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Reversible Inhibition of Anion Exchange in Human Erythrocytes by an Inorganic Disulfonate, Tetrathionate

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Summary. Tetrathionate ($S_4O_6^{--}$) markedly inhibits anion exchange across the human erythrocyte membrane. This phenomenon has been studied in order to obtain further insight into the mechanism of action of reversible inhibitors, in particular disulfonate inhibitors, of anion exchange. Anion fluxes were measured by tracer techniques at equilibrium. The following results were obtained: Tetrathionate, although an inorganic compound, inhibits the self-exchange of sulfate and of divalent organic anions (oxalate, malonate) noncompetitively at K_i values ($\leqq 0.5$ mM) as yet only observed for amphiphilic inhibitors. The inhibitor is effective only from the outside of the cell. The inhibition is temperature-dependent, K_i increasing by a factor of 5 between 5 and 35 °C, and instantaneously and fully reversible. The presence of small monovalent anions (fluoride, bromide, chloride, nitrate, acetate) counteracts inhibition by tetrathionate to a varying and concentration-dependent extent, divalent anions have only a minor effect at high concentrations. Chloride exchange is also inhibited, while glycolate and lactate fluxes are much less sensitive or almost insensitive, in agreement with their alleged transfer by a different transport system. Tetrathionate is unique in its inhibitory action, its structural congeners, peroxodisulfate ($S_2O_8^{--}$) and ethanedisulfonate ($C_2H_4S_2O_6^{--}$) are much less effective.

The results can be interpreted by assuming that tetrathionate inhibits the movement of anions via the inorganic anion exchange system by binding – in a 1:1 stoichiometry – to inhibitory "modifier sites", for which it competes with other anions. These sites are located only on the exofacial surface of the membrane. The high affinity of tetrathionate is probably due to a local excess of π electrons in the region of its central disulfide bond. These may stabilize the binding by their ability to form electron donor-acceptor complexes with membrane sites, thus compensating for the absence of a hydrophobic binding domain in tetrathionate.

Inorganic anions penetrate the erythrocyte membrane predominantly, although not exclusively, by an electrically silent [23, 26, 39], obligatory exchange system [7, 14, 33]. This system translocates various inorganic anions at very different rates [12] and also seems to accept certain organic anions, in particular dicarboxylates [8, 30, 35]. Present concepts on the kinetic properties and the molecular nature of this exchange system have

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largely been derived from studies on chloride and sulfate transfer. Both these processes seem to saturate with similar half-saturation constants [4, 10, 11, 18, 37] in spite of a difference in their maximal transfer rates of about 6 orders of magnitude [10, 37]. In both processes saturation is superimposed by self-inhibition [10, 18, 37]. Fluxes of both anions are sensitive to the concentration and nature of other small anions present [11–13, 37, 40], which inhibit in a mixed, competitive and noncompetitive fashion [11, 37]. These findings have been rationalized in terms of two types of anion binding sites on the transport system, namely a "transport site", responsible for the competition phenomena and an additional "modifier site" [11, 34] with a lower anion affinity, responsible for noncompetitive and self-inhibitory effects.

Anion exchange is sensitive to a number of chemically unrelated amphiphilic organic agents including aliphatic and aromatic carboxylates (e.g., capronate and salicylate), dioxopyrazolidines (e.g., butazolidine), local anesthetics (e.g., tetracaine), sulfonamide diuretics (e.g., furosemide), pyrimidopyrimidines (e.g., dipyridamole), and phloretin [5, 13, 14, 35]. These compounds inhibit reversibly. Concentrations required for half-maximal inhibition range from below 1 μM (e.g., dipyridamole) [14, 35] to more than 1 mM (e.g., salicylate) [14]. The kinetics of inhibition have been shown to be noncompetitive in the few instances studied [35]. With the exception of furosemide [5], however, it is not clear whether such inhibitors interact with the above-mentioned modifier sites or bind to other inhibitor sites.

Since all members of this group have amphiphilic structure, it may be assumed that either the hydrophobic moiety of such molecules provides their high affinity for the membrane while the polar domain produces inhibition, or vice versa. The recent finding [43] that a variety of such inhibitors interferes with the irreversible blockade of anion transfer by an amino-reagent, fluorodinitrobenzene, may indicate a common site of action.

Among these amphiphilic inhibitors, stilbene disulfonates and related compounds have recently attracted particular interest [1, 2, 6, 24, 27, 31, 32, 38, 42]. These compounds can be used as reversible (noncovalent) or irreversible (covalent) inhibitors, depending on the nature of certain side groups. The most powerful irreversible inhibitor of this family, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS)[1] [6, 32], which does not

1 *Abbreviations:*
DAS – 4,4′-diacetamido stilbene-2,2′-disulfonic acid
DIDS – 4,4′-diisothiocyano stilbene-2,2′-disulfonic acid
DNDS – 4,4′-dinitrostilbene-2,2′-disulfonic acid
APMB – 2-(4′-aminophenyl)-6-methyl benzene thiazol-3,7′-disulfonic acid
NAP-taurine – N-(4-azido-2-nitrophenyl) 2-aminoethyl sulfonate
GSH – glutathione

penetrate the erythrocyte membrane and binds to amino groups, has been successfully used to identify the membrane protein probably mediating anion exchange [27, 32, 38, 42]. Reversibly acting members of the family have been used to probe the sidedness of the inhibitor binding sites [1, 24, 31, 42]. They seem to interact, in terms of the above model, with the transport sites [1, 38].

The high affinity of these inhibitors for the anion exchange system has been related to hydrophobic interactions with the membrane, as in case of the other inhibitors [6, 32]. This is, however, not a necessary prerequisite for the activity of disulfonate inhibitors. Results will be reported in the following on a new, efficient reversible disulfonate inhibitor of sulfate and chloride transfer. In contrast to all potent inhibitors of anion transfer known so far, this agent is an *inorganic* disulfonate anion. A characterization of its effects, which are not related to previously demonstrated effects of this compound on the erythrocyte membrane [19], was expected to shed some new light on the mechanism of action of disulfonate inhibitors.

Materials and Methods

Incubation Procedure and Flux Measurements

Experiments were carried out on freshly drawn or 1-day-old human blood from the local blood bank, anticoagulated with heparin. After removal of plasma and buffy coat, the erythrocytes were washed 3 times in 154 mM NaCl. For measurements of sulfate, oxalate, malonate, or glycolate transfer cells were then suspended at a hematocrit of 5% in one of the two following media (extracellular concentrations in mM): (A) NaCl 130, Na_2HPO_4/NaH_2PO_4 6.5; glucose 4.5; sulfate (oxalate, malonate, glycolate) 7.5; pH 7.35. (B) KCl 100; NaCl 30; Na_2HPO_4/NaH_2PO_4 6.5; sulfate (oxalate, glycolate) 7.5; sucrose 27; gramicidin D 5 μg/ml; pH 7.35.

Incubation in medium *A* preserves the characteristic Donnan distribution of anion between cells and medium. In medium *B* cation equilibration is achieved by gramicidin D and colloid osmotic hemolysis prevented by extracellular sucrose. Cells were incubated in medium *B* or similar, gramicidin D and sucrose containing media whenever an increase of cation permeability was to be expected under the experimental conditions chosen, or nonisotonic incubation media were to be used. The presence of gramicidin largely prevents osmotic changes of cell volume. Control experiments had established that gramicidin D *per se* at the concentrations used here neither affects sulfate transfer nor modifies the inhibitory effect of tetrathionate.

For measurements of chloride transfer, cells were suspended in media containing (mM): Na_2HPO_4/NaH_2PO_4, 6.5; sucrose, 27; gramicidin D, 5 μg/ml; and KCl at varying concentrations (400–600 mM); pH 6.4. These conditions were chosen because they allow the resolution of chloride tracer fluxes by the centrifugation technique used in our laboratory.

Equilibration of the cells with these media, loading with 35-S-sulfate, 14-C-oxalate, 14-C-malonate, 14-C-glycolate, and 36-Cl^-, measurements of isotope efflux into isotope-free but otherwise identical media, determination of efflux rate coefficients, and calculation of fluxes followed essentially the protocol given in [15] and [16]. Intracellular concentrations of the anions under study were calculated from their known extracellular concentration and

the distribution ratio of the labeled species, determined after preequilibration from the radioactivities of the supernatant and the packed cells extracted with 0.6 N perchloric acid. Packed cell radioactivities were related to the intracellular water determined by estimation of the dry matter.

In case of $^{36}Cl^-$ efflux, the samples removed for the determination of extracellular radioactivities during the efflux measurements were immediately mixed with phloretin (final concentration 0.25 mM) or salicylate (20 mM). These additives reduce the further release of tracer to extremely low values. The reliability of this inhibitor-stop procedure had been ensured in preliminary experiments.

Quantitative Determination of Tetrathionate

For the quantitation of intracellular concentrations of tetrathionate, packed cells pretreated with iodoacetate in order to block intracellular GSH and incubated with tetrathionate (*see Results*) were washed free of extracellular tetrathionate at 0 °C and lysed with 5 vol of distilled water. The lysate was deproteinized by pressure filtration in an ultrafiltration chamber (Amicon UF 12, Diaflo® Membrane PM 10). 30 µl of the filtrate (containing 15–60 nmol tetrathionate) were incubated for 10 min at room temperature with 1 ml of a 0.2-mM solution (=200 nmol) of glutathione, which cleaves tetrathionate according to the reaction sequence

$$\begin{array}{l} S_4O_6^{--} + GSH \rightarrow GS-S_2O_3^- + S_2O_3^{--} \\ \underline{GS-S_2O_3^- + GSH \rightarrow S_2O_3^{--} + GSSG} \\ S_4O_6^{--} + 2\,GSH \rightarrow S_2O_3^{--} + GSSG \end{array} .$$

The amounts of GSH consumed by the reaction with tetrathionate were quantitated by determination of the concentration of GSH according to [3], before and after this incubation. The proportions of GSH and tetrathionate were such that a maximum of 60 % of the GSH was converted into GSSG. The excess of GSH was required to ensure complete reaction and avoided the interference by the intermediate $GS\text{-}S_2O_3^-$, as was ascertained by calibration curves derived from known mixtures of freshly prepared GSH and tetrathionate solutions. These curves subsequently served to quantitate the unknown tetrathionate concentration of the filtrates.

Materials

Routine chemicals were of the highest purity available. 35-S-sulfate, 14-C-U-oxalate, 2-14-C-malonate, 1-14-C-glycolate and 36-chloride (sodium salts) were obtained from Amersham Buchler. Sodium tetrathionate ($Na_2S_4O_6$) was purchased from Fluka AG, Buchs, potassium peroxodisulfate ($K_2S_2O_8$) and sodium 1,3-benzenedisulfonate from Merck AG, Darmstadt, sodium 1,2-ethane-disulfonate and methane disulfonic acid from Eastman Kodak, gramicidin D was kindly donated by von Heyden-Squibb, Munich.

Results

Influence of Tetrathionate on Sulfate Self-exchange

Figure 1 demonstrates a progressive inhibition of the self-exchange of sulfate in human erythrocytes in the presence of increasing concentrations

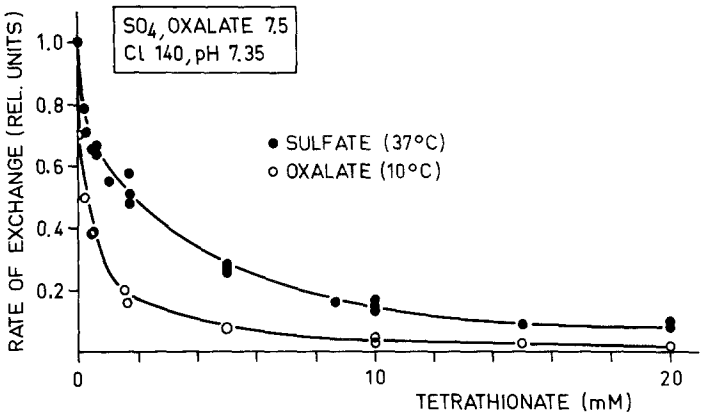

Fig. 1. Influence of tetrathionate on the self-exchange of sulfate and oxalate. Cells were loaded with labeled sulfate or oxalate and the tracer efflux was measured as described in *Methods*. Tetrathionate was present only during the efflux period (15 and 60 min, respectively)

of tetrathionate. Under the conditions of these experiments (7.5 mM $SO_4^=$, 140 mM Cl^-), 50% inhibition requires 1.5 mM of the agent and 20 mM tetrathionate reduces sulfate exchange to 8% of the control value. Even more pronounced inhibition is observed for the self-exchange of oxalate, a dicarboxylate anion which most likely uses the inorganic anion exchange system of the erythrocyte [8, 30]: 50% inhibition requires only 0.2 mM tetrathionate. These differences in inhibition are most likely due to the different temperatures at which the fluxes of sulfate and oxalate were measured: dose response curves for tetrathionate inhibition of oxalate transfer, obtained at 5, 10, and 25 °C, and of malonate transfer, at 35 and 20 °C, reveal a decrease of the concentrations required for 50% inhibition (I_{50} values) with decreasing temperature (Fig. 2).

The effects of tetrathionate are maximal immediately after the cells have come into contact with the inhibitor. This is evident from the efflux kinetics obtained in experiments (Fig. 3*a*) in which tetrathionate was added after a short period of uninhibited tracer efflux. The regression line for the efflux after addition of inhibitor intersects with the line for the uninhibited efflux at the point of the addition of inhibitor, within the time resolution of our experiments.

Inhibition is completely and instantaneously reversible: According to the data in Table 1, the self-exchange of glycolate in cells pretreated with tetrathionate for up to 3 hr is not different from that of untreated controls after only one washing at 0 °C, while the presence of tetrathionate *during* efflux produces inhibition as in case of sulfate and oxalate (*cf.*, however, Fig. 8). The reversal occurs without any time delay as could be de-

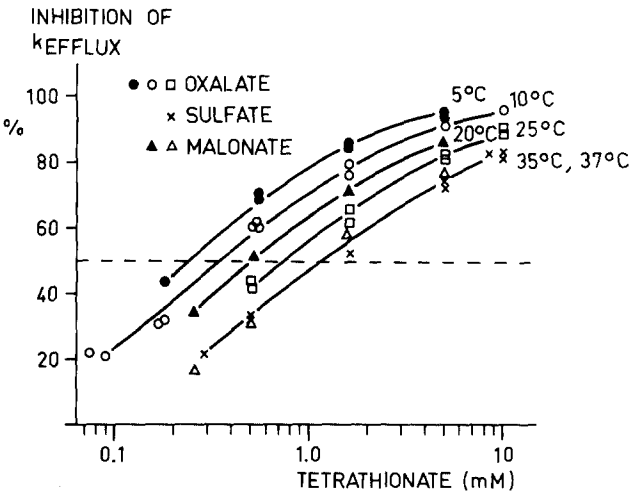

Fig. 2. Dose-response curves for tetrathionate inhibition of anion exchange at different temperatures. Tracer fluxes of oxalate, malonate, and sulfate were measured as described in Fig. 1. For a further evaluation of the data *see* Fig. 11

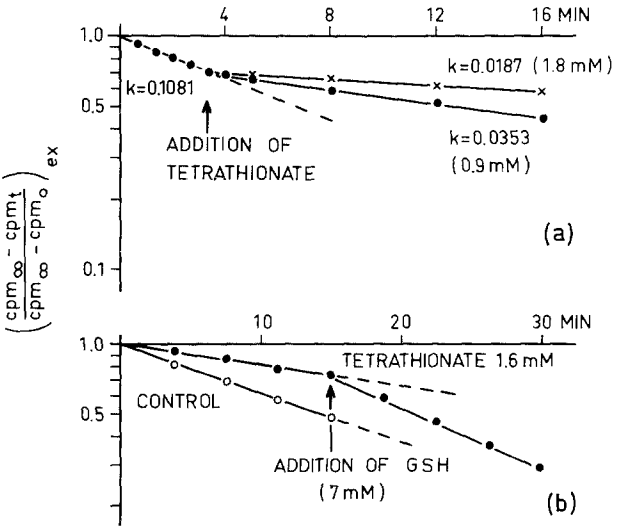

Fig. 3. Time course of the onset and the relief of tetrathionate inhibition of anion exchange. (*a*): Cells were loaded with ^{14}C oxalate in medium *A* and tracer efflux was started by mixing cells with tracer-free medium at 10 °C. After 3.5 min tetrathionate was added at final concentrations of 0.9 and 1.8 mM. Rate coefficients k in min^{-1}. cpm: extracellular radioactivity per 0.5 ml medium, at 0 and t min and ∞, after attainment of tracer equilibrium. (*b*): Cells were loaded with sulfate and tracer efflux initiated without (control) or with 1.6 mM tetrathionate at 37 °C. Glutathione (7 mM) was added after 15 min

Table 1. Reversibility of the tetrathionate effect on anion exchange[a]

	Exposure to tetrathionate (min)	Rate coefficient of glycolate efflux (min^{-1})
A	0	0.0445
B	60	0.0464
C	120	0.0461
D	180	0.0441
E	0	0.0161[b]

[a] Cells were loaded with ^{14}C-glycolate in medium *A*, exposed to tetrathionate (5 mM) for varying periods of time, washed once with tetrathionate-free incubation medium at 0 °C, and resuspended for the efflux measurement in isotope-free incubation medium without (*A* – *D*) or with (*E*) tetrathionate (pH 7.4, 10 °C, Hct 5 %).
[b] Tetrathionate present during efflux measurement.

Table 2. Oxidation of membrane SH-groups during exposure of human erythrocytes to tetrathionate[a]

		Membrane SH-groups (nmol/mg protein)
Controls		68
Tetrathionate 10 mM	60 min	52
	120 min	26
20 mM	60 min	41
	120 min	19

[a] Erythrocytes were incubated (hematocrit 10 %) with tetrathionate in a medium containing (mM): NaCl, 45; KCl, 90; Na_2HPO_4/NaH_2PO_4, 12.5; sucrose, 45; pH 7.4, 37 °C, and subsequently washed 3 times with tetrathionate-free media at 37 °C. Membrane SH-groups and protein content were determined according to [20] and [21].

monstrated by experiments (Fig. 3*b*) in which extracellular tetrathionate was decomposed to thiosulfate, which is not inhibitory (*see* below) during the tracer efflux period by addition of an excess of glutathione (*cf. Methods*). The rate of efflux returned to values even somewhat higher than the controls – for unknown reasons – immediately after addition of the reducing thiol.

The ready reversibility of inhibition by a single washing indicates that the interaction of tetrathionate with the membrane is noncovalent in nature and thus not related to the conversion of membrane SH- into disulfide groups, which occurs in erythrocytes exposed to tetrathionate for prolonged periods of time [19–21]. This covalent modification of the membrane also occurred under the conditions of our efflux measurements, as shown in Table 2, but had only minor, if any, effects on anion exchange (B. Deuticke, C.W.M. Haest & D. Kamp, *unpublished*).

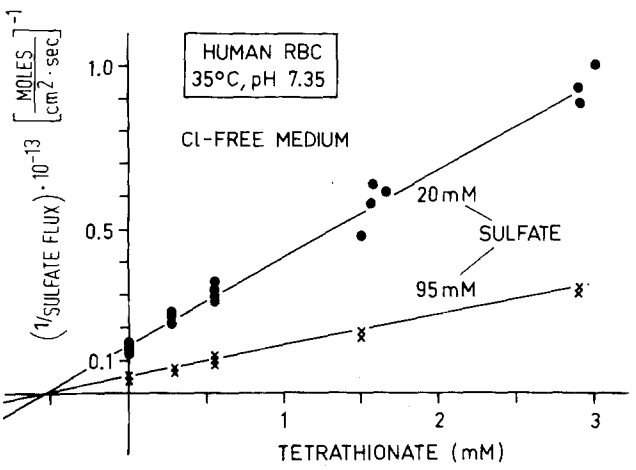

Fig. 4. Dixon plot for the inhibition, by tetrathionate, of sulfate self-exchange. Cells were loaded with sulfate in media containing, except for sulfate at the indicated concentrations (in mM): Na_2HPO_4/NaH_2PO_4 6.25, sucrose 44, gramicidin D 5 μg/ml. Tracer efflux into identical but tracer-free media was measured as described in *Methods*

Kinetic Characteristics of the Inhibition by Tetrathionate

Most of the amphiphilic, reversible inhibitors of anion exchange studied so far act in a noncompetitive fashion [35], while inorganic anions as a rule inhibit competitively at low concentrations [11, 37]. Interestingly, the effect of tetrathionate, which is also an inorganic anion, on sulfate transfer is noncompetitive in nature at low concentrations, as can be concluded from the Dixon plot for tetrathionate inhibition at two different concentrations of sulfate (Fig. 4). From the intersect of the regression lines on the abscissa an inhibitor constant (K_i value) for tetrathionate of 0.5 mM can be derived. This value is lower than that derived from Fig. 1. The reason for the difference becomes clear from Fig. 5*a*. While identical dose-response curves for sulfate exchange are obtained, as expected for a noncompetitive inhibitor, in media containing 20 or 90 mM sulfate as the only anion (except for 6 mM phosphate buffer), addition of chloride to the media shifts the dose-response curves to higher tetrathionate concentrations. This effect was studied in more detail in experiments in which chloride concentrations were varied systematically at constant sulfate concentrations of the media. These variations could be achieved by using media of type *B* (*cf. Methods*), which contain gramicidin D and thus render possible variations of the total salt concentration without major alterations of the cell volume or lysis of the cells.

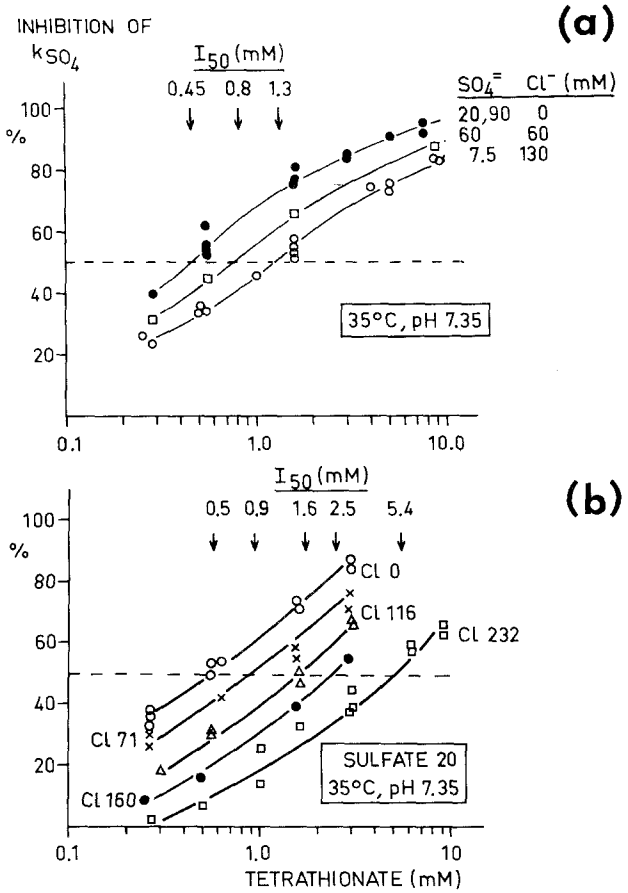

Fig. 5. Dose-response curves for tetrathionate inhibition of sulfate self-exchange at different chloride concentrations. (*a*): Replacement of sulfate by chloride. Media containing, except for sulfate and chloride at the indicated concentrations (in mM): Na_2HPO_4/NaH_2PO_4, 6.25; sucrose, 44. (*b*): Increasing chloride concentrations at constant sulfate concentration. Incubation media containing, except for sulfate (20 mM) and chloride at the indicated concentrations: Na_2HPO_4/NaH_2PO_4, 6.25 mM; sucrose, 44 mM; gramicidin D, 5 μg/ml. I_{50} = concentration required for 50% inhibition

As is evident from Fig. 5*b*, an increase of the extracellular chloride concentration from 0 to 230 mM, which itself reduces sulfate exchange by a factor of 10 (at a sulfate concentration of 20 mM) (data not shown), shifts the I_{50} for tetrathionate from 0.5 to approx. 5 mM. Alternatively, it can be stated that the 50% inhibition of sulfate exchange produced by 0.5 mM tetrathionate can be suppressed almost completely by about 250 mM chloride. Other monovalent inorganic anions also affect tetrathionate inhibition (Fig. 6*a*). Substitution of chloride (130 mM in medium *A*) by nitrate displaces the dose-response curve to higher concentrations, increasing the

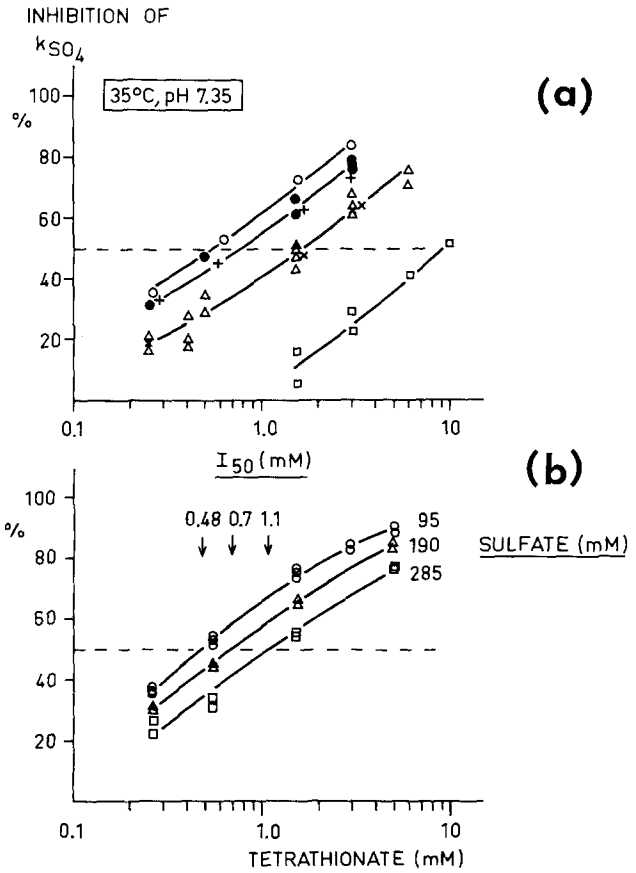

Fig. 6. Influence of various anions on the dose-response curves for tetrathionate inhibition of sulfate self-exchange. (*a*): Media containing (in mM): sulfate, 20; Na_2HPO_4/NaH_2PO_4, 6.25; sucrose, 44; gramicidin D, 5 µg/ml; and no further anion (○) or other anions (● acetate, + fluoride, × bromide, △ chloride, □ nitrate) at 130 mM, pH 7.35, 35 °C. (*b*): Media containing, except for sulfate at the concentrations noted: Na_2HPO_4/NaH_2PO_4, 6.25 mM; sucrose, 44 mM; gramicidin D, 5 µg/ml; pH 7.35, 35 °C

I_{50} value from 1.6 to approx. 10 mM. Acetate and fluoride decrease the I_{50} to 0.8 mM. Since both these I_{50} values are higher than that in media containing no additional anion ($I_{50} = 0.5$ mM), competition between acetate as well as nitrate and tetrathionate can be postulated.

In view of these interferences of monovalent anions with the noncompetitive inhibition of sulfate transfer by tetrathionate, we thought it possible that sulfate might also suppress, at very high concentrations, the inhibitory action of tetrathionate. According to the dose responses curve compiled in Fig. 6*b*, high concentrations of sulfate, in fact, increase the I_{50} value of tetrathionate.

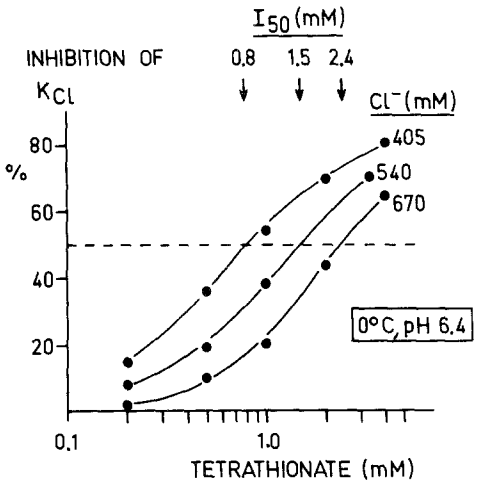

Fig. 7. Influence of tetrathionate on chloride self-exchange. Cells were loaded with ^{36}Cl in media containing, except for KCl at the concentrations noted (in mM): Na_2HPO_4/NaH_2PO_4, 6.25; sucrose, 26; gramicidin D, 5 µg/ml; pH 6.4, 0 °C. Tracer efflux was resolved by using inhibitors of chloride exchange (phloretin, 0.25 mM, salicylate, 20 mM) as "stopping" agents

Influence of Tetrathionate on Chloride Equilibrium Exchange

In view of the suppression by chloride of the tetrathionate effects on sulfate transfer, it was of interest to investigate the influence of tetrathionate on chloride transfer itself. For technical reasons these experiments were carried out at high chloride concentrations, pH 6.4, and 0 °C (for incubation media, *see Methods*). Fig. 7 demonstrates that chloride fluxes are also inhibited by tetrathionate. Again, the I_{50} values are shifted to higher tetrathionate concentration with increasing chloride concentrations.

Substrate Specificity of Tetrathionate Inhibition

From the results demonstrated so far it has become clear that tetrathionate inhibits the exchange of mono- and divalent inorganic anions and of divalent organic anions. In contrast, the transfer of monovalent organic anions penetrating in the ionic form [16] is less sensitive to tetrathionate: glycolate exchange is only inhibited to a maximum of 65%, that of D- and L-lactate only to 15–20% (Fig. 8). This low sensitivity supports the contention [16, 22] that aliphatic monocarboxylates do not, or

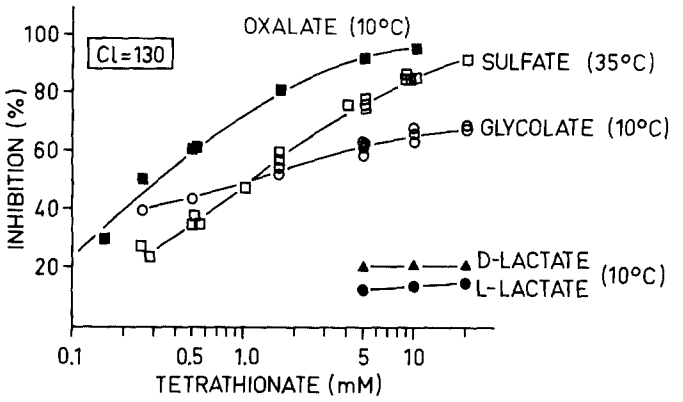

Fig. 8. Influence of tetrathionate on the self-exchange of organic anions. Cells suspended in medium *A*, pH 7.35

Table 3. Rate coefficients of sulfate equilibrium exchange in the presence of various inorganic anions (5 mM), medium *A*, pH 7.35, 37 °C

	K (min^{-1})	Relative to control
Control	0.0345	1.0
Tetrathionate ($S_4O_6^=$)	0.0087	0.25
Thiosulfate ($S_2O_3^=$)	0.0345	1.0
Sulfite ($SO_3^=$)	0.0313	0.91
Thiocyanate (SCN^-)	0.0280	0.81
Perchlorate (ClO_4^-)	0.0267	0.77

only to a limited extent, penetrate the erythrocyte membrane via the pathway of chloride and sulfate.

The Structural Requirements for the Inhibitory Action of Tetrathionate

The high inhibitory potency of tetrathionate raises the question whether structurally similar compounds also have such effects. According to the data compiled in Table 3, related oxy-anions are much less effective. The same is true for a number of even more closely related anions: Tetrathionate can be regarded as a disulfonate in which the two SO_3^--groups are separated by two sulfur atoms. Replacement of these two sulfur atoms by two oxygens, an ethane (or methane) or even a benzene group (Fig. 9) reduces the inhibitory potency of the resulting disulfonates by 1 to 2 orders of magnitude.

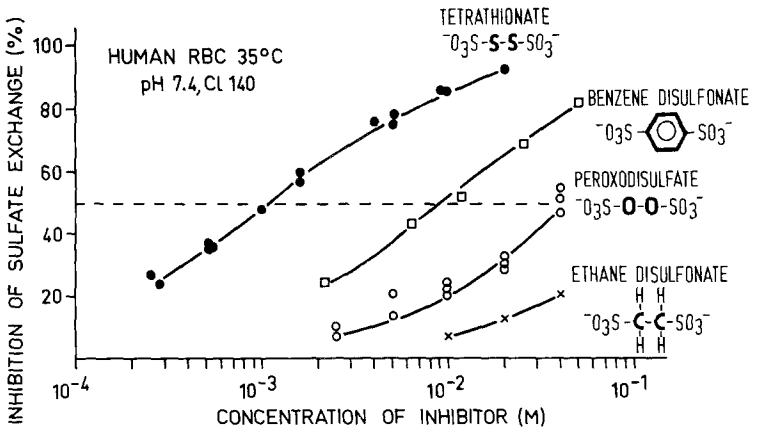

Fig. 9. Influence of tetrathionate and related disulfonate anions on sulfate exchange. Medium *A*, pH 7.45, 35 °C. Inhibitors present only during the efflux period.

Sidedness of the Action of Tetrathionate

The question whether amphiphilic reversible inhibitors of anion can act from both sides of the membrane has recently raised considerable interest. Certain inhibitors (phloretin, DAS, DNDS [1, 24, 28, 36, 38, 42] are only effective if present on the outer membrane surface, others (APMB, NAP-taurine [25, 31, 42]) have been shown to inhibit from both sides of the membrane. In most of these studies the inhibitors were introduced into the cells during hemolysis and fluxes measured in resealed ghosts. Tetrathionate enters the intact erythrocyte with a half time comparable to that of

Table 4. Lack of influence of intracellular tetrathionate on oxalate self-exchange in human erythrocytes[a]

Tetrathionate (mmol/liter cell water)	Rate of oxalate exchange (relative to tetrathionate-free cells)
0	1.00
3.2	1.08
3.9	1.00
5.0	0.95
5.5	1.12
7.5	0.90

[a] Erythrocytes were treated with iodoacetate (20 μmol/ml cells, 45 min, 37 °C) and equilibrated with oxalate and ^{14}C-oxalate at 37 °C in medium *B* as described in *Methods*. Tetrathionate was then added at concentrations of 10 and 15 mM for 90 min (Hct. 35%). After cooling to 0 °C, the cells were washed 3 times with tetrathionate-free medium at 0 °C and oxalate efflux measured at 10 °C as described in *Methods*.

sulfate uptake (15 min at 35 °C [19]), presumably via the anion exchange pathway as indicated by the sensitivity of its uptake to inhibitors like dipyridamole, phloretin, or phenopyrazone [19]. Taking advantage of this comparatively high permeability, we loaded erythrocytes with tetrathionate after pretreatment with iodoacetate in order to block the intracellular glutathione which would convert the inhibitor into thiosulfate. Intracellular concentrations of up to 7.5 mM tetrathionate could thus be achieved, while extracellular tetrathionate was removed by subsequent washing of the cells at 0 °C without detectable loss of intracellular tetrathionate. This indicates a high temperature dependency of tetrathionate transfer, to be expected if this anion moves via the inorganic anion exchange system [14].

The results compiled in Table 4 demonstrate that intracellular tetrathionate, even at very high concentrations[2], has no detectable inhibitory effect on oxalate self-exchange. Obviously, the binding sites for this disulfonate inhibitor are located exclusively outside of the permeability barrier for anions.

Discussion

From the results demonstrated above it has become evident that tetrathionate, an inorganic disulfonate anion, is a powerful, reversible noncompetitive inhibitor of the self-exchange of sulfate and other anions. Its inhibitory potency is much higher than that of comparable divalent inorganic anions such as phosphate, sulfate, or oxalate, which are competitive (or mixed-type) inhibitors of anion exchange with K_i values between 20 and 150 mM [37]. Tetrathionate, with a K_i value of 0.2–0.5 mM (depending on the temperature and measured in the absence of interfering inhibitory anions), even exceeds strongly adsorbing monovalent anions like nitrate or thiocyanate [37], which inhibit sulfate exchange at K_i values of 1.5–3 mM. The K_i values of tetrathionate thus fall among those of amphiphilic inhibitors (e.g., salicylate (1 mM), phenopyrazone (0.75 mM) [14], DAS (0.3 mM) [42], APMB (0.4–0.9 mM) [31]. This indicates a high affinity for binding sites involved in anion transfer.

2 The tetrathionate concentrations given in Table 4 represent those of *free* intracellular tetrathionate, since hemoglobin was removed by ultrafiltration, which will not displace tetrathionate absorbed to hemoglobin. Whether such an absorption occurs has not been studied in our work. The inhibitor did not equilibrate between extra- and intracellular water space during the period of exposure.

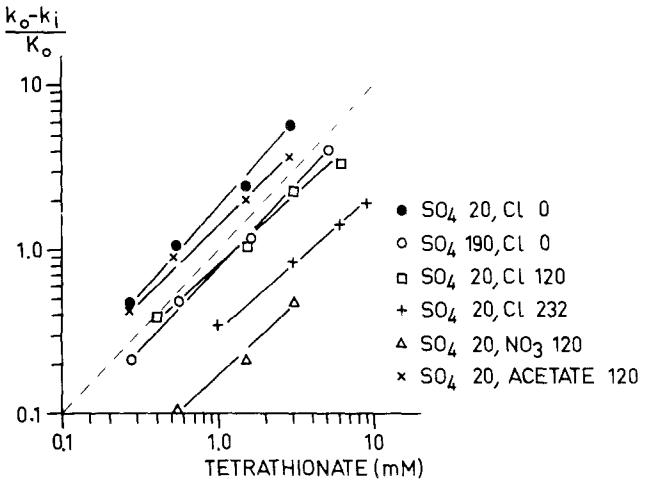

Fig. 10. Double logarithmic plot of the dose-response curve for tetrathionate inhibition under varying conditions of anion milieu, rearranged according to Johnson *et al.* [5]. k_o = rate coefficient in the absence, k_i in the presence, of inhibitor. The dotted line (slope = 1.0) represents the ideal curve for a 1:1 binding of the inhibitor

Stoichiometry of the Binding of Tetrathionate

The stoichiometry of the binding of tetrathionate can be established by plotting $\log (k_o - k_i)/k_i$ *vs.* $\log$ [tetrathionate], where k_o and k_i represent rate coefficients of sulfate self-exchange in the absence and presence of tetrathionate. In this type of diagram [5] the slope gives a measure of the number of inhibitor molecules required to inactivate one transfer site. Under a variety of conditions a slope of approximately 1 was obtained (Fig. 10), indicating that only 1 tetrathionate is required to inactivate 1 transfer site. A similar 1:1 stoichiometry characterizes the inhibition of sulfate transfer by amphiphilic inhibitors, e.g., tetracaine, pyridoxalphosphate, salicylate (B. Deuticke, *unpublished*). As an exception, furosemide seems to interact with more than one site (slope = 1.36 [5]).

Energetics of the Binding of Tetrathionate

The inhibitory action of tetrathionate is characterized by a high temperature dependency. Between 5 and 35 °C the I_{50} value increases by a factor of 5. Plotting $\log I_{50}$ *vs.* $1/T$ in an Arrhenius type diagram and assuming that the I_{50} reflects the reciprocal of the association constant for the binding of tetrathionate to its inhibitor binding site, one obtains a value

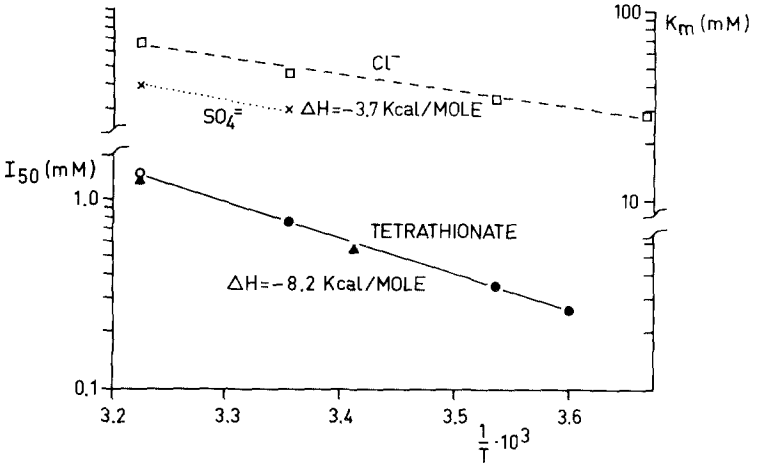

Fig. 11. Temperature dependence of the binding of tetrathionate to its inhibitory site. The I_{50} values obtained from Fig. 2 were plotted semilogarithmic against $1/T$ and the slope calculated by linear regression analysis. ○, sulfate exchange; ●, oxalate exchange; ▲, malonate exchange. For reasons of comparison, the apparent affinity constants of chloride [(□) hatched line] and sulfate [(×) dotted line] self-exchange at different temperatures have been included in the graph

of −8.2 kcal/mole for the heat of association (ΔH) (Fig. 11). A further thermodynamic analysis of tetrathionate binding (as indicated by inhibition) yields values between −4.6 kcal/mole (5 °C) and −4.2 kcal/mole (37 °C) for the free energy (ΔG) and a value of 13 cal/° mole for the entropy (ΔS) of binding. These numbers can be compared with thermodynamic parameters for the high affinity binding of small anions to albumin [34]. For chloride, a ΔG (at 25 °C) of −3.9, a ΔH of −3.0 kcal/mole, and a ΔS of +3.2 cal/° mole has been obtained. For anions adsorbing more strongly (J^-, SCN^-), more negative values for ΔG and ΔH, and negative values for ΔS were reported [34].

Like the binding of the latter anions to albumin, tetrathionate binding to the anion transfer system is characterized by partly compensating changes of enthalpy and entropy, but dominated by ΔH. Provided that K_m values of anion exchange reflect equilibrium constants for anion binding to transfer sites, the binding of tetrathionate can also be compared with the binding of other anions to the exchange system. From K_m values reported by Brahm [4] and Schnell [27] one can calculate the thermodynamic parameters compiled in Table 5 for chloride and sulfate binding. Although all these values are lower than those for tetrathionate, the relative contributions of enthalpy and entropy are the same as in the case of

Table 5. Thermodynamic parameters for the binding of tetrathionate, and of chloride and sulfate, to binding sites on the anion exchange system[a]

	Tetrathionate inhibition		
	ΔH (kcal/mol)	ΔG (kcal/mol)	ΔS (cal/° mol)
5 °C		−4.61	−12.9
25 °C	−8.2	−4.29	−13.1
37 °C		−4.17	−13.0
	Sulfate exchange		
25 °C	−3.7	−2.09	−5.43
38 °C		−2.00	−5.49
	Chloride exchange		
0 °C		−1.95	−6.44
25 °C	−3.7	−1.82	−6.33
38 °C		−1.70	−6.46

[a] ΔH values determined from the graphs in Fig. 11, ΔG calculated from $-RT \cdot \ln 1/I_{50}$ or $+RT \cdot \ln 1/K_m$ [4, 37], and ΔS from $\Delta S = (\Delta H - \Delta G)T$.

tetrathionate. This similarity suggests that the processes governing the binding of the inhibitor tetrathionate and of the transported anions are not greatly different.

Characteristics of the Tetrathionate Binding Site

As mentioned above, the inhibitory effects of small inorganic anions on their own exchange fluxes and on the fluxes of related anions have been interpreted [11] by two types of anion binding sites, a "transport site", giving rise to competition phenomena, and a "modifier site", responsible for noncompetitive inhibition of transport. In terms of this scheme, the noncompetitive tetrathionate inhibition of sulfate exchange could be interpreted by the binding of tetrathionate to a modifier site. It remains an open question whether the same is true for the inhibition of chloride exchange, which could not be measured at low chloride concentrations.

The presence of other anions diminishes the fractional inhibition by tetrathionate in a concentration-dependent fashion (Figs. 5 and 6). This effect is very pronounced for monovalent inorganic anions, which themselves are effective inhibitors of sulfate transfer (e.g., chloride, nitrate), whereas sulfate, even at very high concentrations, has only a minor

influence. The most obvious interpretation for these effects is a competition of the anions with tetrathionate for an inhibitory modifier site[3]. As a consequence, the interference of an anion with the inhibition by tetrathionate could be used as an indicator that this anion itself binds to the modifier site and thereby inhibits sulfate exchange noncompetitively. According to the data compiled in Fig. 6*a*, nitrate and chloride would thus have to be assumed to be noncompetitive inhibitors of sulfate exchange, in addition to their competitive effects demonstrated by Schnell [37]. In contrast, inhibition of sulfate transfer by acetate [37] would have to be classified as a largely competitive process in agreement with the results of Schnell [37], but in contrast to the reported noncompetitive action of this anion on chloride exchange [18].

A quantitative analysis of the effects of sulfate on tetrathionate inhibition in terms of two noncompetitive inhibitors competing for a common "modifier site" was attempted by applying straightforward Michaelis-Menten kinetics. Suitable formulation of this model[4] should provide – as a test – a consistent value for the self-inhibition constant K_{is} of sulfate exchange. Our experimental results could not be fitted to a single value of K_{is}. Mechanisms other than competition must therefore be invoked to explain the increase of the inhibitor constant K_T with increasing

3 In principle, the effect of high sulfate concentrations on the tetrathionate inhibition of sulfate exchange might be due to a weak competition at the transfer site. Quantitatively, however, the decrease of the fractional inhibition with increasing sulfate concentrations is not reconcilable with such a mechanism, as can be shown by calculations on the basis of the K_m values for sulfate exchange presently under discussion [31, 37], and the K_i value for tetrathionate obtained in the present work.

4 Sulfate flux in the presence ($J_{S,T}$) or absence (J_S) of tetrathionate (T) and of self-inhibitory concentrations of sulfate (S) is described by

$$J_{S,T} = J_{max} \frac{s'}{(1+s') \cdot (1+i'_S+i'_T)}$$

where $s' = [SO_4]/K_S$, K_S = apparent half saturation constant of sulfate exchange; $i'_S = [SO_4]/K_{is}$, K_{is} = apparent self-inhibition constant of sulfate exchange; $i'_T = [T]/K_T$, K_T = inhibitor constant of tetrathionate. Values in brackets represent *extracellular* concentrations. The fractional inhibition $I_f = 1 - (J_{S,T}/J_s)$ for tetrathionate inhibition at various sulfate concentrations is given by

$$I_f = \frac{i'_T}{1+i'_T+i'_S}.$$

i'_T values can be calculated using the K_T value determined experimentally in the absence of competing anions. A trial and error procedure can then be used to find out whether a single value of K_{is} fits I_f values obtained at different concentrations of sulfate and tetrathionate.

sulfate concentration. One probable factor is ionic strength, which changes enormously (from 0.095 to 0.855) when the sulfate concentration is raised from 20 to 285 mM.

A further attempt to verify competition of tetrathionate and other anions for a modifier site was based on an evaluation of the influence of chloride on tetrathionate inhibition of sulfate transfer. Since chloride is likely to inhibit sulfate transfer competitively *and* noncompetitively, the kinetic model[5] had to consider two competing noncompetitive inhibitors and additional competitive inhibition by one of the inhibitors. This model was evaluated by trying to find pairs for the competitive and the noncompetitive inhibitor constants (K_{Cl_c}, K_{Cl_n}) for chloride inhibition of sulfate transfer, which would describe the experimental relationship between I_f, the tetrathionate inhibition of sulfate transfer, and the extracellular chloride concentration [Cl]. A fair agreement between theory and experiment could be obtained when K_{Cl_n} was taken to be 40–50 mM and $K_{Cl_c}=100$ mM, but the relationship between I_f and [Cl] was rather insensitive to changes of the latter parameter.

In view of the rather involved procedure used here, inhibitor constants determined in this way cannot be compared with values obtained by other procedures [31]. Our results therefore do not yet allow us to decide whether the modifier site occupied by tetrathionate is identical with the modifier site as defined by Dalmark [11]. A similar ambiguity concerns the question whether the modifier sites occupied by tetrathionate are identical with the sites blocked by other inhibitors, e.g., APMB, furosemide, NAP-taurine, salicylate. Two pieces of indirect evidence indicate that this may be so: Firstly, many of these inhibitors – including tetrathionate – protect the anion exchange system against irreversible inactivation by fluorodinitrobenzene [43]; secondly, the effects of many anionic inhibitors, like those of

5 This situation can be described in terms of Michaelis-Menten kinetics by

$$J_{S,T}=J_{max}\frac{s'}{(1+s')(1+i'_{Cl_n}+i'_T)+i'_{Cl_c}}.$$

The symbols have the same meaning as in Footnote 4, in addition $i'_{Cl_n}=[Cl]/K_{Cl_n}$, K_{Cl_n} = half saturation constant for noncompetitive inhibition of sulfate transfer by chloride; $i'_{Cl_c}=[Cl]/K_{Cl_c}$, K_{Cl_c} = half saturation constant for competitive inhibition of sulfate transfer by chloride.

The fractional inhibition I_f amounts to

$$I_f=\frac{(1+s')\cdot i'_T}{(1+s')\cdot(1+i'_{Cl_n}+i'_T)+i'_{Cl_c}}.$$

This equation can be evaluated for pairs of i'_{Cl_c} and i'_{Cl_n} by iteration procedures.

tetrathionate, have previously been shown to be counteracted by small anions present in the medium [13].

From the low permeability and the immediate effect of tetrathionate one can assume its binding site to be located on or near the exofacial membrane surface. This assumption is also supported by the ineffectiveness of even high concentrations of intracellular tetrathionate. A similar asymmetry of inhibition was previously observed for certain inhibitors of anion transfer (phloretin, DAS, DNDS) [1, 24, 28, 36, 42], while others (APMB, NAP-taurine) [25, 31, 42] also inhibit from inside. Inhibition from inside, however, seems to require much higher concentrations and to be competitive in nature [6]. The same may be true for tetrathionate: On the one hand, its transfer seems to occur via the sulfate exchange system, as indicated by the inhibitor sensitivity [19]. On the other hand, the complete lack of effect of 7.5 mM intracellular tetrathionate on sulfate efflux indicates that the K_i value for its competition with sulfate would have to be greater than 50 mM.

A comparison between tetrathionate, an inorganic disulfonate, and the organic stilbene disulfonates such as DNDS reveals the following situation: Both inhibit – at least with high preference – from the outside of the cell (*cf.* [1]); tetrathionate, however, like many other amphiphilic inhibitors [14] seems to interact with a modifier site, while DNDS [1] and probably its congeners, too [38], act predominantly on a transport site.

Molecular Basis of the High Tetrathionate Affinity of the Anion Exchange System

From the above discussion of the inhibitor constants, it has become evident that tetrathionate is rather unique in its inhibitory potency. The question therefore arises, what makes an inorganic anion such a powerful inhibitor. One feature of major importance would seem to be the SO_3^- groups, which presumbably enable the inhibitor to interact with binding sites structurally similar to, although functionally different from, those blocked by stilbene disulfonates. On the basis of this argument, however, it is difficult to understand the marked differences in effectivity between tetrathionate and its analogues, peroxodisulfate and ethane disulfonate, particularly since the distances between the sulfonate groups are essentially identical for the three anions, as shown by X-ray-crystal-

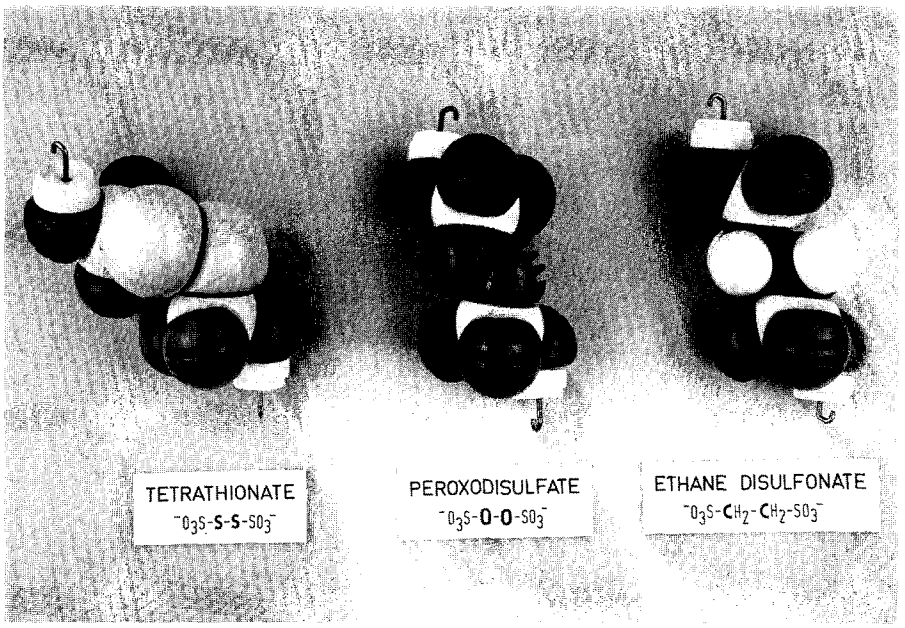

Fig. 12. Molecular configuration of tetrathionate and its $-O-O-$ and $-CH_2-CH_2-$ analogues. The lower halves of the molecules are oriented in the same way. One thus easily recognizes the 90° rotation in tetrathionate's S–S bond (away from the spectator) and the resulting asymmetry which exposes the two bulky S-atoms to one side of the molecule

lographic analyses[6] (tetrathionate, 4.21 Å [17], peroxodisulfate, 3.96 [41], ethane disulfonate, 4.32 Å [29].

Differences in hydrophobicity leading to different affinities to the membrane must also be discarded from the list of possible explanations since the ethane and, in particular, the benzene group are certainly more hydrophobic than the disulfide group and should thus give rise to a higher affinity and consequently a higher inhibitory potency.

The inspection of space-filling models of the three analogues (Fig. 12), however, suggests an interesting explanation: Peroxodisulfate and ethane disulfonate are arranged in a single plane and are in a sense symmetric. Tetrathionate, by virtue of a 90° rotation between the two sulfur atoms, is highly distorted. This rotation probably results from the formation of

6 We are greatly indebted to Dr. H. Formanek, Botanical Institute, University of Munich, for making available to us the X-ray data, for the construction of the spacefilling models (Fig. 12) and for drawing our attention to the structural peculiarities of tetrathionate.

π bonds, arising from the interaction of electrons in the *p*-orbitals and the *d*-orbitals of the two central sulfur atoms. By virtue of these π bonds leading to a local excess of electrons, the disulfide group behaves as a weak Lewis base, able to interact with Lewis or Brønsted acids having a deficit of free electrons or an excess of protons. Due to this Lewis base property, which is essentially absent in peroxodisulfate and ethane disulfonate, tetrathionate may be able to bind with high affinity to the anion transfer system, which contains clusters of positively charged groups [32] behaving as Lewis acids.

Thus, in order to make a compound a potent reversible inhibitor of anion exchange in the erythrocyte, a lipophilic moiety of the inhibitor molecule seems not be to be an absolute requirement. Weak interactions between electron pair acceptors (Lewis acids) and donors (Lewis bases) seem to be able to provide sufficient stability to complexes between inhibitors and the modifier sites of the anion exchange system and to fully compensate for the absence of hydrophobic domains in tetrathionate.

It is difficult to decide at present to which extent this type of interaction contributes to the binding of the more complex organic inhibitors. Indications for the relevance of electron donor-acceptor complexes for the binding of such inhibitors of anion transfer, however, have been put forward, while this work was in completion, by other investigators [2, 9]. Moreover, we have recently shown that the cycloalkyl analogues of aromatic inhibitors of anion transfer are less effective (B. Deuticke, *unpublished*).

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