

A Kinetic Study of Sulphate Transport in Rat Liver Mitochondria

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The kinetics of sulphate uptake catalysed by the dicarboxylate carrier were measured. The K_m value for sulphate is about 0.3 mM. A completely competitive relation exists between the influxes of sulphate and malonate, whereas the relation between sulphate and phosphate is of the mixed type. The inhibition of sulphate influx by mersalyl and bathophenanthroline is similar to that of malonate influx and different from the inhibition of phosphate influx. It is considered that sulphate and malonate probably bind to the same locus on the carrier, whereas phosphate occupies a different site. The possible implications of this conclusion are discussed.

The original work of Chappell and co-workers (see Chappell, 1968) showed that phosphate and certain *cis*-dicarboxylate anions, e.g. malate, succinate and malonate, are substrates of the dicarboxylate carrier in mitochondria. This carrier is inhibited by substrate analogues, e.g. 2-*n*-butylmalonate (Robinson & Chappell, 1967), thiol reagents, e.g. mersalyl (Meijer *et al.*, 1970), and metal-complexing agents, e.g. bathophenanthroline (Tyler & Newton, 1970; Passarella *et al.*, 1973). Palmieri *et al.* (1971) obtained evidence from kinetic studies to suggest that there are separate binding sites for phosphate and dicarboxylate anions.

It has been shown recently that the known substrates of this carrier exchange with sulphate, sulphite and thiosulphate in a butylmalonate- and mersalyl-sensitive manner, from which it was concluded that sulphate, sulphite and thiosulphate are also transported (Crompton *et al.*, 1974*a,b*). If this interpretation is correct, an inhibitory relationship should exist between the transport of these anions and the other recognized substrates. Further, the nature of such an inhibition ought to elucidate the relation between the binding of the sulphur-containing anions and the phosphate and dicarboxylate anions, and possibly allow the number of binding sites to be further evaluated.

The present study examines the transport of sulphate and the effect on this process of two other types of substrate, phosphate and malonate, and inhibitors of the dicarboxylate carrier. It is concluded that sulphate and dicarboxylate anions probably share a common binding site on the dicarboxylate carrier, distinct from the phosphate-binding site.

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Experimental

Materials

[³⁵S]Sulphuric acid, [1-¹⁴C]malonate, [³²P]phosphoric acid, [1,5-¹⁴C]citrate, [5-¹⁴C]oxoglutarate and ³H₂O were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. *N*-Ethylmaleimide, mersalyl and bathophenanthroline-sulphonate were bought from Sigma Chemical Co., St. Louis, Mo., U.S.A. 2-Phenylsuccinate was obtained from K & K Laboratories, Plainview, N.Y., U.S.A. and 2-*n*-butylmalonate was from Aldrich, Milwaukee, Wis., U.S.A. Rat liver mitochondria were prepared and their protein content was determined as described previously (Crompton *et al.*, 1974*a*).

Procedure used to load mitochondria with phosphate or malate

Mitochondria (containing 40–50 mg of protein) were incubated at 15–20°C in 10 ml of medium, pH 6.8, containing 100 mM-KCl, 20 mM-Tris-HCl, 1 mM-EDTA, 20 µg of rotenone, and either 0.5 mM-potassium phosphate or 0.5 mM-potassium malate, depending on which of these was loaded. After 2 min, the suspension was diluted to 50 ml with ice-cold medium (as above) and the mitochondria were sedimented by centrifugation at 8000 g for 8 min at 0–2°C. The supernatant was removed as completely as possible and the mitochondria were suspended in medium (as above; approx. 40 mg of mitochondrial protein/ml). Mitochondria that were loaded in this way and suspended in reaction medium (as described below) contained 14–20 mM-phosphate or malate, whereas the extramitochondrial concentration of phosphate or malate was less than 10 µM.

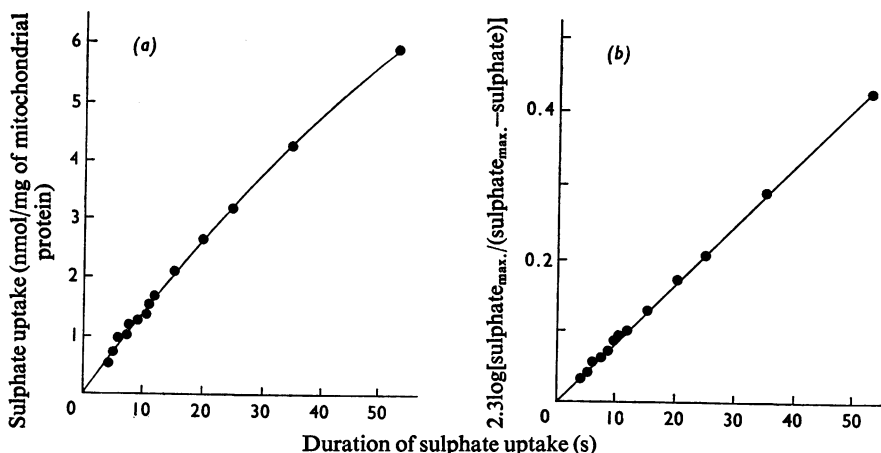


Fig. 1. Sulphate influx with time into liver mitochondria

Phosphate-loaded mitochondria were used. Further details are given in the Experimental Section. In *b*, sulphate and sulphate_{max.} refer to the amounts of sulphate influx after the time specified on the abscissa and after equilibrium was reached (see the text).

Measurement of the kinetics of substrate uptake

Mitochondria (containing approx. 2 mg of protein), loaded with either phosphate or malate, were suspended in 0.9 ml of medium, pH 7.0, containing 0.20 M sucrose, 20 mM Tris-HCl, 10 mM KCl, 1 mM MgCl₂, 1 μg of rotenone and about 1 μCi of ³H₂O; the suspension was maintained at 3°C. When phosphate-loaded mitochondria were used the suspension also contained 2 mM *N*-ethylmaleimide. After 1 min, the exchange reaction was begun by quickly adding the radioactive substrate (approx. 0.1 μCi) and terminated 8 s later (unless otherwise indicated) by rapidly introducing an inhibitor. The compounds used to inhibit the carriers were as follows (see Palmieri *et al.*, 1971, 1972*a,b*): dicarboxylate carrier, 20 mM butylmalonate; oxoglutarate carrier, 20 mM phenylsuccinate; tricarboxylate carrier, 20 mM benzene-1,2,3-tricarboxylate; phosphate carrier, 2 mM mersalyl. The mitochondria were sedimented immediately by centrifugation for 2 min in an Eppendorf bench centrifuge (model 3200) operating at 15000 rev./min. The supernatant was removed and the pellet was extracted with 0.5 ml of 0.3 M HClO₄. The radioactivity contents of the supernatants and the pellet extracts were determined by liquid-scintillation spectrometry as described by Crompton *et al.* (1974*a*).

Each test determination (described above) was accompanied by a control experiment in which the sequence of addition of substrate and inhibitor was reversed, i.e. inhibitor was added 8 s before the substrate. The control experiments were done to determine the amounts of substrate in the extra-mitochondrial space of the pellets plus any which

permeated the intramitochondrial space in the presence of inhibitor (i.e. after the 8 s reaction period). In fact, the inhibitors usually prevented completely the access of substrates to the intramitochondrial space as defined by the sucrose-impermeable space of the mitochondrial pellet.

Knowledge of these quantities permitted the amounts of substrate uptake into the intramitochondrial compartment during the 8 s reaction period to be calculated, and this uptake was used as the rate of substrate influx. This is justified, since the rates of uptake of sulphate (see the Results section) and the other substrates used are constant for at least 8 s under the experimental conditions described above (see Palmieri *et al.*, 1971, 1972*a,b*).

The effects of other metabolites on the rates of substrate uptake were measured by adding these metabolites simultaneously with the substrate in both test and control determinations.

Results

Characteristics of sulphate uptake

Fig. 1(*a*) shows the time-course of sulphate uptake by phosphate-loaded mitochondria. Sulphate uptake is linear with time (*t*) under these experimental conditions for at least 10 s. Equilibrium was reached after 5 min incubation, when the maximum amount of 17.1 μmol of sulphate/mg of mitochondrial protein was taken up.

Sulphate uptake with time was further analysed by the treatment of Pfaff *et al.* (1969) for the case where translocation occurs by strict exchange between exogenous and endogenous anions [as in sulphate

transport (Crompton *et al.*, 1974a)]; this treatment also requires that the anion content in the extra-mitochondrial volume is much larger than the endogenous content of exchangeable anions, as in the present study. It is predicted that the net transfer of a radioactively labelled anion between the exogenous and endogenous pools might obey a relation of the form:

$$2.3 \log \left(\frac{A_{\max.}}{A_{\max.} - A} \right) = kt$$

where $A_{\max.}$ is the uptake of the labelled anion at equilibrium, A is the uptake of labelled anion at time t and k is a first-order constant. The constant k is equal to the translocation activity v (zero-order) divided by $A_{\max.}$. This relation is observed by the carrier-mediated transport of adenine nucleotides (Pfaff *et al.*, 1969), glutamate (Bradford & McGivan, 1973), oxoglutarate (Palmieri *et al.*, 1972b) and citrate (Palmieri *et al.*, 1972a).

Fig. 1(b) shows that the influx of labelled sulphate proceeds according to this equation. The value of k was 0.48 min^{-1} and the corresponding value of v was $8.2 \text{ nmol/min per mg}$ of mitochondrial protein at 2°C .

The dependence of the rate of sulphate influx on the extramitochondrial sulphate concentration produces linear reciprocal plots (see Figs. 3, 5 and 6). In a

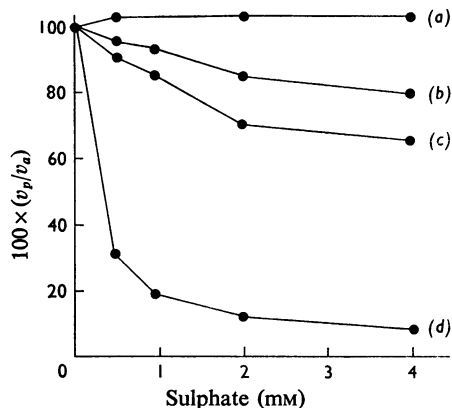


Fig. 2. Effect of sulphate on the rate of anion uptake by the different anion-transporting systems of liver mitochondria

Details are given in the Experimental section. The symbols v_p and v_a refer to the rate of substrate influx in the presence and absence of sulphate respectively. (a) 0.2 mM -phosphate influx into phosphate-loaded mitochondria in the presence of 20 mM -butylmalonate and the absence of N -ethylmaleimide (phosphate-carrier activity); (b) $50 \mu\text{M}$ -oxoglutarate influx into malate-loaded mitochondria (oxoglutarate-carrier activity); (c) 0.12 mM -citrate influx into malate-loaded mitochondria (tricarboxylate-carrier activity); (d) 1.5 mM -phosphate influx into malate-loaded mitochondria in the presence of 2 mM - N -ethylmaleimide (dicarboxylate-carrier activity).

series of 10 experiments conducted at 3°C and $\text{pH } 7.0$, the $V_{\max.}$ values varied between 10 and $16 \text{ nmol/min per mg}$ of mitochondrial protein and the K_m values were between 0.22 and 0.36 mM .

Inhibition of anion-transporting systems by sulphate

Fig. 2 shows the effect of increasing concentrations of sulphate on the rate of substrate uptake by different anion-transporting systems of rat liver mitochondria. Phosphate influx, catalysed by the phosphate carrier, was measured in the presence of butylmalonate to prevent phosphate transport by the dicarboxylate carrier (Robinson & Chappell, 1967). Conversely, phosphate transport by the dicarboxylate carrier was determined with N -ethylmaleimide present to inhibit the phosphate carrier (Meijer *et al.*, 1970). One potential mechanism whereby sulphate might inhibit the activity of a carrier is by altering its substrate affinity. Therefore to obtain a more meaningful comparison between the effects of sulphate on the activity of the different carriers, the substrate concentration used for the assay of each carrier was approximately equal to its K_m value for that substrate (see Palmieri *et al.*, 1971, 1972a,b; Passarella *et al.*, 1973).

Sulphate markedly inhibits the activity of the dicarboxylate carrier. In contrast, sulphate has a small effect on the activity of the oxoglutarate and tricarboxylate carriers, and does not inhibit the phosphate carrier.

Since inhibition by sulphate is presumably a consequence of sulphate binding, it seems that sulphate is bound strongly by the dicarboxylate carrier, but binds weakly, or not at all, to the tricarboxylate, oxoglutarate and phosphate carriers. This supports the previous conclusion that sulphate is transported by the dicarboxylate carrier, but not by the phosphate, oxoglutarate and tricarboxylate carriers (Crompton *et al.*, 1974a).

Mutual inhibition between sulphate, phosphate and malonate influx

Figs. 3(a) and 3(b) report the effect of two substrates of the dicarboxylate carrier, phosphate and malonate, on the rate of sulphate influx. The data are presented as double-reciprocal plots. Both malonate (Fig. 3a) and phosphate (Fig. 3b) inhibit sulphate transport, but the nature of their inhibition differs; malonate inhibits competitively, whereas the inhibition by phosphate is of the mixed type. The K_i values of malonate (0.27 – 0.29 mM) are reasonably close to its K_m value (0.2 mM ; Fig. 4a).

A similar distinction is found between the effects of sulphate on the transport of malonate and phosphate by the dicarboxylate carrier (Figs. 4a and 4b respectively). Sulphate is a competitive inhibitor of malonate transport, but causes mixed inhibition of phosphate transport. The K_i value of sulphate (0.31 – 0.41 mM ;

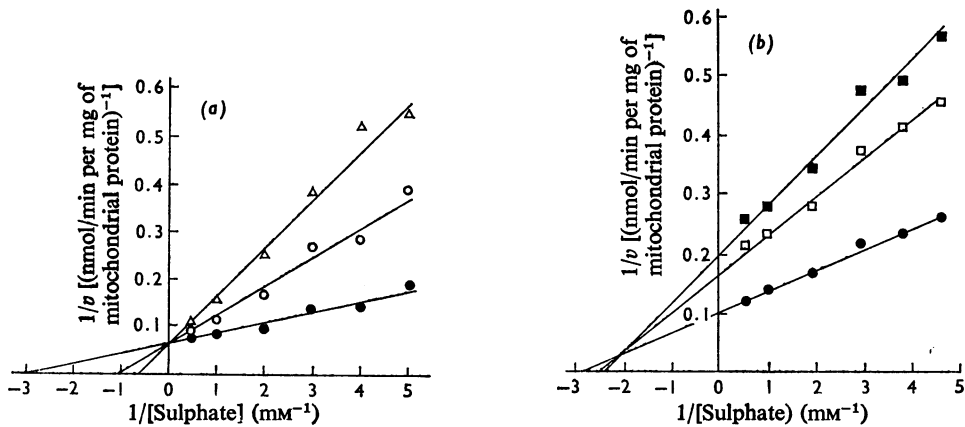


Fig. 3. Inhibition of sulphate influx by malonate (a) and phosphate (b)

Phosphate-loaded mitochondria were used. Further details are given in the Experimental section. Additions: ●, none; ○, 0.4 mm-malonate; △, 1 mm-malonate; □, 1 mm-phosphate; ■, 2 mm-phosphate.

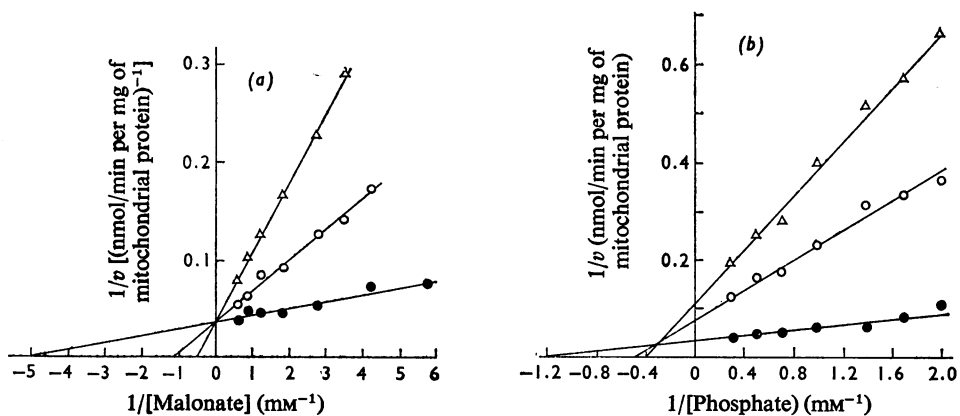


Fig. 4. Inhibition of the influx of malonate (a) and phosphate (b) by sulphate

Phosphate-loaded mitochondria were used. Further details are given in the Experimental section. Additions: ●, none; ○, 1.5 mm-sulphate (a) or 0.5 mm-sulphate (b); △, 3 mm-sulphate (a) or 1 mm-sulphate (b).

from Fig. 4a) is similar to its K_m value. Thus there is a competitive relation between the transport of sulphate and malonate, and mixed inhibition between sulphate and phosphate transport.

Effect of inhibitors of the dicarboxylate carrier on sulphate transport

Fig. 5 shows the effect of two known inhibitors of the dicarboxylate carrier on sulphate transport. The substrate analogue, butylmalonate, and the metal-complexing agent, bathophenanthrolinesulphonate, inhibit sulphate uptake competitively. Butylmalonate and bathophenanthrolinesulphonate are also com-

petitive inhibitors of malonate transport, but they inhibit phosphate transport by the dicarboxylate carrier in a mixed manner (Palmieri *et al.*, 1971; Passarella *et al.*, 1973). Inhibition by bathophenanthrolinesulphonate was shown to be dependent on its capacity to complex metal ions, since addition of low amounts of Fe^{2+} or Co^{2+} (less than 1 ng-ion/nmol of bathophenanthrolinesulphonate) removed the inhibition of phosphate, malonate and sulphate influx (Passarella *et al.*, 1973; M. Crompton, unpublished work).

Inhibition by the thiol reagent mersalyl is largely competitive when added simultaneously with sul-

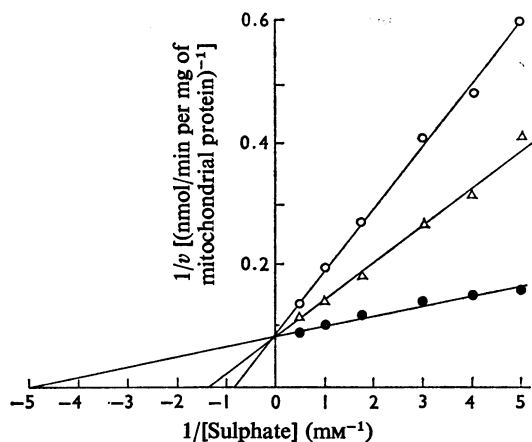


Fig. 5. Inhibition of sulphate influx by butylmalonate and bathophenanthrolinesulphonate

Phosphate-loaded mitochondria were used. Further details are given in the Experimental section. Additions: ●, none; ○, 8 μ M-butylmalonate; Δ , 50 μ M-bathophenanthrolinesulphonate.

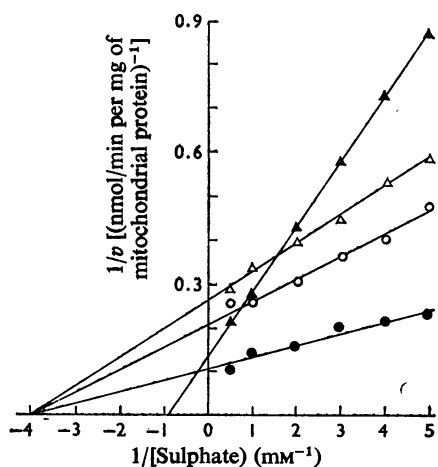


Fig. 6. Inhibition of sulphate influx by mersalyl

Phosphate-loaded mitochondria were used. Symbols: ●, no additions; ▲, 0.25 mM-mersalyl added simultaneously with sulphate; ○, 20 μ M-mersalyl added 15s before sulphate; Δ , 20 μ M-mersalyl added 30s before sulphate. Further details are given in the Experimental section.

phate (Fig. 6). However, when mitochondria are preincubated for 15s or 30s in the presence of mersalyl before the addition of sulphate, the inhibition by mersalyl becomes non-competitive. The intermediate case, in which the reaction of mersalyl with the carrier is clearly incomplete (i.e. after 15s

preincubation), presumably contains both competitive and non-competitive elements. The competitive element is not apparent, probably because the mersalyl concentration used was only one-twelfth of that used when mersalyl was added together with sulphate.

Mersalyl also inhibits competitively the transport of both malonate and phosphate by the dicarboxylate carrier when added simultaneously with these substrates to the mitochondria. However, the inhibition of malonate uptake, but not phosphate uptake, becomes non-competitive after preincubation with mersalyl (Palmieri *et al.*, 1974).

In summary, the effects of butylmalonate, bathophenanthrolinesulphonate and mersalyl on the transport of sulphate are similar in nature to their effects on malonate transport, and differ from those on phosphate transport.

Further characterization of the inhibition of sulphate transport by dicarboxylate anions, bathophenanthrolinesulphonate and mersalyl

The inhibitions of sulphate uptake by malonate, butylmalonate, bathophenanthrolinesulphonate and mersalyl were further investigated to distinguish between the completely and partially competitive types of inhibition. Reciprocal plots (Fig. 7) were made of the fractional inhibition (i) against inhibitor concentration at a fixed sulphate concentration, as advised by Webb (1963, p. 160). The fractional inhibition is defined as

$$i = 1 - \left(\frac{v_i}{v} \right)$$

where v_i and v are the rates of sulphate uptake in the presence of and absence of inhibitor [see Webb (1963) p. 57]. Completely competitive inhibitions give lines which intersect the ordinate at unity, whereas partial inhibitions produce higher intercepts.

The plots with malonate, butylmalonate, bathophenanthrolinesulphonate and mersalyl all intersect the ordinate at, or very close to, unity and it seems probable that these substances are completely competitive inhibitors of sulphate transport.

Discussion

The present results support the previous proposal that sulphate is transported by the dicarboxylate carrier of rat liver mitochondria (Crompton *et al.*, 1974a,b). It required that the influxes of sulphate, phosphate and malonate be mutually inhibitory, and this has been verified.

It seems that sulphate binds significantly only to the dicarboxylate carrier. The possible existence of different binding sites for phosphate and dicarboxylate anions on this carrier (Palmieri *et al.*, 1971, 1974; Passarella *et al.*, 1973) raises the question of the locus

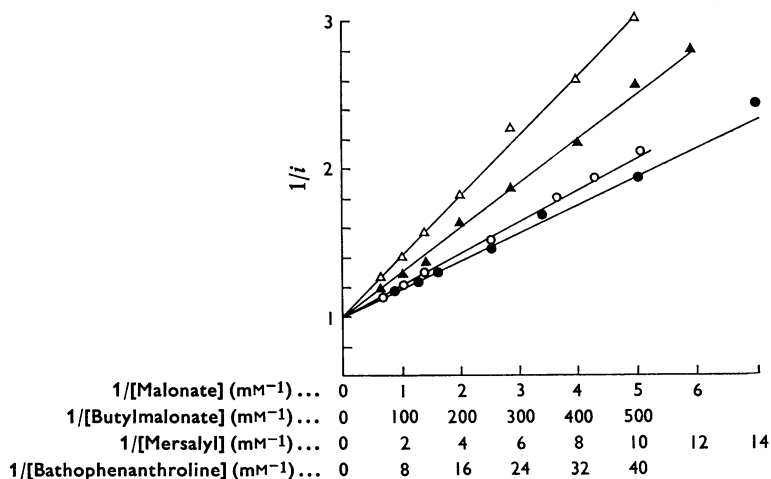


Fig. 7. Inhibition of sulphate influx by various concentrations of malonate, butylmalonate, bathophenanthroline sulphate and mersalyl

The rates of influx of 0.4mM-sulphate into phosphate-loaded mitochondria were measured. All inhibitors were added simultaneously with sulphate. The symbol i refers to the fractional inhibition (see the text). Symbols: ●, with mersalyl; ○, with bathophenanthroline sulphate; ▲, with malonate; △, with butylmalonate.

of sulphate binding. The inhibition between phosphate and sulphate is of the mixed type. Inhibition at infinite concentrations of substrate (phosphate or sulphate) implies that the binding sites for phosphate and those for sulphate may be different, so that a ternary complex, carrier-phosphate-sulphate, may be formed, which translocates at a decreased rate, or not at all, with respect to the carrier-sulphate and carrier-phosphate complexes. If this is indeed the case, the competitive element of the mixed-type inhibition suggests that the binding of sulphate and phosphate are interdependent processes in which the binding of phosphate to one site decreases the affinity of the sulphate-binding site for sulphate, and vice versa. This could result from proximity of two binding sites. As discussed later, there is evidence that the affinity of the carrier for phosphate is also decreased by the binding of mersalyl at, or near to, the sulphate-binding site.

In contrast, there is a completely competitive relation between the transport of sulphate and malonate. It is not certain whether the apparent mutually exclusive binding of sulphate and malonate results from their binding to the same site. However, a common binding site is favoured by the similarity between effects of butylmalonate, bathophenanthroline and mersalyl on sulphate and malonate transport. Butylmalonate, which is a competitive inhibitor of dicarboxylate uptake (Chappell, 1969) and presumably interacts with the dicarboxylate-binding site,

inhibits sulphate uptake completely competitively. Similarly, bathophenanthroline sulphate is a purely competitive inhibitor of uptake of both dicarboxylate (Tyler & Newton, 1970; Passarella *et al.*, 1973) and sulphate which, since the inhibition is reversed by Fe²⁺ or Co²⁺, suggests at least that both the sulphate and dicarboxylate binding sites may contain metal ions. For mersalyl, inhibition of sulphate and malonate influx is completely competitive initially, but becomes non-competitive with time. The non-competitive inactivation is evidently a slow process [inactivation is incomplete after 15 s incubation with mersalyl (Fig. 6); for inhibition of malonate uptake, see Palmieri *et al.* (1974)]. Thus when substrate and mersalyl are added together, the influxes are measured before equilibrium is reached, and a formally competitive inhibition of sulphate and dicarboxylate uptake might result if mersalyl combines with the sites occupied by these substrates, since the latter would thereby decrease the rate of mersalyl binding [see Webb (1963) p. 771]. This interpretation has been applied to the observed effects of mersalyl on phosphate and dicarboxylate transport by the dicarboxylate carrier (Palmieri *et al.*, 1974) and indeed was experimentally supported, since the rate of development of mersalyl inhibition is inhibited competitively by malonate (but is not inhibited by phosphate). Since mersalyl forms a strong complex with thiols it would be expected, once reacted, to prevent sulphate and dicarboxylate binding non-

competitively. The competitive inhibition of phosphate uptake by mersalyl, even after preincubation, has been interpreted to indicate that the binding of mersalyl decreases the affinity for phosphate by inducing a change in the conformation of the phosphate-binding site (Palmieri *et al.*, 1974). It is perhaps important to note that this hypothesis would mean that the mobility of the phosphate-carrier complex is unaffected by the binding of mersalyl, whereas it is clearly affected by the binding of sulphate and dicarboxylate anions (as evidenced by the mixed inhibition between phosphate and dicarboxylate or sulphate uptake).

In conclusion, there is evidence that thiol groups and metal ion(s) may be located at or near the binding site for *cis*-dicarboxylate anions and sulphate. Although it is premature to infer mechanisms for the binding of these anions, the data reported suggest that any such mechanism may have to account for discrimination between phosphate and sulphate, which differ minimally in structure. For example, although electrostatic interactions may well be involved in binding, the intercharge distances in SO_4^{2-} and HPO_4^{2-} are about 0.24 nm and 0.25 nm respectively [calculated assuming regular tetrahedra with the S-O and P-O bonds of length 0.149 nm and 0.154 nm respectively; from Pauling (1960)] and, therefore, would offer no possible basis for selection between the two anions; it should be noted that HPO_4^{2-} is probably the form in which phosphate is transported (Papa *et al.*, 1971).

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