INS may then react with the intracellular aspect of the membrane protein and distort the kinetic analysis in the present study.

Several isothiocyanates have been known to behave as reversible inhibitors of anion transport in human erythrocytes (Cabantchik & Rothstein, 1972, 1974; Lepke *et al.*, 1976; Zaki *et al.*, 1975). The reversible effect of isothiocyanates on the flux constant (k_r) appears to be quite a weak one in the case of INDS (Table 1). For k values that are as close to the k_{max} value as possible, i.e., where nearly all of the inhibitor binding sites are reversibly complexed with the isothiocyanate, the flux constant has dropped to only about 1.00/hr. This finding indicates that

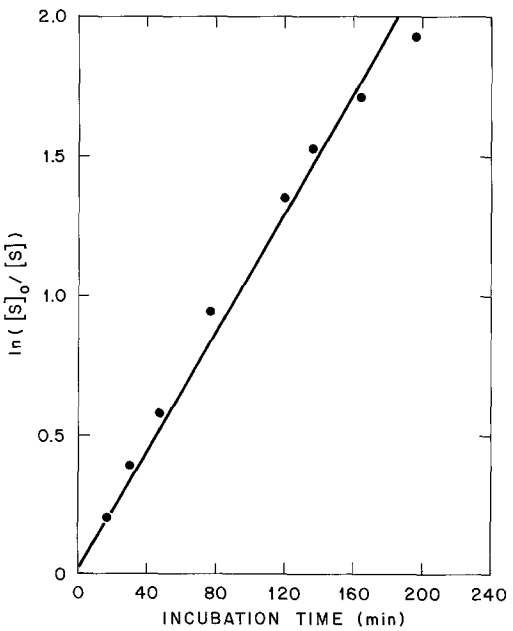


Fig. 5. Sulfate efflux from red cells in the presence of 0.075 mM INS

the sulfate binding sites and the INDS binding sites on the red cell membrane are not identical. With INS (Table 2) the flux constant drops to about 0.2/hr. A strong reversible inhibition effect is observed for INS in the case where sulfate efflux is measured with 0.075 mM INS in the flux medium. A plot (Fig. 5) according to Eq. (6) gives a straight line with a slope of $0.64\,hr^{-1}$. The absence of an exponential curve would suggest there may be no appreciable irreversible inhibition.

Sulfate Efflux in Human Red Cells which had been Pretreated with Isothiocyanates

The kinetic analysis employed above considers the time-dependent diminution of sulfate efflux in erythrocytes incubated in the presence of the isothiocyanates to arise from the development of the irreversible inhibition of the sulfate exchange process. The validity of this analysis can be verified independently. Studies were carried out using cells which had been exposed to the inhibitor, INDS, but were washed to remove reversibly bound inhibitor before determining the flux constant ($k_{SO_4, inh.}$).

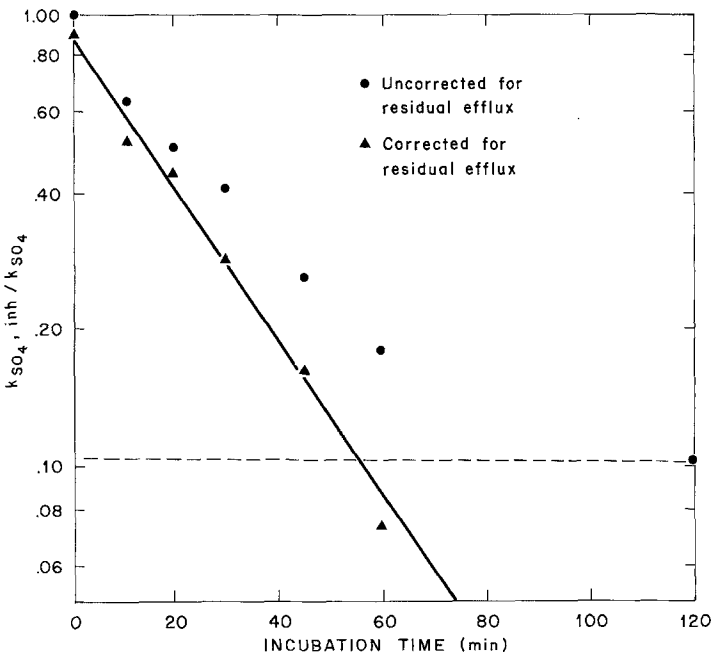


Fig. 6. Inactivation of sulfate efflux in red cells pretreated for different lengths of time with INDS. The change in sulfate efflux represents the extent of inhibition due to the irreversible binding component of INDS. *See text* for details

In this type of experiment the inhibition of sulfate transport is completely irreversible. A plot of $\log(k_{SO_4,\,inh.}/k_{SO_4})$ *vs.* incubation time, for 0.5 mM of INDS is shown in Fig. 6. It will be seen that the inactivation of sulfate efflux levels off at 10.8% of the control flux constant, k_{SO_4}. When this 10.8% residual activity value is subtracted from the percent activity at each time, t, and the result plotted on a log scale once more, a straight line relationship is obtained (Levy *et al.*, 1963), since

$$\ln(k_{SO_4,\,inh.}/k_{SO_4})_{corrected} = -kt. \tag{12}$$

The value for k from these studies should agree with the value obtained from Eq. (7). Figure 6 gives $k = 2.34 \pm 0.20\,hr^{-1}$. This value is 15.8% less than the value for k obtained when 0.5 mM INDS is present in the flux medium (Table 1). The agreement between the two estimates of the k for irreversible inhibition is considered satisfactory since the difference falls within experimental variation. Furthermore, the straight line plot in Fig. 6 demonstrates that the inactivation of the transport system is a single exponential function of time. The other two isothiocyanates also gave straight line plots for Eq. (12).

Stability of Isothiocyanates in Sulfate-Chloride Medium

The above analysis assumes that the isothiocyanates used remain at constant concentration and are not hydrolyzed during the course of their reaction with the red cell membranes. To test this assumption, the isothiocyanates were incubated in the 10 mM sulfate medium at 37 °C for 3 hr, at the end of which time the $^{35}SO_4$-loaded red cells were added and sulfate efflux determined. With INDS at 0.6 mM, INS at 0.2 mM, and IBS at 0.15 mM, the k values obtained were 2.03/hr, 3.24/hr and 2.71/hr, respectively. These values are approximately 20% smaller than the corresponding values when the isothiocyanate was dissolved in the sulfate-chloride medium immediately before the beginning of the flux determination (Tables 1 and 2). The isothiocyanates will hydrolyze to the corresponding amino-arylsulfonates which are not known to bind covalently to the red cell membranes, but they are known to act as reversible inhibitors (Cabantchik & Rothstein, 1972, 1974; Zaki *et al.*, 1975).

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