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The Kinetics of Anion Equilibrium Exchange across the Red Blood Cell Membrane as Measured by Means of ^{35}S Thiocyanate

Steen Dissing*, Leonardo Romano**, and Hermann Passow

Max-Planck-Institut für Biophysik, Frankfurt am Main, Federal Republic of Germany

Summary. Up to a SCN^- concentration of about 110 mM, the concentration dependence of SCN^- equilibrium exchange in human red cell ghosts can be represented by the superimposition of two flux components. One component shows saturation kinetics, the other does not. The saturable component has an activation enthalpy of 105 kJ/mole, exhibits a *trans* acceleration by Cl^- and can be inhibited by H_2DIDS. The nonsaturable component has a much lower activation enthalpy of 33 kJ/mole, is slightly reduced in *trans* acceleration experiments with Cl^- and insensitive to H_2DIDS but susceptible to inhibition by phloretin. At SCN^- concentrations exceeding 110 mM, the saturable component undergoes irreversible self inhibition while the nonsaturable component remains unaltered.

The half saturation concentration of the saturable flux component increases with decreasing pH from 3.0 mM at pH 7.4 to 13.3 mM at pH 6.0. Over this pH range, the maximal flux is only slightly increased from 19×10^{-12} to 22×10^{-12} moles $\times$ cm^{-2} $\times$ sec^{-1}. The nonsaturable flux component also increases slightly.

In accordance with previous observations of Wieth (*J. Physiol. (London)* **207**:563–580, 1970), we find that SCN^- increases K^+ and Na^+ permeability. The induced cation-permeability is considerably smaller than the SCN^- exchange and the latter does not show the paradoxical temperature dependence that is known to pertain to the former.

Key Words: Red blood cell, erythrocyte, anion transport, thiocyanate

The kinetics of inorganic anion transport across the red blood cell membrane have been studied extensively. Most of the transport takes place by way of an exchange mechanism that does not contribute to the conductance of the membrane (Harris & Pressman, 1967; Scarpa, Ceccheto & Azzone, 1970; Hunter, 1971) and thus resembles the tightly coupled ion exchange system first proposed by Ussing (1948).

For all inorganic anion species studied so far (e.g., halides, bicarbonate, sulfate), the exchange system shows saturation kinetics with half saturation between 10–60 mM and self inhibition at more elevated concentrations (Gunn, Dalmark, Tosteson & Wieth, 1973; Wood & Passow, 1974; Dalmark, 1975; Schnell, Gerhardt & Schöppe-Fredenburg, 1977; Wieth, 1979). The superimposition of saturation kinetics and self inhibition has made it difficult, so far, to obtain reliable estimates of the kinetic parameters that describe the transport system (Passow, Pring, Legrum-Schuhmann & Zaki, 1977; Barzilay & Cabantchik, 1979). If one assumes that the kinetics are simply governed by a combination of substrate anions with a transfer site and an inhibitory modifier site (Dalmark, 1975*a*, *b*, 1976), one has to derive from the curve, relating transport to substrate concentration, four kinetic constants: Two apparent K's and two limiting rates, *viz.* the transport rates of the maximally activated and the maximally inhibited system. Moreover, more realistic models of anion transport should take into account that the affinities of the transfer site as well as of the modifier site are bound to change when the transfer site moves from the inner to the outer membrane surface (Gunn & Fröhlich, 1979; Knauf et al., 1980; Passow et al., 1980*a*). In view of these problems, it seemed desirable to search for an anion species for which an unambiguous distinction between saturation kinetics and self inhibition can be achieved. It was known from previous work (Wieth, 1970*a*; Schnell et al., 1977; Deuticke, v. Bentheim, Berger & Kamp, 1978) that low concentrations of SCN^- anions are capable of producing competitive inhibition of sulfate transport. Thus, we decided to study SCN^- transport in

* *Present address:* Department of Physiology, Yale University, New Haven, Connecticut.

** *Present address:* Istituto Fisiologia Generale, Università de Messina, Sicily, Italy.

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some detail and, in the present paper, we shall demonstrate that the half saturation concentration for the thiocyanate anion (about 3.0 mM) is indeed much lower than for the other anion species studied so far. Saturation with SCN^- leads to an extended plateau where the rate of SCN^- exchange becomes independent of the substrate concentration and where inhibition, by very high SCN^- concentrations, represents an irreversible process that can be unequivocally distinguished from the saturation process.

The exchange of monovalent and divalent inorganic anions across the red cell membrane takes place at enormously different rates (10^2–10^5 fold) and shows different pH dependences (for review, *see* Dalmark, 1976). The thiocyanate anion fits into this rule. Like other monovalent anion species, it exchanges at a high rate with a pH dependence that is more similar to that of Cl transport than that of SO_4 transport. However, in contrast to the behavior of other anion species, the half saturation concentration for SCN^- is low and the plateau at saturation is much better defined. It is possible, therefore, not only to study the pH dependence at low SCN^- concentrations in the initial linear portion of the saturation curve, but also at high SCN^- concentrations on a reasonably well-defined plateau.

Like other anion species, SCN^- not only penetrates across the red blood cell membrane via the anion exchange system but also through a parallel pathway that is insensitive to the action of H_2DIDS. This pathway is likely to be identical with the so-called conductance pathway. In contrast to other anion species, the penetration across the H_2DIDS-insensitive pathway can be easily measured at equal SCN^- concentrations on both surfaces of the membrane where electrical driving forces can be neglected. SCN^- movements via this pathway are linearly related to the SCN^- concentration and show a different pH dependence and a very much lower temperature dependence than the movements mediated by the exchange pathway.

Materials and Methods

Human blood (type O, Rh^+) from healthy donors was obtained from the blood bank of the Red Cross, stored in acid-citrate-dextrose and used three to five days after withdrawal.

Ghosts were prepared according to the principles discussed by Bodemann and Passow (1972). Thrice-washed red cells (50% suspension in 166 mM NaCl solution) were hemolyzed at 0°C by 1:20 dilution in a well-stirred medium containing 4 mM $MgSO_4$, 1.6 mM CH_3COOH. After 5 min, sufficient Na_3EDTA was added to obtain a final concentration of 20 mM. The pH was adjusted to 7.4 with 0.1 N NaOH. The hemolysate was then allowed to stay on ice for 15 min. Subsequently, equal volumes of the hemolysate were placed in 35 ml Sorvall centrifuge vials and centrifuged at 20,000 rpm ($60,000 \times g$) for 5 min. The pellets were resuspended in EDTA-buffered (20 mM) media containing KSCN or other salts at the desired concentrations. After two washes at 0°C, the sedimented ghosts were resuspended in their respective washing media and a trace of $^{35}SCN^-$ was added. For resealing, the ghost suspensions were now transferred to 37°C and incubated at that temperature for 45 min. For the experiments with $^{42}K^+$ and $^{22}Na^+$, the isotopes were added at 0°C 15 min before the initiation of the resealing period. Ghosts equilibrated with ^{35}SCN were sedimented and used for flux measurements without further washes, while the ghosts that had been equilibrated with the radioactive cations were washed twice to remove interstitial radioactivity.

For H_2DIDS treatment, the resealed ghosts were washed thrice and then exposed in the presence of the radio-isotope to the inhibitor at a concentration of 25 or 50 μM for 30 min at 37°C.

For the flux measurements, 0.25 ml of the packed (5 min at $60,000 \times g$) resealed ghosts from each batch were collected in a 1-ml propylene syringe (ASIK I/S). The isotope efflux was initiated by injecting the ghosts into the appropriate buffer (10 ml) that was rapidly stirred by a Teflon-coated magnet. The number of cells in the final suspension was determined by means of a Coulter Counter (Coulter Electronics Model F).

For measuring the fast SCN^- fluxes, the inhibitor stop technique of Ku, Jennings and Passow (1979) was applied. In 3-sec intervals with a repeating syringe (SMI, Emeryville, Calif., or Henke-Sass, Wolf GmbH, Tuttlingen), 0.5 ml samples were withdrawn and injected into 0.5 ml ice-cold buffer containing 50 μM H_2DIDS and 300 μM phloretin. This mixture inhibits the $^{35}SCN^-$ efflux at all SCN^- concentrations used by at least 96%. It should be noted that the presence of phloretin in addition to H_2DIDS is essential, especially for measuring SCN equilibrium exchange at high SCN concentrations, where a considerable fraction of the SCN flux cannot be inhibited by H_2DIDS. For example, when measured at 100 mM SCN, H_2DIDS alone inhibits SCN equilibrium exchange by 84%. The remaining flux is inhibited by some additional 75% when phloretin is added, yielding a total inhibition of 96%.

After collecting eight samples, the suspensions that had been diluted with the stopping solution were centrifuged in an Eppendorf centrifuge (model 5412) for 2 min. 0.5 ml of the supernatants were pipetted into scintillation vials and counted after addition of a scintillation fluid (instagel, Packard). $^{42}K^+$ was determined by adding water to the vials and counting the Cerenkov radiation.

The tracer release from H_2DIDS pretreated cells was determined by pipetting 0.5 ml aliquots into a stopping solution that contained 300 μM phloretin and immediately subsequent centrifugation.

Unless stated otherwise, tracer efflux was measured at equilibrium, i.e., under conditions where the composition of the media inside and outside the ghosts was identical. Under these conditions, efflux of the radioactive isotope follows a single exponential. The rate constants for efflux were calculated by means of a nonlinear least squares curve fitting program.

Calculation of Fluxes

Rate constants were corrected for the decrease in cell volume that follows the equilibration with increasing salt concentrations prior to resealing (Funder & Wieth, 1976). Volume determinations were performed by measuring hematocrit values. For this purpose, aliquots taken from each vial, and containing equal numbers of ghosts, were suspended in about equal volumes of medium to obtain a hematocrit of approximately 50%. The precise hemato-

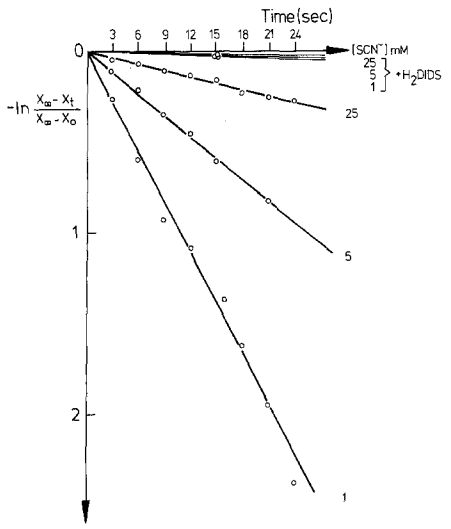

Fig. 1. Time course of $^{35}SCN^-$ release from ghosts. The media inside and outside the ghosts had the same composition. They contained 20 mM EDTA at pH 7.4 plus the KSCN concentrations indicated on the abscissa. Hematocrit, 2.5%; temperature, 4°C. The straight lines represent computer fits to a single exponential

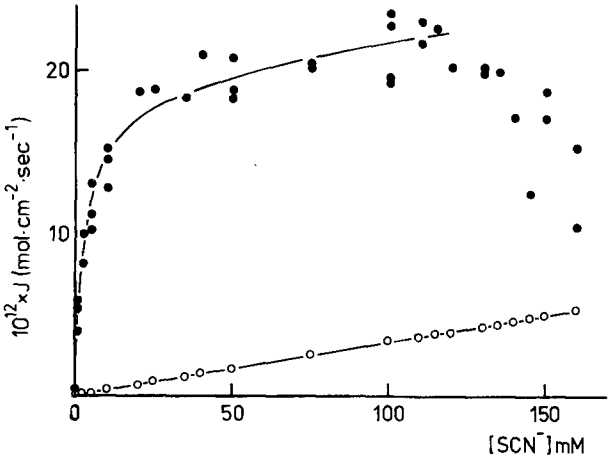

Fig. 2. SCN^- equilibrium exchange in erythrocyte ghosts as a function of the SCN^- concentration. Ghosts containing 20 mM EDTA at pH 7.40 and the SCN^- concentrations indicated on the abscissa were suspended in buffers that had the same composition as the ghost interior. The SCN^- efflux was measured by means of the inhibitor stop-technique (*see* Fig. 1). Closed symbols: untreated ghosts. Open symbols: ghosts pretreated with 25 μM H_2DIDS suspended in buffers containing 50 μM H_2DIDS

crit value was then determined by centrifugation in hematocrit tubes at 17,000 rpm (Sorvall SS 34) for 30 min. These tubes had been developed by Dr. P. Wood in our laboratory and were kindly supplied by him.

Absolute values of the fluxes in mol/cm²/sec were calculated on the basis of the following considerations: For each run, the number of ghosts per sample is known from the measurements with the Coulter Counter. From the isotope concentrations X_0 and X_∞ in the supernatant at times $t=0$ and $t=\infty$, respectively, it is possible to calculate the mean cellular volume (MCV) for each population of ghosts.

$$\text{MCV} = \frac{0.25}{N}\left(1 - \frac{X_0}{X_\infty}\right)$$ where N is equal to the number of ghosts.

The average ghost volume was 88 μm³ at 0.1 mM KSCN, 20 mM EDTA, pH 7.4, and 45 μm³ at 150 mM KSCN, 20 mM EDTA, pH 7.4.

The unidirectional fluxes were calculated from the equation

$$J = k \times \frac{\text{MCV}}{A} \times C_i \text{ mol/cm}^2\text{/sec}$$

where k = the rate constant in sec^{-1}, A = the surface area of a human red blood cell (1.42×10^{-6} cm², *see* Westerman, Pierce & Jensen, 1961; La Celle, 1972), and C_i = the anion concentration in mol/cm³.

The flux can also be expressed as mol/3.1×10^{13} cells/sec (*see*, e.g., Funder & Wieth, 1976), by multiplying the flux (in mol/cm²/sec) by the factor 4.4×10^7 cm², which is the surface area of 3.1×10^{13} cells.

For every run, it was ascertained that the $^{35}SCN^-$ efflux actually followed a single exponential, and hence that the ghosts behaved as a kinetically homogenous population. Cell size determinations by means of a Coulter Counter with a hydrodynamic focussing system showed a Gaussian size distribution of the ghosts prepared at KSCN concentrations between 0–170 mM KSCN.

Results

1. *Dependence of SCN^- Equilibrium Exchange on SCN^- Concentration*

The time course of release of $^{35}SCN^-$ from the ghosts into the media that have the same composition as the ghost interior follows a single exponential. On a semi log plot, the data points pass through the origin, indicating that the exchange takes place in a population of uniformly sealed ghosts (Fig. 1).

When the SCN concentration is increased at equal concentration levels inside and outside the ghosts, the flux increases steeply at lower SCN^- concentrations and less steeply at higher concentrations, with a tendency to approach a straight-line relationship above 50 mM (Fig. 2).

Up to about 110 mM SCN^-, the curve relating SCN^- flux (J) to SCN^- concentration (S) can be represented by a superimposition of a saturable and nonsaturable component:

$$J = \frac{J_{max} \cdot S}{K_{1/2} + S} + \gamma S$$

where J_{max}, $K_{1/2}$ and γ indicate, respectively, the limiting value of the flux approached at saturation, the half-saturation constant, and a proportionality factor that describes the linear relationship between flux and concentration of the nonsaturating component. The numerical values of these parameters were obtained by fitting the equation to the data points by means of a nonlinear curve fitting pro-

Table 1. Kinetic parameters of SCN^- equilibrium exchange (4 °C)

pH	$V_{max} \times 10^{12}$ (mol × cm^{-2} × sec^{-1})	$K_{1/2}$ (mM)	$\gamma \times 10^8$ (cm × sec^{-1})
7.4	19.0	3.0	3.3
6.0	22.1	13.3	10.6

The parameter values were derived by means of a nonlinear curve fitting method.

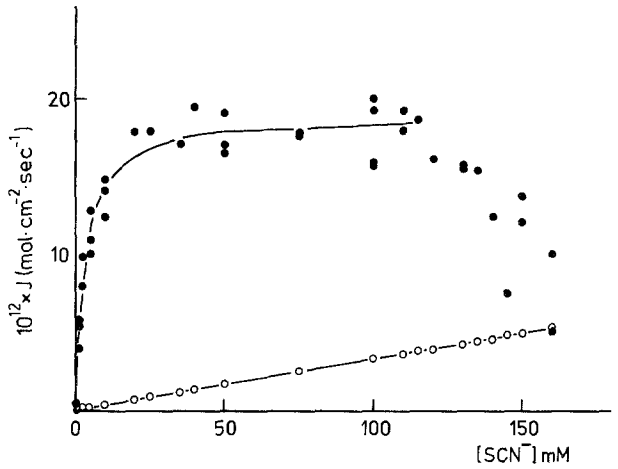

Fig. 3. H_2DIDS sensitive (●) and insensitive (○) SCN^- movements in erythrocyte ghosts as a function of the SCN^- concentration, 4 °C, pH 7.40. The H_2DIDS-sensitive fluxes were calculated from the data in Fig. 2 by subtracting the H_2DIDS-insensitive flux from the total flux. The curve for the H_2DIDS-sensitive flux was fitted to the data points using the first term of the equation:

$$J = \frac{J_{max} \cdot S}{K_{1/2} + S} + \gamma \cdot S$$

The numerical values of the constants J_{max}, $K_{1/2}$ and γ are listed in Table 1

Table 2. Inhibition of SCN^- equilibrium exchange by H_2DIDS[a]

Time of incubation with 20 μM H_2DIDS (min)	Flux (per cent)	
	No H_2DIDS in efflux medium	50 μM H_2DIDS in efflux medium
0	100.0	67.0
10	75.0	27.3
30	29.1	16.4
60	14.6	13.6
90	16.4	13.2
120	14.6	12.7

[a] Prior to the flux measurements, the ghosts had been incubated at 37 °C in the presence of 20 μM H_2DIDS in a medium consisting of 80 mM KSCN and 20 mM EDTA, pH 7.4, for the times indicated in the first column. ^{35}SCN efflux was measured at 4 °C after removal of excess H_2DIDS in the absence (2nd column) or presence (3rd column) of 50 μM H_2DIDS in the external medium.

gram. They are listed in Table 1. The drawn curves in the figure were calculated by inserting the calculated values in the above equation.

The curve fitting procedure had been applied to the data points up to 110 mM. Above this concentration, inhibition takes place. A closer inspection of the data points in Fig. 2 indicates that the choice of 110 mM as an upper limit for the evaluation of the data in terms of the equation might appear to be somewhat arbitrary. One could postulate, for instance, that above 50 mM a plateau is reached that represents the resultant of a further increasing saturation of the transport system by the substrate and an earlier onset of self inhibition. Observations described below on the inhibition by H_2DIDS, the temperature dependence, the counter acceleration by Cl, and the irreversible character of SCN self inhibition at concentrations exceeding 110 mM suggest, however, that the computer fit to the equation as plotted in the figure yields a more meaningful result.

2. H_2DIDS-Sensitive vs. H_2DIDS-Insensitive SCN^- Flux

The justification for subdividing the SCN^- transport up to an SCN^- concentration of about 110 mM into two components is derived from the observations presented in Fig. 3 and Table 2. It is shown in Table 2 that the specific inhibitor of anion exchange, H_2DIDS (Cabantchik & Rothstein, 1972; Lepke, Fasold, Pring & Passow, 1976), produces a maximal effect at less than complete inhibition of SCN^- equilibrium exchange. The residual flux that remains after exposure of the ghosts to a maximally inhibiting concentration of H_2DIDS (50 μm), is a linear function of the SCN^- concentration (Figs. 2 and 3). The slope of the straight line that relates SCN^- flux through the H_2DIDS-insensitive pathway to SCN^- concentration is nearly indistinguishable from the slope of the linear portion of the relationship between flux and concentration that extends nearly up to the upper end of the SCN^- concentration range studied in ghosts that had not been treated with H_2DIDS. After subtraction of the H_2DIDS-insensitive fluxes from the total flux, a saturation curve is obtained. The H_2DIDS-sensitive flux component reaches 95 % of the maximal value at an SCN^- concentration of 50 mM (Fig. 3).

A further and most convincing justification for discriminating between the two pathways comes from measurements of the temperature dependence of the H_2DIDS-sensitive and insensitive SCN^- fluxes (Fig. 4). The H_2DIDS-sensitive flux exhibits an apparent activation enthalpy of about 104.6 kJ/mol and thus shows a similarly high value for the ex-

change of other inorganic anion species (monovalent and divalent) for which activation enthalpies in the range 117–146 kJ/mol have been reported (Passow, 1961; Deuticke, 1970; Dalmark & Wieth, 1972; Funder & Wieth, 1976; Brahm, 1977; Wieth, 1979). In contrast, the apparent activation enthalpy of the H_2DIDS-insensitive efflux amounts to about 33.5 kJ/mol.

As a final piece of evidence for the different properties of the two pathways, we shall consider the *trans*-acceleration of the total SCN^- efflux and the inhibition of the H_2DIDS-insensitive SCN^- efflux by external Cl^-.

At a concentration of 100 mM, the equilibrium exchange of Cl^- is about 15 times faster than that of SCN^-. One would expect, therefore, that the H_2DIDS-sensitive SCN^- efflux should be enhanced when measured in an iso-osmotic medium that contains Cl^- rather than SCN^- itself. This has actually been observed. Ghosts containing 100 mM KSCN, 20 mM EDTA, released SCN^- into an SCN^- medium of identical composition with a rate constant of 0.384 min^{-1}. When the external medium contained 100 mM KCl in place of 100 mM KSCN, the rate constant was 1.00 min^{-1}, i.e., 2.8 times higher.

In the experiment described above, total SCN^- efflux was measured, i.e., the sum of the H_2DIDS sensitive and insensitive fluxes. In a similar experiment with H_2DIDS-treated ghosts, a reduction of the rate constant for efflux into the KCl-containing medium was observed. The efflux amounted to 0.56 times the rate of efflux into the corresponding KSCN-containing medium. Thus, Cl^- causes a *trans*-acceleration of the H_2DIDS-sensitive pathway and an inhibition of the H_2DIDS-insensitive pathway.

3. *Self Inhibition at High SCN^- Concentrations*

The inhibition of SCN^- efflux at the upper end of the SCN^- concentration range studied shows a considerable scatter. The differences between the H_2DIDS sensitive and insensitive fluxes become smaller and errors associated with the difference formation make a separate investigation of the two flux components difficult. However, from the very size of the effect, it is clear that inhibition of the exchange pathway plays an important role. Nevertheless, the self inhibition of SCN^- exchange differs in one most important aspect from that observed with chloride; the effect of SCN^- is irreversible, while that of chloride is not.

The irreversible character of the inhibition by SCN^- is illustrated by the experiments presented in Table 3. Ghosts had been resealed by incubation for 60 min at 37 °C in media that contained either KCl

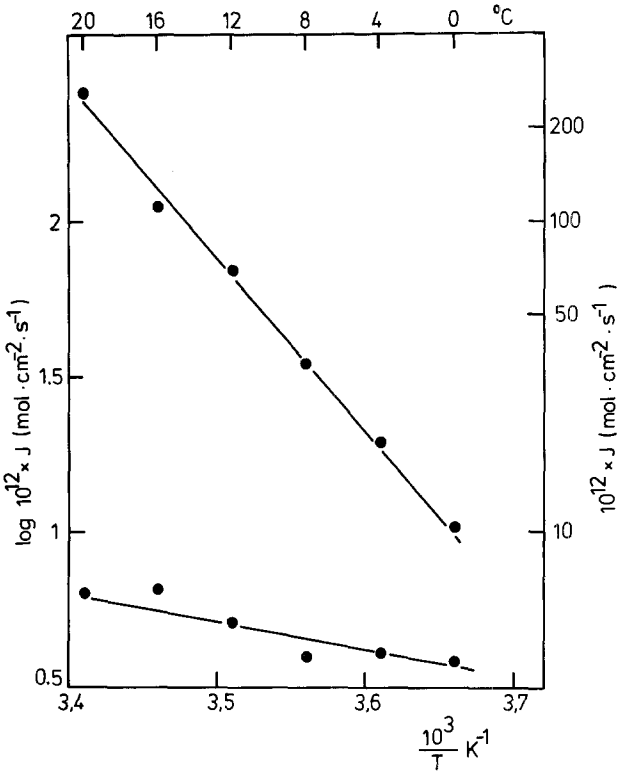

Fig. 4. Arrhenius diagram of the H_2DIDS-sensitive (upper curve) and insensitive SCN^- flux (lower curve). *Ordinate:* $\log_{10}$ of SCN^- flux · 10^{12}. *Abscissa;* the reciprocal absolute temperature in °K. The H_2DIDS-sensitive flux was calculated by substracting the H_2DIDS-insensitive flux from the total flux at the same temperature. The activation energies were calculated from the slopes of the straight lines and amounted to 105 kJ/mol and 33.5 kJ/mol for H_2DIDS-sensitive and insensitive fluxes, respectively. Ghosts and media contained 100 mM KSCN, 20 mM EDTA at pH 7.40

Table 3. Effects of high concentrations of SCN^- and Cl^- on SCN^- equilibrium exchange[a]

	Resealing for 60 min, 37 °C in (mM)	Incubation for 60 min, 37 °C, in (mM)	Wash twice with (mM)	$^{35}SCN^-$ equilibrium exchange, $10^2 \cdot k$ (sec^{-1})
a	160, KCl	–	160, KCl 1, KSCN	5.17
b	160, KSCN	–		3.38
c	160, KCl	160, KSCN		4.54
d	160, KSCN	160, KCl		3.54
e	110, KCl	–	110, KCl 1, KSCN	7.02
f	110, KSCN	–		7.69
g	110, KCl	110, KSCN		7.42
h	110, KSCN	110, KCl		7.46

[a] Ghosts were resealed in media that contained 20 mM EDTA, pH 7.40, plus the KCl or KSCN concentrations indicated in the table. SCN^- equilibrium exchange was measured at 4 °C after resealing (*a*, *b*, *e*, *f*) or after another incubation of the resealed ghosts at 37 °C in KSCN (*c*, *g*) or KCl (*d*, *h*)-containing media.

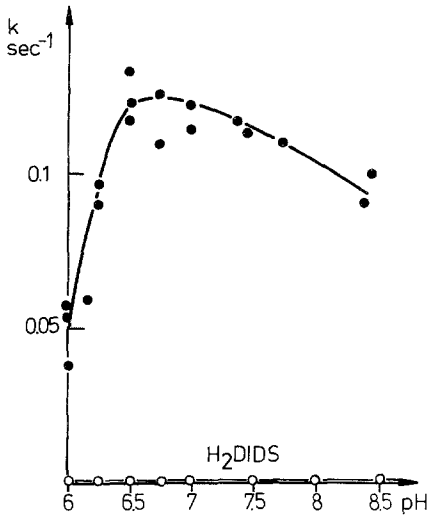

Fig. 5. pH dependence of H_2DIDS-sensitive (upper curve) and insensitive (lower curve) SCN^- fluxes. SCN^- concentration was 0.25 mM. Otherwise the experimental conditions were identical to those described in the legend to Fig. 6

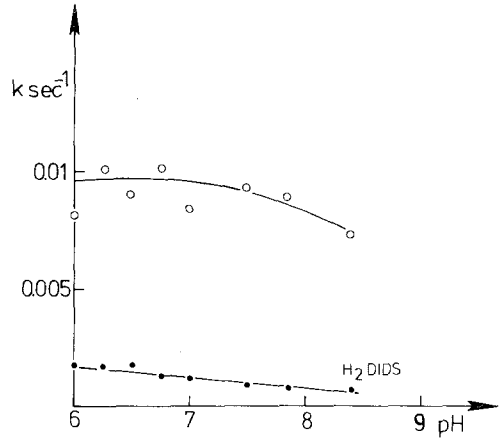

Fig. 6. pH dependence of H_2DIDS-sensitive (○) and insensitive (●) SCN^- fluxes as measured at equal concentrations of the buffer inside and outside the ghosts (50 mM KSCN, 20 mM EDTA, 4°C). pH equilibration by two consecutive resuspensions (15 min) in buffers of the appropriate pH values

or KSCN as the principal electrolyte. They were then washed twice in media that contained 1 mM KSCN plus additional KCl as the principal electrolyte and were subjected to measurements of SCN^- flux (Table 3, *a*, *b*, *e*, *f*). The rates observed in the ghosts that had been resealed in a medium containing 160 mM KSCN were about 35% lower than in those resealed in a medium containing the same concentration of KCl. The effect of resealing in 160 mM KSCN medium cannot be reversed by subsequent incubation for one hour in iso-osmotic KCl medium (Table 3, *d*).

The irreversible effect of SCN^- is not only noticeable when the ghosts are resealed in a KSCN medium. Ghosts that had first been resealed in KCl medium and then incubated in an iso-osmotic KSCN medium also show a decrease of the SCN^- flux as subsequently measured under standard conditions (Table 3, *a* and *c*). The decrease is, however, somewhat smaller than observed after exposure of the ghosts to the SCN^- during the resealing period. If similar experiments are performed at 110 instead of 160 mM SCN^-, no irreversible modification of the anion transport can be seen (Table 3, *e–h*). It may be mentioned, however, that exposure to Cl^- concentrations that cause considerable self inhibition of Cl^- flux do not produce an irreversible modification of the Cl^- exchange pathway (not shown).

4. The Use of SCN^- for the Study of the pH Dependence of Anion Equilibrium Exchange

The pH dependence of anion exchange has been repeatedly studied (Passow, 1961; Pfleger, Rummel & Seifen, 1967; Gunn et al., 1973), most extensively by Dalmark (1975) and Schnell et al. (1977), who measured at a range of pH values the fluxes of Cl^- and SO_4^{2-} as functions of the concentrations of the respective anion species. It was observed that both saturation and self inhibition of the transport system were affected, but an unambiguous quantitative evaluation of the data was not possible.

To demonstrate the advantage of using an anion species that shows well-defined saturation kinetics, we measured the pH dependence of SCN^- equilibrium exchange at two different SCN^- concentrations: (1) at a concentration that amounted to about 1/10 of $K_{1/2}$ and (2) at another at which the transport system is saturated to at least 80% (50 mM) but where there is little if any self inhibition. At low substrate concentration, the flux passes through a maximum at pH 6.5 with a steep decrease towards lower pH's and a less pronounced decrease towards higher pH's (Fig. 5). At the nearly saturating substrate concentration, there is little if any pH dependence between pH 6 and 7 and, perhaps a slight decrease of SCN^- flux at pH's above 8 (Fig. 6). Thus, the binding of the anion rather than the turnover of the fully-loaded transport system seems to govern the pH dependence of SCN^- equilibrium exchange.

The conclusions drawn from the experiments in Figs. 5 and 6 are corroborated by the results represented in Table 1. It is shown in that table that the V_{max} values at pH 6.0 and 7.4 as determined by measuring the concentration dependence of SCN equilibrium exchange do not differ by more than

15%, while the $K_{1/2}$ values differ by about a factor of 3.

In Figs. 5 and 6, the pH dependence of the H_2DIDS-insensitive flux components are also plotted. The results obtained at low SCN^- do not permit drawing conclusions. The H_2DIDS-insensitive fraction is too small for the calculation of reliable values. At the high SCN^- concentration, the H_2DIDS-insensitive flux can be determined with reasonable accuracy. There exists a slight pH dependence that is clearly distinct from that of the *unsaturated* H_2DIDS-sensitive exchange system (Fig. 5).

Mutual Interactions of SO_4^{2-} and SCN^- at the H_2DIDS-Sensitive Pathway

The data on SCN^- transport are confined to low temperatures where SCN^- fluxes can be easily measured by the inhibitor stop technique of Ku et al. (1979). Nevertheless, some idea of the behavior of SCN^- transport at more elevated temperatures can be obtained by measuring the inhibition of SO_4^{2-} equilibrium exchange by SCN^-. Since both anion species compete for the same transfer site (Schnell et al., 1977; Deuticke, 1970) one may assume that the apparent K_I value for the inhibition of SO_4^{2-} equilibrium exchange by SCN^- as measured at a negligibly small SO_4^{2-} concentration should be roughly equal to the apparent K_m value of SCN^- transport. Figure 7 shows that in the range pH 7.4 to at least 8.5 the apparent K_I for the inhibition of SO_4^{2-} equilibrium exchange as measured at 0.1 mM SO_4^{2-} and a temperature of 37°C is independent of pH and amounts to 2.2 mM. Below pH 7.4, there is a steep increase of the apparent K_I, suggesting that $K_{1/2}$ for SCN^- transport increases with decreasing pH much more steeply than at 4°C.

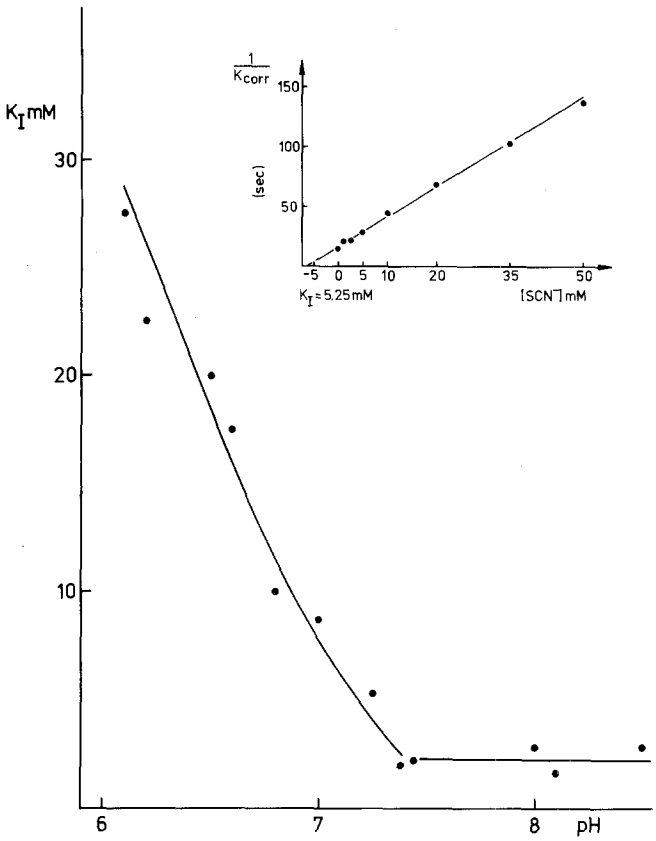

Fig. 7. Inhibition of SO_4^{2-} equilibrium exchange by SCN^- at 37°C as a function of pH. Inhibition is expressed in terms of apparent K_I values (ordinate) that have been determined at the pH values indicated on the abscissa by measuring SO_4^{2-} equilibrium exchange at a range of SCN^- concentrations. At each pH, the media contained 20 mM EDTA, 0.1 mM Na_2SO_4 and KSCN at eight different concentrations. The apparent K_I values were determined from Dixon plots in which the reciprocal value of the volume-corrected rate constants for the H_2DIDS-sensitive component of SO_4^{2-} flux was plotted against the SCN^- concentration. An example for a Dixon plot is represented in the inset. It pertains to the data point at pH 7.24

The H_2DIDS-Insensitive Pathway

The H_2DIDS-sensitive component of SCN^- efflux is obviously related to the band 3 mediated anion exchange. It remains open whether or not the H_2DIDS-insensitive SCN^- equilibrium exchange only proceeds via the so-called conductance pathway that is also available to other anion species or if at least some of the SCN^- penetrates via an additional pathway that is specific for SCN^- or induced by that anion itself. The rate of SCN^- transport across the H_2DIDS-insensitive pathway exceeds the rate of Cl^- penetration across the conductance pathway as defined, e.g., by Hunter's (1971) procedure for the measurement of P_{Cl}; P_{SCN} as calculated from our data is about 0.075 min^{-1} while the H_2DIDS-insensitive fraction of P_{Cl} is about 0.012–0.015 min^{-1} (Knauf, Fuhrmann, Rothstein & Rothstein, 1977; Kaplan, Pring & Passow, 1980; Knauf & Law, 1980). Thus, the penetration rates differ considerably but are still of the same order of magnitude.

Attempts to measure proton fluxes that could be associated with the H_2DIDS-insensitive net SCN^- flux into a Cl^- medium of pH 7 with low buffer capacity (in the absence of CO_2 and the presence of 1 mM acetazolamide) showed no measurable pH changes, even when determined under conditions where the measured $^{35}SCN^-$ efflux leads one to expect a reduction of the pH from 7.0 to 3.3.

It has been described by Wieth (1970*b*) that high concentrations of SCN^- increase the permeability of the red cell membrane to K^+ and Na^+. Our observations confirm this (Fig. 8) and show a similar paradoxical temperature dependence as previously

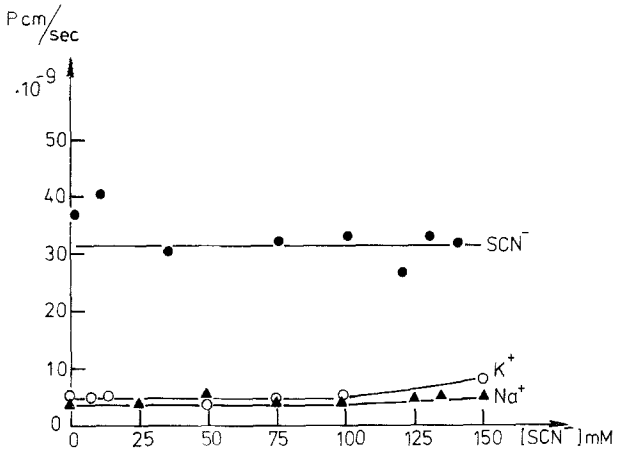

Fig. 8. Penetration rates for SCN^-, Na^+ and K^+ as measured at equal composition of the media inside and outside the ghosts. The media consisted of 20 mM EDTA, pH 7.4, and the SCN^- concentrations indicated on the abscissa. The latter concentration was varied by the addition of either KSCN or NaSCN. Prior to the flux measurements, the ghosts were pretreated with 25 μM H_2DIDS. The efflux medium contained 50 μM H_2DIDS. The permeability was calculated according to: $P = \frac{MCV}{A} \cdot k$[cm/sec], where MCV = mean cellular volume, A = area of a red cell (1.42×10^{-6} cm^2), k = the rate constant for the appearance of $^{35}SCN^-$, ^{42}K or ^{22}Na in the medium (sec^{-1})

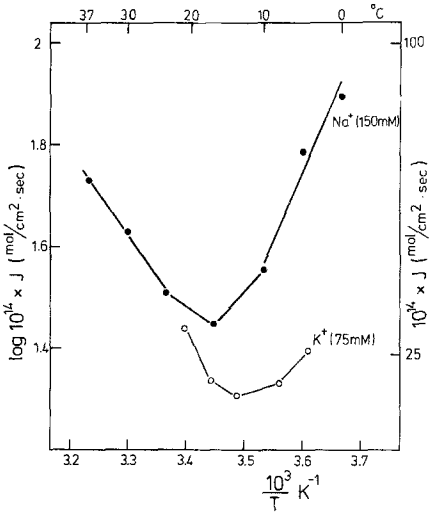

Fig. 9. Arrhenius diagram for Na^+ or K^+ efflux as measured at equal concentrations of either NaSCN or KSCN inside and outside the ghosts. Activation energies were calculated for the ascending and descending branches of the curves. The following values were obtained: For K^+: 4–8 °C: −30.1 kJ/mol; 18–21 °C: +38.1 kJ/mol; Na^+: 0–10 °C: −51.1 kJ/mol; 25–37 °C: +31.4 kJ/mol

reported by this author (Fig. 9). The absolute values of P_K and P_{Cl} that can be calculated from our data (Fig. 8) show that within the concentration range in which measurements of the H_2DIDS-insensitive fluxes are meaningful, the SCN^--induced changes of P_K are small as compared to the absolute value of P_{SCN} and hence are unlikely to contribute significantly to the P_{SCN} value that refers to the H_2DIDS-insensitive SCN^- equilibrium exchange. Moreover, the temperature dependence of the H_2DIDS-insensitive flux yields a straight line in an Arrhenius plot (Fig. 4) while the cation fluxes do not (Fig. 9). Thus, the high value of P_{SCN} derived from our data does not reflect contributions of a diffusion of HSCN or of a transport of SCN^- together with K^+ or Na^+.

Discussion

Our results indicate that the kinetics of SCN^- permeation can be described on the assumption that there exists (1) a H_2DIDS-sensitive pathway that shows saturation with a half saturation constant $K_{1/2}$ of about 3.0 mM at pH 7.4 and little if any self inhibition up to an SCN^- concentration of 110 mM and (2) a H_2DIDS-sensitive pathway that shows a linear relationship between flux and concentration over the whole range from 0–100 mM. Above 110 mM, an irreversible inhibition of the H_2DIDS-sensitive pathway takes place.

1. The H_2DIDS-Sensitive Pathway

The $K_{1/2}$ value of 3.0 mM is about one third of that for HCO_3^- (10 mM), the lowest value reported in the literature on inorganic anion transport (Wieth, 1979). However, in contrast to the $K_{1/2}$ value for SCN^-, the value for HCO_3^- does not seem to reflect a true measure for the saturation of the anion exchange system. Similar to the halides and sulfate, HCO_3^- shows a pronounced self inhibition that seems to become quite effective before the transport system is saturated and operates at maximal capacity. Attempts to determine quantitatively the effects of self inhibition on the relationship between transport and anion concentration have been made for sulfate transport by using equations that reflect the superimposition of saturation and inhibition (Schnell et al., 1977; Passow et al., 1977; Barzilay & Cabantchik, 1979). It has been shown that the $K_{1/2}$'s calculated are about $2\frac{1}{2}$ times higher than the $K_{1/2}$'s determined on the assumption that the maximum in the curves relating flux to concentration represents saturation of the transport system. Thus it is likely that even the $K_{1/2}$ value for HCO_3^- represents an underestimate and that SCN^- assumes a position within the anion species tested so far that is even more exceptional than the available $K_{1/2}$ values would suggest. The SCN^- transport is also rather unique with respect to its low capacity for self in-

hibition. For HCO_3^- and the other anion species, self inhibition is quite pronounced at the concentrations at which SCN saturates.

It has been repeatedly pointed out that the transport protein mediates anion exchange neither like a pore nor like a diffusable carrier (e.g., Passow et al., 1980*a*). Instead, the anion translocation is brought about by a *cis-trans* isomerization of the stationary transport protein that moves the bound anion from one surface of the membrane to the other. The kinetics of such a system have been dealt with first by Patlak in 1957, before the discovery of the first transport protein, and subsequently by many other workers. If it assumed that the binding of the substrate is fast as compared to the *cis-trans* isomerization and that the latter takes place in a single step, one obtains for the concentration dependence of the efflux as measured at Donnan equilibrium an equation (Passow et al., 1980*a*) that is formally identical with the equation of Michaelis and Menten:

$$J_{SCN} = V_{max} \cdot \frac{a}{K_{\frac{1}{2}} + a} \tag{1}$$

where

$$V_{max}^{SCN} \frac{k_{12s} \cdot k_{21s}}{k_{12s} + k_{21s}} = k_{12s} \cdot \frac{1}{1 + \frac{k_{12s}}{K_{21s}}} = k_{12s}\alpha \tag{2}$$

and

$$K_{\frac{1}{2}} = \alpha K_{101} + (1-\alpha) K_{11}. \tag{3}$$

$\frac{k_{12s}}{k_{21s}}$ represents the distribution ratio of the substrate loaded *cis* and *trans* conformers, k_{12s} and k_{21s} denote the rates of transition of the loaded form of the transport protein from *trans* to *cis* conformation and vice versa, respectively. K_{11} and K_{101} indicate the affinities of the substrate to the transport protein in *trans* and *cis* conformation, respectively. The equations neglect slippage (i.e., isomerizations without bound substrate) and thus can only be applied if the anion concentration is high enough to ensure that the number of isomerizations per time unit of the loaded form of the protein far exceeds the number of isomerizations without substrate. This condition is fulfilled for monovalent anions down to very low concentrations.

The observation that V_{max} is nearly independent of pH over the range pH 6-8 indicates that either k_{12s} and k_{21s} are both pH independent or that any change of the rate of transition from *trans* to *cis* is associated with a change of the distribution ratio of *cis* and *trans* conformers that approximately compensates for this change. This latter possibility needs to be emphasized since for Cl^- transport in intact red cells, or in ghosts in which the transport system is largely (although not completely) saturated, the flux decreases with decreasing pH (Dalmark, 1975; Gunn & Fröhlich, 1980; Wieth, Brahm & Funder, 1980). This points to the existence of a proton binding modifier site that acts by altering k_{12} and/or k_{21}.

The increase of transport rate with decreasing pH between pH 8.5 and 7.0 that is observed at low SCN^- concentrations is rather similar to that observed by Brahm (1977) for Cl^- transport at 38 °C. It would be compatible with a cotransport of protons and SCN^- ions, although, of course, it would not prove the case. The sharp decrease of SCN^- transport at pH's below 7.0 is similar to that observed with other monovalent anions, such as Cl^- or I^-, (Gunn et al., 1973; Wood & Passow, 1974; Dalmark, 1975; Funder & Wieth, 1976; and others) and can certainly not be attributed to an H^+ binding that is associated with a cotransport. Instead, one could postulate that the protonation of a modifier site (i.e., a site that is not a substrate binding site) is involved which is allosterically linked to the substrate binding site. Our data show that decreasing the pH from 6.75 to 6.0 increases the $K_{1/2}$ value for SCN^- transport somewhat less that threefold.

An interpretation of these findings in terms of Eq. (3) encounters the difficulty that $K_{1/2}$ depends on both and the affinity of the substrate to the *cis* and *trans* conformers of the transport protein. A change of the affinity of the substrate binding site that is induced by protonation of an allosterically linked modifier site seems, however, a likely interpretation.

A stationary transport protein can mediate the same type of countertransport as a diffusable carrier. For the description of the SCN^- efflux from SCN-containing ghosts into an all Cl^- medium, Eqs. (1) to (3) remain valid, except that the coefficient k_{21s} that designates the rate constant for the *cis-trans* transition of SCN^- has to be replaced by the coefficient k_{21c} which designates the rate coefficient for the influx of Cl^-. The Cl^- equilibrium exchange takes place at a rate that exceeds the V_{max} for SCN^- equilibrium exchange by a factor of at least 24. In the countertransport experiment (SCN^- efflux *vs.* Cl^- influx) V_{max} for SCN^- efflux is still about 2.8 times higher than the V_{max} for SCN^- equilibrium exchange. From these observations, it is possible to deduce (by applying the appropriate forms of Eq. (2)) that both the rate of isomerization from *cis* to *trans*

Table 4. Rate constants for H_2DIDS-intensitive $^{35}SCN^-$ efflux at equal concentrations of ghosts and medium as measured in the presence or absence of 1 μM valinomycin (75 mM NaSCN or 75 mM KSCN, 20 mM EDTA at pH 7.40, 4 °C)[a]

		$k \cdot sec^{-1} \cdot 10^4$
NaSCN	20.6 ± 1.0	21.4 ± 2.0 (+Val)
KSCN	24.4 ± 0.6	50.7 ± 4.9 (+Val)

[a] Valinomycin was added to the ghosts at 37 °C. The ghosts were then packed at 4 °C and subsequently resuspended in their final medium that also contained valinomycin at the concentration indicated.

and the rate of isomerization in the opposite direction are lower for SCN than for Cl.[1]

2. *The H_2DIDS-Insensitive Pathway*

The anion movements that contribute to the conductance of the red cell membrane can be subdivided into two fractions. One is H_2DIDS-sensitive and probably reflects "slippage", i.e., *cis-trans* transitions of the unloaded form of the anion gate and possibly some diffusion through the open anion gate. The other is H_2DIDS-insensitive and probably pertains to anion diffusion across a parallel pathway or to anion transport by a highly unsaturated transport system with slippage at a rate that is equal to the rate of anion transfer. Our determinations of the H_2DIDS-insensitive SCN^- equilibrium exchange refer to the latter and hence is not directly comparable to most of the estimates of anion penetration that contribute to the conductance that encompasses both the H_2DIDS-sensitive as well as insensitive portions. Cl^- and SO_4 fluxes that contribute to the conductance of the membrane are inhibited about 50–65%, respectively, by H_2DIDS or SITS (Knauf et al., 1977; Knauf & Law, 1980; Kaplan et al., 1980). If one assumes that similar percentages apply to other anion species, too, one could conclude, on the basis of the data published by Hunter (1979) that P^*_{SCN} (the star refers to the H_2DIDS-insensitive conductance pathway) is about four times higher than P^*_{Cl} or $P^*_{HCO_3^-}$ but lower than P_I^* by a factor of 0.3. Like I^-, SCN^- should also be suitable for studies of the conductance pathway in the absence of electrical fields under the conditions of equilibrium exchange. Unfortunately, it was not possible to check the estimates of P^*_{SCN} that are based on measurements of equilibrium exchange against others where the electrical field contributes to the driving force (e.g., by Hunter's method). The reason is that valinomycin, which is required to render the membrane cation permeable and thus to provide an electrical driving force, increases the H_2DIDS-insensitive SCN^- flux by a factor of about 2 when ghosts and medium contain equal concentrations of KSCN (Table 4). Thus, if the need should arise for an independent method for the measurement of P^*_{SCN}, it would be necessary to use a different technique.

[1] It should be recalled that Eq. (2), and hence this conclusion, is based on the assumption that the isomerization of the transport protein, rather than the reaction with the substrate, is rate limiting.

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