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Reaction of the Glucose Carrier of Erythrocytes with Sodium Tetrathionate: Evidence for Inward-Facing and Outward-Facing Carrier Conformations

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Summary. Sodium tetrathionate reacts with the glucose carrier of human erythrocytes at a rate which is greatly altered in the presence of competitive inhibitors of glucose transport. Inhibitors bound to the carrier on the outer surface of the membrane, either at the substrate site (maltose) or at the external inhibition site (phloretin and phlorizin), more than double the reaction rate. Inhibitors bound at the internal inhibition site (cytochalasin B and androstenedione), protect the system against tetrathionate. After treatment with tetrathionate, the maximum transport rate falls to less than one-third, and the properties of the binding sites are modified in unexpected ways. The affinity of externally bound inhibitors rises: phloretin is bound up to seven times more strongly and phlorizin and maltose twice as strongly. The affinity of cytochalasin B, bound at the internal inhibition site, falls to half while that of androstenedione is little changed. The affinity of external glucose falls slightly. Androstenedione prevents both the fall in transport activity and the increase in phloretin affinity produced by tetrathionate. An inhibitor of anion transport has no effect on the reaction. The observations support the following conclusions: (1) Tetrathionate produces its effects on the glucose transport system by reacting with the carrier on the outer surface of the membrane. (2) The carrier assumes distinct inward-facing and outward-facing conformations, and tetrathionate reacts with only the outward-facing form. (3) The thiol group with which tetrathionate is presumed to react is not present in either the substrate site or the internal or external inhibitor site. (4) In binding asymmetrically to the carrier, a reversible inhibitor shifts the carrier partition between inner and outer forms and thereby raises or lowers the rate of tetrathionate reaction with the system. (5) Reaction with tetrathionate converts the carrier to an altered state in which the conformation at all three binding sites is changed and the rate of carrier reorientation is reduced.

Key Words tetrathionate · glucose carrier · inhibition · carrier sites · phloretin · cytochalasin B

Introduction

Sodium tetrathionate reacts with thiol groups in proteins to give a negatively charged S-sulfocysteine derivative, and the reaction is reversed by thiols [14, 19, 33, 36]. Bloch [6] has shown that tetrathionate reacts with the glucose carrier of human erythrocytes, producing a partial inhibition of transport; as expected, the activity of the transport system is almost completely restored by treatment with mercaptoethanol. The reaction is first order in tetrathionate concentration, which suggested that a single thiol group in the carrier is involved in the inhibition. The rate of the reaction increases in the presence of maltose, indicating that the reactive thiol group is probably located outside the substrate site, where maltose binds; and indeed the affinity for glucose is little changed after treatment [6].

Two aspects of the tetrathionate reaction have now been investigated further. First, the effects of reversible competitive inhibitors on the rate of the reaction were determined: the inhibitors were chosen for study because they bind either at the external substrate site (maltose), the external inhibitor site (phloretin and phlorizin), or the internal inhibitor site (cytochalasin B and androstenedione). Second, the affinity of the transport system for each of the reversible inhibitors was measured before and after treatment with tetrathionate. The observations provide new evidence for distinct inward-facing and outward-facing carrier conformations and reveal unexpected properties of the inhibition sites.

Materials and Methods

Sodium tetrathionate was synthesized by iodine oxidation of sodium thiosulfate, following the procedure of Gilman et al. [13]. Other chemicals were obtained commercially and were the best grade available. Water was glass distilled. Human blood, outdated, was obtained from a blood-bank.

Washed red cells were treated at 25°C with a solution of sodium tetrathionate, 132 mM, dissolved in 104 mM sodium phosphate buffer, pH 7.5, either in the presence or absence of glucose. In the case of treatment in the absence of glucose, one volume of packed cells was suspended in four volumes of tetrathionate-phosphate solution. After a period of incubation, the cells were rapidly sedimented in a clinical centrifuge, the super-

Table 1. The effects of reversible competitive inhibitors on reaction of the glucose carrier with sodium tetrathionate (132 mM, pH 7.5, 25°C)[a]

Inhibitor	Concentration (mM)	K_i (mM)	Where bound		Relative exit time
			Outside	Inside	
—					1.83 ± 0.036
Maltose	53	13	+		2.88 ± 0.056
Phloretin	6×10^{-3}	5.6×10^{-4}	+		3.03 ± 0.083
Phlorizin	2.8	0.13	+		2.97 ± 0.073
Cytochalasin B	16×10^{-3}	2.8×10^{-4}		+	0.96 ± 0.024
Androstenedione	0.57	2.4×10^{-2}		+	0.92 ± 0.019

[a] Cells (20% suspension) were incubated for 40 min in the presence or absence of an inhibitor, after which they were washed free of the incubation medium and loaded with glucose. The rates of glucose exit were determined by light scattering (*see* Materials and Methods). The exit times for the treated cells relative to that for the untreated control are listed. The exit time is the reciprocal of the maximum transport rate. K_i values were measured at 25°C.

natant was removed, and at a recorded time the cells were resuspended in 10 volumes of ice-cold isotonic NaCl solution (0.9%). They were washed once more in the same solution and then suspended in 4 volumes of 145 mM glucose in isotonic salt and incubated for 1 hr at 37°C.

In the case of treatment in the presence of glucose, cells were pre-equilibrated with 145 mM glucose in 104 mM sodium phosphate buffer, pH 7.5, for 40–60 min. at 37°C. The cells were collected and resuspended, 1 volume to 4, in either glucose-phosphate solution (the control) or tetrathionate-glucose-phosphate. The suspensions were incubated for a predetermined period at 25°C and sedimented; the cells were then washed three times in glucose-salt solution (145 mM glucose in 0.9% NaCl).

Rates of glucose exit were determined by the light-scattering method [35, 39, 40], with the injection of 0.4 ml of a 20% cell suspension into 66 ml of salt solution maintained at either 25 or 29°C. Exit times, which are the reciprocals of the maximum transport rates, were calculated from the initially linear segment of the exit tracings. The half-saturation constant of a reversible inhibitor was found from the rate of glucose exit into solutions of increasing inhibitor concentration, and the constant for the substrate from the net rate of exit into solutions of increasing glucose concentration.

Results

Tetrathionate Treatment in the Presence of Reversible Inhibitors of Glucose Transport

Cells were incubated with 132 mM sodium tetrathionate in the absence of glucose, as described above, but in the presence of several reversible competitive inhibitors. The results of one such experiment are given in Table 1. After 40 min in tetrathionate, in the absence of any reversible inhibitor, the glucose exit time nearly doubled (the exit time is the reciprocal of the rate of transport). In the presence of maltose, phloretin, or phlorizin, the inactivation was more extensive, the exit times being three times as long as in the control. By contrast, cytochalasin B and androstenedione prevented tetrathionate from inhibiting the system.

When the time course of inactivation was examined (Fig. 1), phloretin appeared to be more effective than maltose, both in accelerating the inactivation rate and in reducing the limiting transport activity. To find the inactivation rates, the data in Fig. 1 were plotted according to a rate equation which takes into account the residual activity of the system:

$$\log\left(\frac{v}{v_o} - \frac{v_f}{v_o}\right) = \log\left(1 - \frac{v_f}{v_o}\right) - kt \qquad (1)$$

where v is the measured rate at time t, v_o the rate for the intact system (at zero time), and v_f the final activity remaining after reaction with tetrathionate. The exit times shown in Fig. 1 are the reciprocals of the rates. Inactivation rate constants (k) were as follows: phloretin, 1.63 ± 0.05 $\text{min}^{-1}\ \text{M}^{-1}$; maltose, 0.95 ± 0.11 $\text{min}^{-1}\ \text{M}^{-1}$; the limiting residual transport activity was 15.3 ± 0.06% and 19.8 ± 1.4% with phloretin and maltose, respectively. The rate of inactivation in the control cannot be calculated from these data, because the times are too short to give the final activity of the system, but in similar experiments carried out for a longer period of time, the inactivation rate constant was 0.29 ± 0.01 $\text{min}^{-1}\ \text{M}^{-1}$ (Fig. 2); the limiting activity ranged between 20 and 30% in different samples of red cells. The inactivation constants are calculated for a first-order dependence of the rate on the concentration of tetrathionate, as demonstrated by Bloch [6].

The protection given by androstenedione was examined in several different cell samples (from different donors), and in some cases was less complete

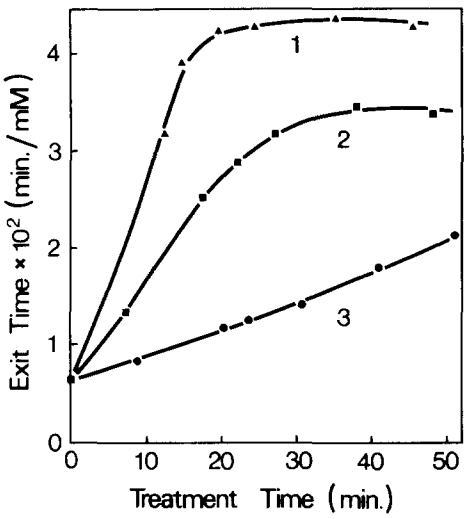

Fig. 1. The time course of inhibition of glucose exit by 132 mM sodium tetrathionate, pH 7.5, 25°C. The reaction was carried out either in the presence of 16.4 μM phloretin (curve *1*) or 45 mM maltose (curve *2*), or in the absence of reversible inhibitors (curve *3*). The rates of inactivation in this and companion experiments, calculated by least squares analysis of plots according to Eq. (1), were as follows: phloretin, 1.63 ± 0.05 min^{-1} M^{-1}; maltose, 0.95 ± 0.11 min^{-1} M^{-1}. Calculated residual activities, as a percentage of the rate in the untreated system, were: phloretin 15.3 ± 0.6%; maltose 19.8 ± 1.4%. The exit times, determined at 25°C, are reciprocals of the maximum rates of glucose exit (124 mM glucose)

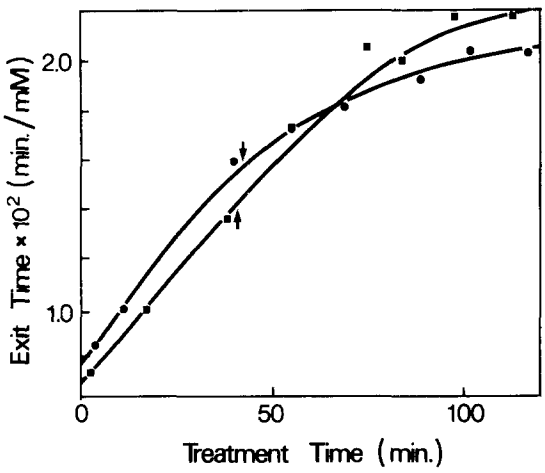

Fig. 2. Tetrathionate inhibition in the presence or absence of an inhibitor of anion transport (4-acetamido-4′-isothiocyanostilbene-2,2′disulphonic acid). Cells were preincubated with glucose with or without the anion inhibitor (SITS), at a concentration of 0.58 mM, for 1 hr; following this they were treated with 132 mM sodium tetrathionate, again in the presence or absence of the same concentration of SITS (*see* text). Circles represent glucose exit times for cells treated in the presence of SITS, and squares those treated in its absence. From plots of the data according to Eq. (1) the inactivation rates in the presence and absence of SITS were 0.295 ± 0.013 min^{-1} M^{-1} and 0.292 ± 0.012 min^{-1} M^{-1}, respectively. The arrows in the figure show the points at which 50% of the final inactivation had been achieved

than in Table 1. For example, after a 2-hr treatment in tetrathionate the residual activity in another experiment was 35 ± 0.7%, and with 0.59 mM androstenedione it was 86 ± 2.9%; considering that the inactivation is at most partial, this indicates strong though incomplete protection.

Tetrathionate Treatment in the Presence of an Inhibitor of Anion Transport (SITS, 4-Acetamido-4′-Isothiocyanostilbene-2,2′-Disulphonic Acid)

Cells were pre-incubated with glucose for 1 hr at 37°C as described under Materials and Methods, in one case in the presence of 0.58 mM SITS and in the other in its absence. The solutions were then replaced with a glucose-tetrathionate solution, again with or without 0.58 mM SITS, and incubated at 25°C. The rates of glucose exit were determined at intervals by the light-scattering method, at 25°C, without removing the tetrathionate solution. The course of inactivation is shown in Fig. 2, where the anion exchange inhibitor is seen to have had no effect on the tetrathionate reaction. It was also shown that preincubation of glucose-loaded cells with 0.5 mM SITS for 1 hr at 37°C did not significantly affect the rate of glucose exit or the affinity for phloretin.

The Affinity of the Modified System for Glucose and Reversible Inhibitors

The behavior of inhibitors was examined after a 2½-hr treatment of the cells in glucose-tetrathionate solutions. Control cells were incubated over the same period in the absence of tetrathionate. Rates of glucose exit were measured at 29°C as a matter of convenience, since inhibited rates are more precisely determined at this temperature than at 25°C. The results, in Fig. 3*A–F*, show that the affinity of the inhibitors, and of glucose, was altered after treatment. The same trends were observed at 25°C. The results on a single batch of cells, where the effects of different inhibitors are directly comparable, are given by the following ratios (each ratio is the half-saturating concentration of an inhibitor in untreated relative to that in treated cells): phloretin, 7.17 ± 0.18; phlorizin, 1.47 ± 0.03; maltose, 1.92 ± 0.04; external glucose, 0.892 ± 0.016; cytochalasin B, 0.463 ± 0.007. Results obtained with blood from

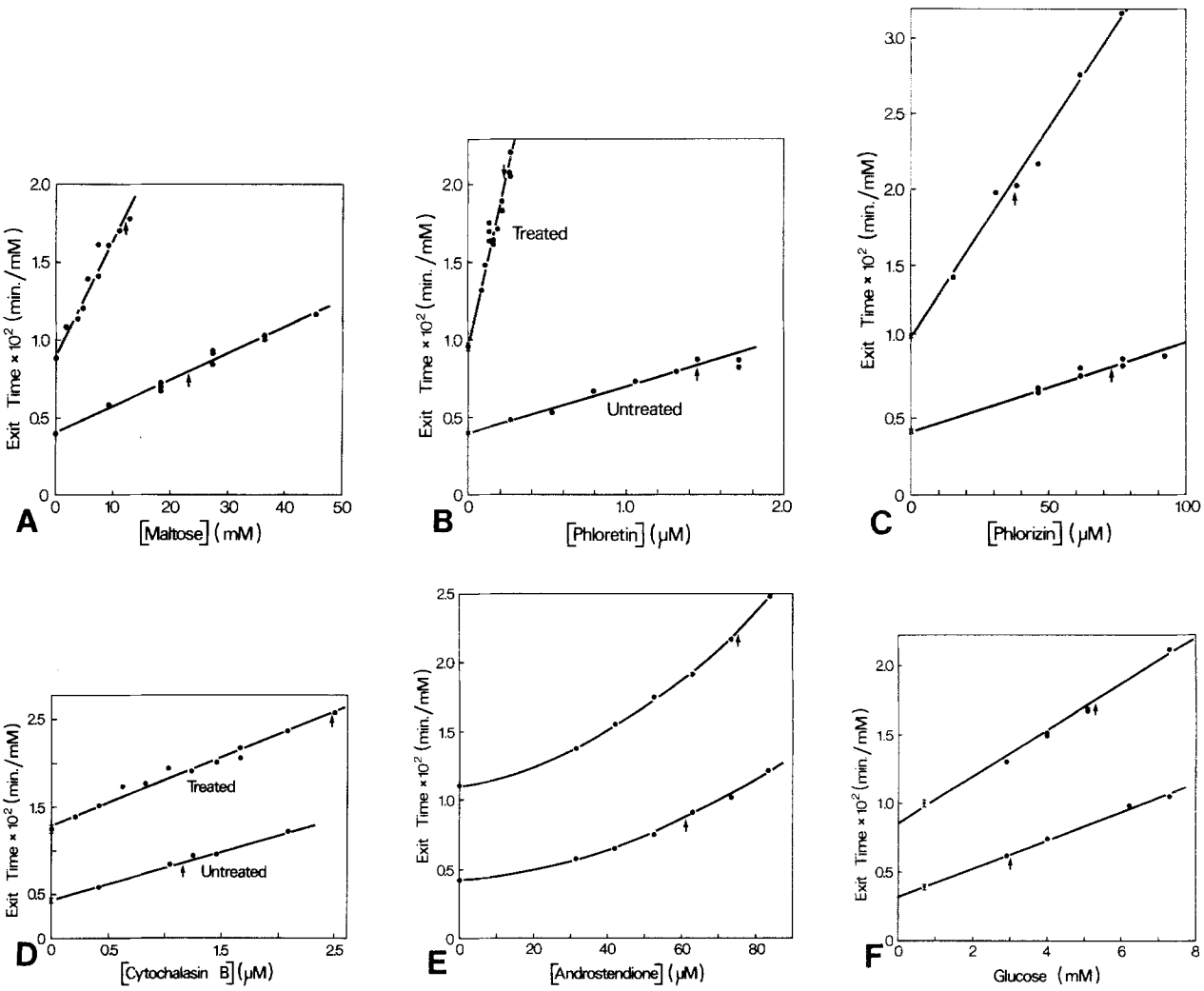

Fig. 3. Behavior of the glucose transport system before and after treatment with 132 mM sodium tetrathionate. The experimental procedure is described in the caption to Table 2 and in the text. The inhibition of glucose exit at 29°C by varying concentrations of reversible inhibitors is shown (*A–E*), as well as the inhibition of net glucose exit by external glucose [Sen-Widdas experiment (*F*)]. In each case the lower line is a plot of glucose exit times for the untreated system and the upper line is for the system after reaction with tetrathionate. From a least-squares analysis, the half saturation constants are as follows: maltose (*A*): control 23.0 ± 0.07 mM, treated 11.9 ± 0.33 mM; phloretin (*B*): control 1.45 ± 0.026 μM, treated 0.228 ± 0.0036 μM; phlorizin (*C*): control 73.0 ± 0.90 μM, treated 37.9 ± 0.09 μM; cytochalasin B (*D*): control 1.16 ± 0.01 μM, treated 2.48 ± 0.07 μM; androstenedione (*E*): control 61 μM, treated 85 μM; glucose (*F*): control 3.01 ± 0.015 mM; treated 5.32 ± 0.048 mM. A statistical analysis was not carried out for androstenedione because the plots are curved rather than linear

different donors are summarized in Table 2, and the variation among samples is seen to be larger than the uncertainty of individual measurements.

The Behavior of the System when Treated with Tetrathionate in the Presence of Androstenedione

Androstenedione prevents tetrathionate from inhibiting transport (Table 1) and the question now asked is whether it also prevents tetrathionate from modifying the affinity of the system for inhibitors. Cells were treated for two hours with tetrathionate-glucose solutions (*see* Materials and Methods) either in the presence or absence of androstenedione (0.56 mM). In its absence the affinity for phloretin rose by a factor of 2.75 ± 0.05, but in its presence there was no significant change, the factor now being 1.03 ± 0.02.

Discussion

The Binding of Reversible Inhibitors

The inhibitors used in this study act by a competitive mechanism; i.e., when the substrate and inhibi-

tor are in a position to add to the carrier on the same side of the membrane, they compete with one another [3, 12, 22, 24, 27, 32]. The inhibitors were selected because they are bound asymmetrically, at either the external substrate or inhibitor site, or the internal inhibitor site.

The question of where maltose binds is particularly simple, since this substrate analog is not transported by the system and cannot enter the cell [30] and therefore could not bind on the inner surface of membrane. That it is bound only on the outer surface is shown, too, by the fact that it competes with glucose in the external medium [24] but not with glucose inside the cell [25]. Further, maltose protects the system against inactivation by butanedione, a property shared with substrates but not with cytochalasin B or phloretin [26]. This, taken together with the structural similarity to the substrate, indicates that maltose is bound at the substrate site.

The behavior of cytochalasin B is quite different, since it does not bind on the outer surface of the membrane, but rapidly enters the cell and binds with high affinity to a site in the inward-facing carrier [2, 3, 10]. The main evidence is that the inhibition is competitive in equilibrium exchange and zero *trans* exit, and noncompetitive in zero *trans* entry, and from an analysis of the carrier model [29] this implies asymmetric binding; specifically, binding inside but not outside. Androstenedione is similar, in that it binds predominantly at an inner site; like cytochalasin B, it does not compete with external substrate, but does compete with substrate inside the cell [27]. Cytochalasin B and androstenedione strongly protect the system against inactivation by 2,4-fluorodinitrobenzene, which substrates do not do [5, 7, 21, 24, 26]; the protection appears to be steric in nature, arguing that the inhibitor site is separate from the substrate site [26], though some authors have suggested, on the basis of model building studies with different cytochalasins, that they overlap [17, 43].

The behavior of phloretin is different from that of either maltose or cytochalasin B. Although phloretin rapidly penetrates the cell membrane [12, 20], it only adds to the outward-facing carrier; the evidence for this is that the inhibition is competitive in entry and noncompetitive in exit, just the reverse of that with cytochalasin B [25]. The sites for these inhibitors, therefore, lie at opposite poles of the carrier molecule (which has been shown to span the membrane [1, 15]): the phloretin site is on the outer surface, the cytochalasin B site on the inner.

Phlorizin, the glucoside of phloretin, inhibits in exactly the same way as phloretin, though for a different reason. Experimentally, the inhibition is competitive in zero *trans* entry, and noncompetitive in zero *trans* exit (R.M. Krupka, *unpublished results*), showing that the inhibitor is bound on the outer but not the inner surface of the membrane. Phlorizin, unlike phloretin, fails to penetrate the red cell membrane [31], probably because of the polar glucose substituent in the molecule, and therefore when added to the external solution can only bind outside. However, if present inside the cell it also binds to the inward-facing carrier, as shown by the fact that it inhibits xylose transport about equally whether sealed in ghosts or present in the suspending medium [31, 38]. Phlorizin's ability to add to the carrier on either side may be explained by its low affinity; the inhibition constant is 290 μM, compared with 0.66 μM for phloretin [24]. With this high concentration, binding should be relatively nonspecific: at the phloretin site on the outer surface of the membrane, and possibly at the cytochalasin B site on the inner surface. Several steroids of low affinity have also been shown to bind on both sides [27].

Table 2. Effects of tetrathionate treatment on the affinity of the carrier for competitive inhibitors and glucose[a]

Compound	Ratio of half-saturating concentrations in untreated relative to treated cells
Maltose	2.11 ± 0.06, 1.92 ± 0.04
Phloretin	6.37 ± 0.15, 7.17 ± 0.18, 4.06 ± 0.07, 3.74 ± 0.13, 2.75 ± 0.05
Phlorizin	1.93 ± 0.03, 1.47 ± 0.03
Cytochalasin B	0.50 ± 0.013, 0.47 ± 0.014, 0.46 ± 0.01, 0.38 ± 0.01
Androstenedione	0.97, 0.81
Glucose	0.89 ± 0.02, 0.81 ± 0.01, 0.57 ± 0.006

[a] Cells (20% suspension) were incubated with 132 mM sodium tetrathionate in solutions containing 145 mM glucose and 104 mM sodium phosphate buffer, pH 7.5, 25°C, for 2½ hr. The cells were then washed in a solution of 145 mM glucose in 0.9% NaCl, and rates of glucose exit were determined by the light-scattering method at 29°C with varying concentrations of an inhibitor or glucose in the external solution. Half-saturation constants were determined from a least-squares analysis of the linear plots of exit time *versus* concentration (*see* Fig. 3*A–D* and *F*). In the case of androstenedione, where the plot curved upwards (Fig. 3*E*; [20]) a statistical analysis was not carried out.

The ratios of half-saturating concentrations for untreated cells relative to those treated with tetrathionate are listed above. A ratio greater than unity indicates that the affinity for an inhibitor rises after treatment, and a ratio less than unity, that it falls. Variation in the ratio for a given inhibitor appears to be dependent on the properties of cells obtained from different donors, as suggested by the fact that the differences are larger than the uncertainty of individual measurements.

The Site of Tetrathionate Reaction

Tetrathionate, a divalent anion, is unlikely to diffuse passively across a lipid membrane, but it has

been reported to enter red cells with a halftime of about 15 min when present at 1 mM [18]. Its entry is blocked by several different inhibitors of the anion exchange system of these cells [18], and it has been shown to inhibit the transport of other anions by competing for the anion exchange carrier [9, 23]. Hence, tetrathionate uptake is probably dependent on the anion exchanger. Nevertheless, an inhibitor of anion transport, SITS [8], had no effect on the rate of inactivation (Fig. 2). Further, since tetrathionate enters slowly, reaction on the internal surface of the membrane should become faster over a period of time, particularly at the high concentrations of tetrathionate required for inhibition (132 mM), but no significant lag period in the reaction was seen (Figs. 1 and 2). Almost certainly, therefore, the inhibitory reaction of tetrathionate is restricted to the external surface of the cell membrane.

That tetrathionate reacts with some group (probably a thiol group [6]) closely associated with the carrier is indicated by the specificity of the effects of different inhibitors on the rate of the reaction: the effects vary according to whether the inhibitors are bound to external or internal sites, and after reaction, the affinity of the carrier for the inhibitors varies in a specific manner, which also depends on where they bind. The observations may be summarized as follows. Competitive inhibitors bound to the carrier on the external surface of the membrane, namely, maltose and phloretin, greatly increase the rate of the reaction (Fig. 1), whereas inhibitors bound at the internal inhibition site, cytochalasin B and androstenedione, block the tetrathionate reaction (Table 1). The substrate, under equilibrium conditions where it would be bound to both the inner and outer sites, has little effect on the reaction rate, reducing it by roughly 15–20% [6]. After treatment of the system with tetrathionate, the affinity of externally bound inhibitors increases and the affinity of external glucose falls slightly; the affinity of cytochalasin B falls to half while that of androstenedione is unchanged, though both are bound at the internal inhibition site (Table 2).

It is also observed that androstenedione prevents tetrathionate from either reducing the transport rate or increasing the affinity for phloretin, suggesting that both effects result from the same modification of the carrier structure.

Although tetrathionate appears to react directly with the carrier, the reaction is outside any of the three main binding areas. The internal inhibition site may be ruled out for two reasons: first, the evidence shows that the reaction occurs on the external surface of the membrane; and, second, the affinity of this site for androstenedione is little changed after reaction. The substrate site is ruled out by several findings: first, the rate of tetrathionate reaction is only slightly reduced in the presence of a saturating concentration of equilibrated glucose [6] and is substantially increased in the presence of maltose; and, second, the affinity for glucose is similar before and after reaction, while the affinity for maltose is doubled. The external inhibition site may also be ruled out, because phloretin and phlorizin, which are bound here, increase the rate of the reaction and after reaction are far more strongly bound to the carrier than before. It follows that tetrathionate reacts outside these sites, but in a region of the carrier exposed to the external solution in the outward-facing carrier.

The basis of enhanced affinity at the external binding sites is unclear. The introduction of a negative charge on the external surface of the carrier, as a result of tetrathionate reaction, would not be expected to have this effect, since maltose is a neutral molecule, and phloretin in its ionized form is an anion (pK_a = 7.3 [20]). Nor can a shift in the distribution of the free carrier or carrier-substrate complex between inward-facing and outward-facing forms explain the affinity changes: the equation for the rate of transport [29] could account for a uniform increase in the affinity of externally bound inhibitors and a uniform decrease in the affinity of internally bound inhibitors, but, as Table 2 shows, this is not found. Nor could the existence of several carrier types with different affinities for inhibitors explain the observations; in such a hypothesis, low affinity forms might be preferentially inactivated by tetrathionate, leaving the high affinity forms functional, but nonlinearity would then be seen in plots of $1/v$ versus $[I]$ for the intact system, contrary to the observations (Fig. 3*A–C*; [3, 24]). At present, the best hypothesis would seem to be that the tetrathionate reaction stabilizes an altered carrier conformation with a changed configuration at all three binding sites and a reduced rate of reorientation in the membrane.

The distortions at each site are seen to have specific effects on individual ligands. Thus, the enhancement in the affinity of phloretin is greater than that of its glucoside phlorizin; presumably the glucose residue in the latter, which interferes with binding of the phloretin moiety to the intact carrier, limits the strength of additional interactions in the modified carrier. The difference in the effects on cytochalasin B and androstenedione, both bound at the inner site, could also be explained by some such hypothesis. Interpreting the effects on glucose and maltose, which are bound at the substrate site, is less certain; the differences could be due either to

the ligands binding in overlapping but not identical regions of the carrier, or to the measured half-saturation constants (one for a substrate, the other for a nontransported analog) having a different dependence on carrier reorientation rates [29].

Impermeant Thiol Reagents

That the glucose carrier is dependent on a thiol group exposed on the outer surface of the membrane is supported by other observations. Van Steveninck et al. [44] reached this conclusion on finding that *p*-chloromercuriphenylsulfonic acid and chlormerodrin, reagents that do not enter the cell, block glucose transport. Batt et al. [4] found that the impermeant reagents glutathione-maleimide and dextran-maleimide also block glucose transport. Cytochalasin B protected the system against glutathione-maleimide, while glucose had only a small protective effect (though glucose appeared to give more protection against dextran-maleimide). N-maleoylmethionine sulfone, an impermeant thiol reagent investigated by Roberts et al. [38], blocked transport, and again cytochalasin B protected the activity of the system. None of the reagents was shown to inhibit completely. The findings resemble those with tetrathionate, and the same external thiol group may be involved in all cases. It will be of interest to determine the effects of maltose and phloretin on these reagents and also to examine the binding of inhibitors to the modified carrier.

Effects of Reversible Inhibitors on the Tetrathionate Reaction

The thiol group which reacts with tetrathionate becomes exposed when a competitive inhibitor is bound to the carrier on the external surface of the membrane, either at the substrate site or the inhibitor site, but becomes unreactive when an inhibitor is bound at the internal surface (Table 1). This suggests that internal and external inhibitors stabilize two different carrier conformations, one inward-facing and the other outward-facing. In the absence of inhibitors or substrates, the system is an equilibrium mixture of these carrier forms, and the rate of its reaction with tetrathionate depends on the proportion of the outer form. In the presence of an externally bound inhibitor, the carrier is drawn into the outward-facing form, causing the reaction rate to increase; in the presence of an internally bound inhibitor, it is drawn into the inward-facing form, causing the rate to fall to zero. The protection given by inhibitors bound to the inner site is therefore not steric in nature, but depends on their effect on the carrier equilibrium.

Whether the externally bound inhibitors phloretin and maltose stabilize the same carrier conformation, and whether all outward-facing forms, free or an inhibitor complex, react with tetrathionate at the same rate, is still uncertain. Different outward-facing conformations are suggested by the apparently unequal activities of the system after reaction in the presence of either phloretin or maltose (Fig. 1). The progress of inactivation in the presence of the two inhibitors was also different (Fig. 1), though this could be because phloretin (16.4 μM) is more nearly saturating than maltose (45 mM): the inhibition constants (K_i) are 0.56 μM and 13 mM, respectively, so that $[I]/K_i$ is 29 for phloretin and only 3.5 for maltose.

Inward-Facing and Outward-Facing Carrier Conformations in Relation to Transport

The effects of asymmetrically bound inhibitors on the tetrathionate reaction were seen to implicate two carrier conformations that are interconvertible: in one conformation an inhibitor site is exposed on the outer surface of the membrane, and in the other a site is exposed on the inner surface. As the inhibitors are competitive, it is likely that the substrate site, along with an inhibitor site, is exposed on one side at a time. Hence, these conformations may be directly involved in transport, and substrate transfer may occur when the carrier, with a substrate molecule attached, is converted from one to the other. No more direct evidence is available at present to link the two conformations to the process of transport, but evidence of another kind supports such a mechanism: a ternary complex of the carrier with two inhibitor molecules, one bound to the inner site and one to the outer, could not be detected; this was the case (a) in transport studies on whole cells, involving cytochalasin B and either phloretin [28] or maltose (R.M. Krupka, *unpublished results*); (b) in binding studies on red cell membranes, involving cytochalasin B or propyl-β-D-glucopyranoside (bound inside) and 4,6-O-ethylidene-D-glucose or phloretin (bound outside) [16, 22]; and (c) in binding studies on the isolated carrier, involving cytochalasin B and phloretin [41]. It follows that transport probably depends on a substrate site which alternately appears on opposite sides of the membrane, rather than on a pair of substrate sites which are simultaneously available on both sides [11]. The glucose carrier has been shown to span the membrane, [1, 15] and therefore should alternately as-

sume inward-facing and outward-facing conformations. It is these two forms of the carrier that tetrathionate appears to distinguish.

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