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# The Transport of Sulphate and Sulphite in Rat Liver Mitochondria

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1. The mechanism of sulphite and sulphate permeation into rat liver mitochondria was investigated. 2. Extramitochondrial sulphite and sulphate elicit efflux of intramitochondrial phosphate, malate, succinate and malonate. The sulphate-dependent effluxes and the sulphite-dependent efflux of dicarboxylate anions are inhibited by butylmalonate, phenylsuccinate and mersalyl. Inhibition of the phosphate efflux produced by sulphite is caused by mersalyl alone and by *N*-ethylmaleimide and butylmalonate when present together. 3. External sulphite and sulphate cause efflux of intramitochondrial sulphate, and this is inhibited by butylmalonate, phenylsuccinate and mersalyl. 4. External sulphite and sulphate do not cause efflux of oxoglutarate or citrate. 5. Mitochondria swell when suspended in an iso-osmotic solution of ammonium sulphite; this is not inhibited by *N*-ethylmaleimide or mersalyl. 6. Low concentrations of sulphite, but not sulphate, produce mitochondrial swelling in iso-osmotic solutions of ammonium malate, succinate, malonate, sulphate, or phosphate in the presence of *N*-ethylmaleimide. 7. It is concluded that both sulphite and sulphate may be transported by the dicarboxylate carrier of rat liver mitochondria and also that sulphite may permeate by an additional mechanism; the latter may involve the permeation of sulphurous acid or $SO_2$ or an exchange of the sulphite anion for hydroxyl ion(s).

The permeability of rat kidney and liver mitochondria to sulphate has been demonstrated by Winters *et al.* (1962) and Rasmussen *et al.* (1964), although little is known of the mechanism whereby sulphate permeates. The work of Chappell & Haarhoff (1967) provided evidence that sulphate influx does not occur in exchange for hydroxyl ions or together with protons. In contrast, Mitchell & Moyle (1969) have suggested that sulphate does permeate with protons, although from the data presented it appears that the rate of permeation according to this mechanism is very slow. Winters *et al.* (1962) observed that rat kidney mitochondria are able to accumulate radioactive sulphate from the ambient medium, in excess of that which may be explained by isotope equilibration with the endogenous sulphate of the mitochondria. This may indicate that sulphate is able to exchange with endogenous metabolites in addition to sulphate. In fact Rasmussen *et al.* (1964) showed that the amounts of radioactive sulphate, phosphate and arsenate taken up by rat liver mitochondria after 20min incubation are mutually exclusive, which suggests that sulphate, phosphate and arsenate are able to exchange with the same intramitochondrial anions. Phosphate, malate and succinate are recognized counter-anions of phosphate influx (Chappell, 1968; Palmieri *et al.*, 1971). With arsenate, there is evidence that this anion may be transported by the phosphate carrier (Chappell, 1968; Tyler, 1969) and hence might be presumed to exchange with phosphate.

The permeability of mitochondria to sulphite has not been studied previously. However, sulphite is an intermediate produced in liver mitochondria during the degradation of cysteine to sulphate (Singer & Kearney, 1956). In this sequence of reactions, cysteinesulphinate transaminates with oxoglutarate or oxaloacetate to yield glutamate or aspartate, and sulphinylpyruvate, which spontaneously hydrolyses into pyruvate and sulphite (see Meister, 1965). Since the transamination is catalysed by aspartate aminotransferase (Singer & Kearney, 1955), this reaction occurs in the mitochondrial matrix (Klingenberg & Pfaff, 1966; Schnaitman & Greenawalt, 1968) and hence forms sulphite in this compartment. However, the oxidation of sulphite to sulphate, by sulphite oxidase, takes place in the intermembrane space of the mitochondria (Wattiaux-De Coninck & Wattiaux, 1971) and this implies that sulphite formed during cysteinesulphinate degradation must permeate outwards across the inner membrane to be oxidized.

The present paper reports experiments concerned with the counter-anions and carriers involved in sulphite and sulphate permeation in rat liver mitochondria. Evidence suggests that both sulphite and sulphate may be transported by the dicarboxylate carrier, and further, that there is an additional mechanism for sulphite permeation in which sulphite

movement occurs in exchange for hydroxyl ions or by a process equivalent to this.

## Experimental

### *Chemicals*

[$^{32}$P]Phosphoric acid, [$^{35}$S]sulphuric acid, [1,5-$^{14}$C]citrate, [5-$^{14}$C]oxoglutarate, [1-$^{14}$C]malonate, [1,4-$^{14}$C]succinate and [U-$^{14}$C]malate were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). *N*-Ethylmaleimide and mersalyl acid were bought from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and 2-phenylsuccinate was from K & K Laboratories (Plainview, N.Y., U.S.A.). 2-Butylmalonate was kindly supplied by Professor J. B. Chappell, University of Bristol.

Sulphuric acid and sulphurous acid were obtained from Bracco Industria Chimica (Milan, Italy) and Carlo Erba (Milan, Italy) respectively. The $pK_2$ value of sulphurous acid (estimated $pK_2$ of 1M soln. at 23°C, 6.65) is much higher than its $pK_1$ value and both $pK$ values of sulphuric acid. Hence, titration of the first dissociation of sulphurous acid (to pH3.0) also includes titration of both dissociations of any sulphuric acid present as an impurity. Accordingly, the limit of contamination of sulphurous acid by sulphuric acid was estimated from the amounts of alkali needed to titrate the two dissociations of the sulphurous acid solution. This titration indicated that the 1M-sulphurous acid used contained less than 15mM-sulphuric acid. A titration of the sulphuric acid solution did not detect the presence of any sulphurous acid.

### *Isolation of mitochondria*

Rat liver mitochondria were prepared as described by Klingenberg & Slenczka (1959) and suspended finally in 0.25M-sucrose containing 20mM-Tris–HCl and 1mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate] (approx. 50mg of mitochondrial protein/ml); final pH7.2.

### *Determination of mitochondrial protein*

Protein was measured by a modification of the biuret procedure (Kroger & Klingenberg, 1966) with bovine plasma albumin (Sigma Chemical Co.) as standard.

### *Mitochondrial swelling*

The rate of mitochondrial swelling was monitored by recording the decrease in $E_{623}$ as described by Chappell and Crofts (1966), with an Eppendorf photometer model 1101M.

### *Sulphite oxidase*

The sulphite oxidase activity of the mitochondrial preparation was measured at 10°C with an oxygen electrode in a reaction medium (1.8ml) containing 100mM-KCl, 20mM-Tris–HCl, 1mM-EGTA, 1mM-KCN and 2mg of mitochondrial protein. A stream of air was bubbled through the reaction medium for 30min at 10°C before use; the oxygen content of this solution was taken to be 0.89$\mu$g-atoms of oxygen/ml at 10°C (calculated from Umbreit *et al.*, 1964).

### *Procedure used to load mitochondria with labelled metabolites*

Mitochondria (containing 40–50mg of protein) were incubated for 2min at room temperature in 10ml of medium, pH6.8, containing 100mM-KCl, 20mM-Tris–HCl, 1mM-EGTA, 20$\mu$g of rotenone and the potassium salt of the metabolite (2mM) to be loaded. The mitochondria were washed in the medium without the metabolite and then resuspended (40–50mg of mitochondrial protein/ml). After this, the labelled metabolite (carrier-free; approx. 1$\mu$Ci/ml of mitochondrial suspension) was introduced into the mitochondrial suspension. Equilibration of the radioisotope between the extramitochondrial and intramitochondrial pools of the metabolite was obtained after 2min incubation at 8°C. Further details of the procedure are exactly as described by Palmieri *et al.* (1972*b*), with the following modifications: (*a*) 2mM-arsenite was present only when oxoglutarate was loaded; (*b*) the loading medium for phosphate contained oligomycin (25$\mu$g/ml of mitochondrial suspension). After this loading procedure, the intramitochondrial concentration of labelled metabolite varied between 7 and 20mM.

### *Measurement of the exchange between intramitochondrial labelled substrates and external anions*

Metabolite-loaded mitochondria (50$\mu$l; see above) were suspended in 1ml of medium, pH6.8, containing 100mM-KCl, 20mM-Tris–HCl, 1mM-EGTA and 1mM-KCN, and maintained at 8°C. In some reactions, the medium included the inhibitors *N*-ethylmaleimide, butylmalonate, phenylsuccinate or mersalyl as indicated in the Tables. After 2min incubation, the exchange reaction was initiated by addition of unlabelled anion (external anion) and terminated 1 or 2min later (unless otherwise specified) by centrifugation for 1min in an Eppendorf bench centrifuge (model 3200) operating at 15000rev./min. After this, the supernatant was removed as completely as possible, and the pellet was extracted with 0.3M-$HClO_4$. The radioactivity content of the extracts was determined by liquid-scintillation spectrometry in a scintillation solution prepared as follows: 1.4 litres

of toluene, 0.6 litre of monomethylethylene and 8g of PPO (2,5-diphenyloxazole).

If the radioactivity content of the mitochondrial extract in the presence and absence of external anion is represented by $C_p$ and $C_a$ respectively, then

$$\% \text{ exchange} = 100 \frac{(C_a - C_p)}{C_a}$$

Mitochondria that were loaded with phosphate and sulphate underwent a slow loss (approx. 4–9%/min) of labelled metabolite from the intramitochondrial compartment in the absence of added external anion during incubation in the reaction medium at 8°C. All experimental results have been corrected for this spontaneous leakage by conducting parallel experiments in the absence of external anion in each case. There was no detectable spontaneous efflux of malonate, oxoglutarate or citrate.

## Results

### *Mitochondrial swelling*

*Swelling in sulphite, sulphate and phosphate salts.* Fig. 1(*a*) shows that rat liver mitochondria swell rapidly in 120mM-$(NH_4)_2SO_3$. There is negligible swelling in $(NH_4)_2SO_4$, $K_2SO_4$ or $K_2SO_3$. The rate of swelling in $K_2SO_3$ is stimulated somewhat by the addition of valinomycin, which allows electrogenic permeation of $K^+$ (Henderson *et al.*, 1969) but the rate is slow in comparison with that observed in $(NH_4)_2SO_3$. The swelling in $(NH_4)_2SO_3$ is not inhibited (rather, it is increased slightly) by 2mM-*N*-ethylmaleimide or 0.1mM-mersalyl.

These results were compared with the rate of swelling of the same mitochondrial preparation when suspended in 120mM-ammonium phosphate (Fig. 1*b*). The initial rate of swelling is about 65% of that

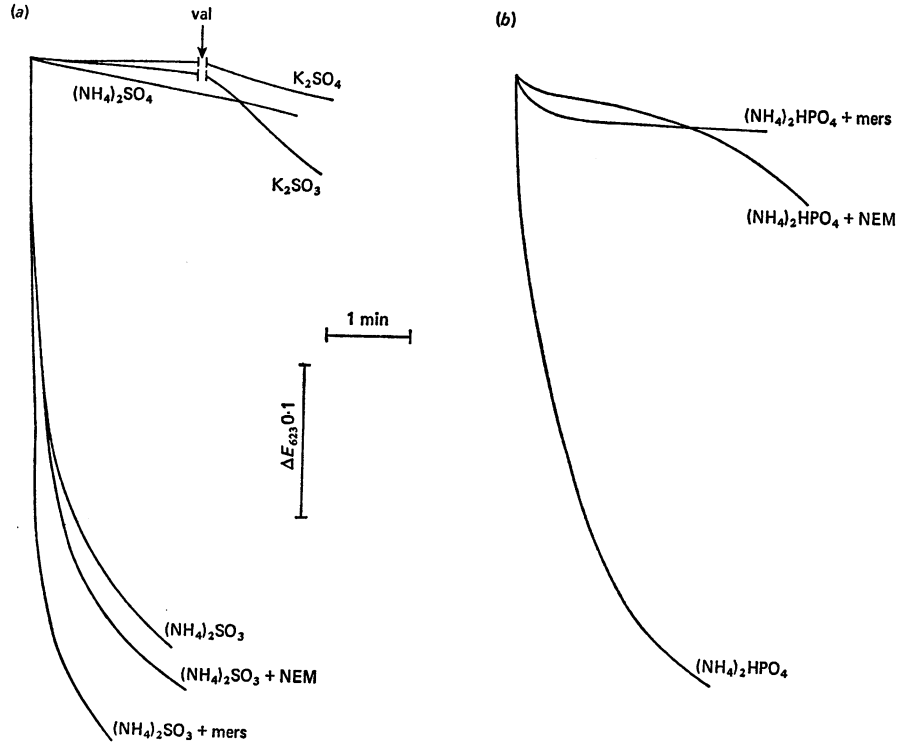



Fig. 1. *Swelling of liver mitochondria in sulphite, sulphate and phosphate salts*

The incubation media contained the ammonium or potassium salt of sulphurous, sulphuric or phosphoric acid (as indicated), concentration 120mM, and 20mM-Tris–HCl, 0.5mM-EDTA, 1mM-KCN, and 2.4mg of mitochondrial protein. Some incubations included 2mM-*N*-ethylmaleimide (NEM), 0.1mM-mersalyl (mers), or 1 $\mu$g of valinomycin (val). Final volume, 2.5ml; pH7.4; temperature, 24°C. The initial absorbance was 1.23.

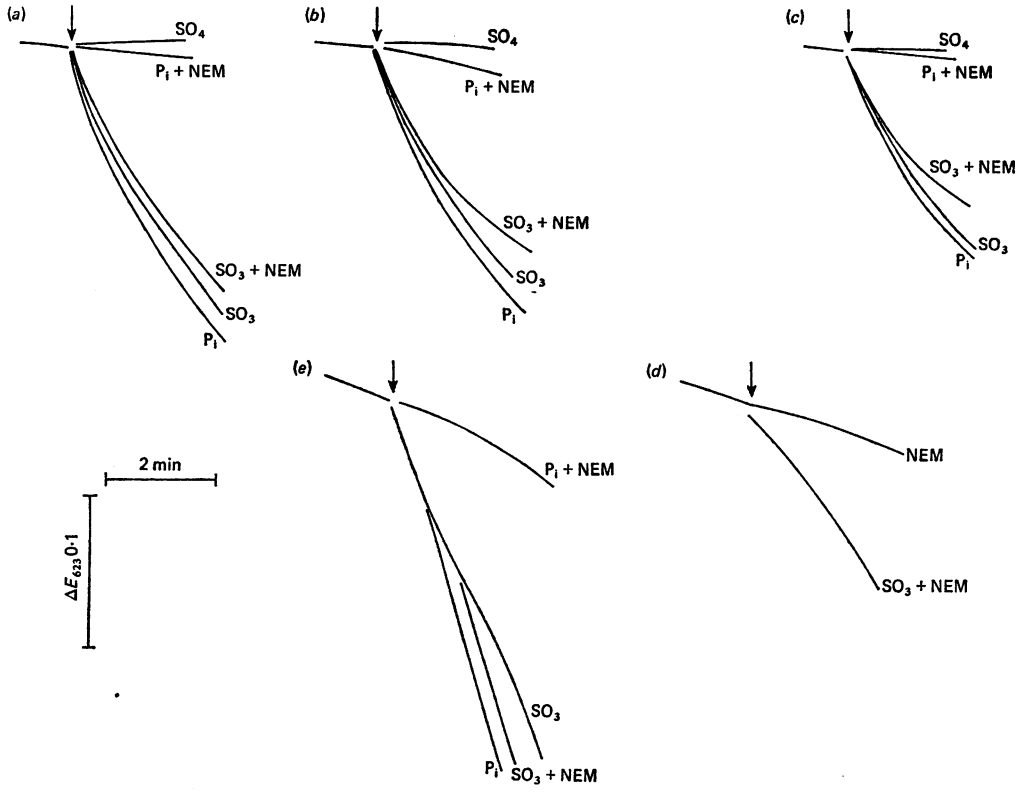



Fig. 2. *Swelling of liver mitochondria in ammonium malate, succinate, malonate, sulphate and phosphate*

The incubation media contained either 100mM-ammonium malate (*a*), succinate (*b*), malonate (*c*), phosphate (*d*) or sulphate (*e*), 20mM-Tris–HCl, 0.5mM-EDTA, 1mM-KCN, 2.5mg of mitochondrial protein and, in some cases, 2mM-*N*-ethylmaleimide (NEM); volume, 2.5ml. At the arrows, 50$\mu$l of 250mM-ammonium phosphate ($P_i$), sulphate ($SO_4$) or sulphite ($SO_3$) was added (final concentration 5mM). Temperature, 24°C; pH7.4. The initial absorbance was 1.25.

observed in $(NH_4)_2SO_3$, and is inhibited by 0.1mM-mersalyl and, temporarily, by 2mM-*N*-ethylmaleimide, which agrees with the known ability of these compounds to inhibit $P_i$-hydroxyl exchange (Fonyo & Bessman, 1968; Tyler, 1968, 1969; Meijer *et al.*, 1970). Other experiments (not shown) were carried out in which mitochondria were preincubated with 10mM-*N*-ethylmaleimide or 0.5mM-mersalyl for 2min at room temperature in the absence of phosphate or sulphite (making the final concentrations of *N*-ethylmaleimide and mersalyl 2mM- and 0.1mM respectively). However, these treatments did not affect the degree of inhibition observed with ammonium phosphate or the lack of inhibition with $(NH_4)_2SO_3$.

Since it is probable that ammonia enters mitochondria as $NH_3$ (Chappell & Crofts, 1966), the above results indicate that sulphite, but not sulphate, is able to permeate the mitochondrial membrane together with protons or in exchange for hydroxyl ions; a further possibility is that $SO_2$ is permeant. Since *N*-ethylmaleimide and mersalyl do not inhibit $(NH_4)_2SO_3$ influx, it appears that sulphite permeation is not catalysed by the phosphate carrier.

*Stimulation of swelling in various ammonium salts by sulphite, sulphate and phosphate.* Figs. 2(*a*)–2(*c*) report the swelling of rat liver mitochondria when suspended in 100mM-ammonium malate, succinate or malonate. Little swelling occurs until either 5mM-ammonium phosphate or $(NH_4)_2SO_3$, but not 5mM-$(NH_4)_2SO_4$, is added. Inclusion of 2mM-*N*-ethylmaleimide completely prevents the ability of phosphate to initiate swelling, but has little effect on the swelling elicited by sulphite. The low concentration (5mM) of ammonium phosphate or sulphite added was insufficient in itself to produce the swelling response observed; thus, when these additions were made to a suspension of mitochondria in 120mM-KCl, instead of the ammonium salt of the dicarboxylic acid there was no detectable swelling. This is also an important point

with respect to the remaining swelling experiments described below.

The stimulation of swelling by phosphate and sulphite may be explained by assuming an influx of ammonium phosphate or sulphite (as in Fig. 1), and an efflux of phosphate or sulphite in exchange for malate, succinate or malonate; the net influx would be the ammonium salt of the dicarboxylic acid. This explanation appears to be valid for phosphate, when the exchange with a dicarboxylate anion is catalysed by the dicarboxylate carrier (Chappell & Haarhoff, 1967; Chappell, 1968). Since sulphite behaves similarly, it is possible that sulphite also is a substrate for this carrier.

Although rat liver mitochondria oxidize sulphite to sulphate, it is unlikely that the stimulation of swelling by sulphite depends on its conversion into sulphate (thereby reflecting a sulphate–dicarboxylate exchange), since the enzyme catalysing this reaction, sulphite oxidase, is located in the intermembrane space of rat liver mitochondria (Wattiaux-De Coninck & Wattiaux, 1971) and would produce sulphate outside the inner mitochondrial membrane.

The prevention of phosphate-dependent swelling by *N*-ethylmaleimide is understandable, since this compound specifically inhibits the phosphate carrier (Meijer *et al.*, 1970). The sulphite-dependent swelling, however, is affected only slightly, which is in agreement with the observed insensitivity of $(NH_4)_2SO_3$ influx to *N*-ethylmaleimide (Fig. 1*a*).

If the apparent exchange between sulphite and dicarboxylate anions is catalysed by the dicarboxylate carrier, a further exchange, between sulphite and phosphate, would be predicted since phosphate is also a substrate for this carrier. Fig. 2(*d*) reports an experiment in which mitochondria were suspended in 120mM-ammonium phosphate in the presence of *N*-ethylmaleimide to inhibit $P_i$-hydroxyl exchange (as in Fig. 1*b*). The rate of mitochondrial swelling is stimulated by the addition of 5mM-$(NH_4)_2SO_3$, in accordance with the postulate that sulphite may be transported by the dicarboxylate carrier.

The inability of sulphate to elicit swelling in ammonium malate, succinate or malonate would be expected, since $(NH_4)_2SO_4$ itself is impermeant (Fig. 1*a*), and does not exclude the possibility that sulphate is able to exchange with dicarboxylate anions. Fig. 2(*e*) shows that rat liver mitochondria may be induced to swell in 120mM-$(NH_4)_2SO_4$ by the addition of either 5mM-ammonium phosphate or $(NH_4)_2SO_3$. A possible interpretation is that ammonium phosphate or sulphite enters the mitochondria, and that sulphate is able to permeate in exchange for both intramitochondrial phosphate and sulphite. As in Figs. 2(*a*)–2(*c*), the swelling initiated by sulphite is only slightly repressed by *N*-ethylmaleimide, in contrast with the marked inhibition of the phosphate-dependent swelling.

*Swelling in ammonium citrate.* The ability of sulphite and sulphate to exchange with citrate was also investigated (Fig. 3). From the control experiments it is evident that both phosphate and malate are required for swelling to occur in 80mM-ammonium citrate. In this case, the presence of phosphate allows malate to permeate (as in Fig. 2*a*) and citrate enters in exchange for intramitochondrial malate (Chappell & Haarhoff, 1967). However, no swelling takes place when malate is replaced by either sulphate (which permeates in the presence of phosphate; Fig. 2*e*) or sulphite, and this suggests that neither sulphate nor sulphite is able to exchange with citrate. Swelling does occur if phosphate is replaced by sulphite, but not by sulphate, which agrees with the previous conclusion that $(NH_4)_2SO_3$ [but not $(NH_4)_2SO_4$] permits entry of malate (Fig. 2*a*).

*Swelling in ammonium oxoglutarate.* Fig. 4 reports the ability of sulphite and sulphate to exchange with oxoglutarate. In the control experiment (Fig. 4*a*), ammonium malate enters the mitochondria in the presence of phosphate (as in Fig. 2*a*), and oxoglutarate is transported by the oxoglutarate carrier in exchange for intramitochondrial malate (Meijer & Tager, 1966; Robinson & Chappell, 1967). To demonstrate the oxoglutarate carrier by the swelling technique it was necessary to incubate the mitochondria in the presence of phosphate, malate and arsenite (which inhibits oxoglutarate dehydrogenase), and to initiate swelling by addition of a relatively low concentration of ammonium oxoglutarate. The addition of oxoglutarate produced no swelling in the absence of either phosphate, malate or arsenite.

No swelling occurs when sulphite replaces malate (Fig. 4*b*). Since $(NH_4)_2SO_3$ is permeant, this result indicates that external oxoglutarate cannot exchange with intramitochondrial sulphite. The result of substituting sulphate for malate (Fig. 4*c*) is more difficult to interpret, since a slow rate of swelling is observed in the absence of oxoglutarate. However, this rate of swelling is not increased by addition of ammonium oxoglutarate. Other experiments (not shown) have indicated that the continuous swelling in the presence of sulphate and phosphate depends on the inclusion of arsenite; the reason for this is not known.

### *Exchange between intramitochondrial labelled metabolites and extramitochondrial anions*

Mitochondria were loaded with various labelled metabolites, and the ability of externally added sulphite and sulphate to exchange with the labelled intramitochondrial metabolites was tested in the presence and absence of various inhibitors. Control experiments were conducted in which the external anion added (unlabelled) was the same as the internal labelled anion; these were done to determine the

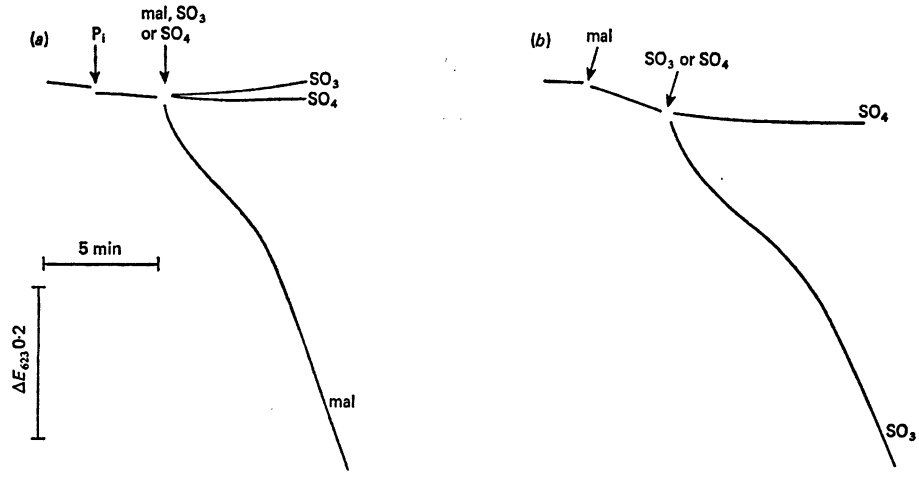



Fig. 3. *Swelling of liver mitochondria in ammonium citrate*

The incubation medium contained 80mM-ammonium citrate, 20mM-Tris–HCl, 0.5mM-EDTA, 1mM-KCN and 2.2mg of mitochondrial protein; volume, 2.5ml. At the arrows, 50$\mu$l of 250mM-ammonium phosphate ($P_i$), malate (mal), sulphite ($SO_3$), or sulphate ($SO_4$) were added (final concentration, 5mM). Temperature, 24°C; pH7.4. The initial absorbance was 1.14.

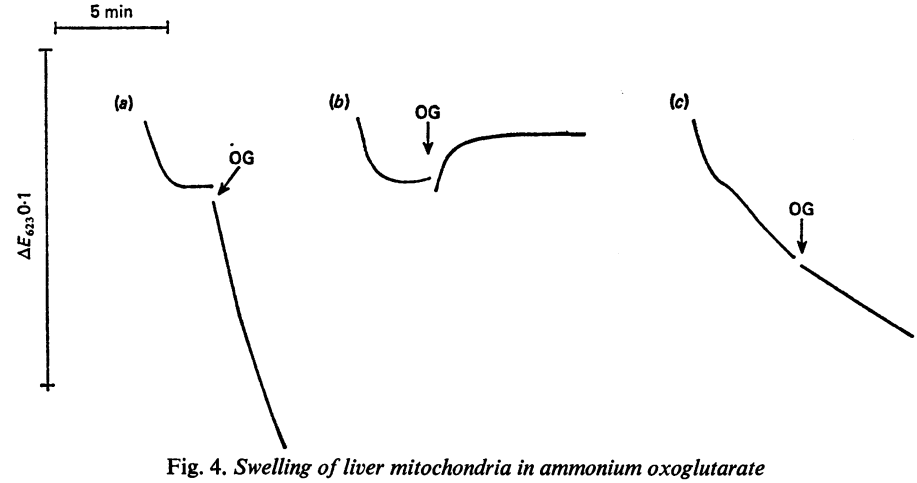



Fig. 4. *Swelling of liver mitochondria in ammonium oxoglutarate*

The incubation media contained either 10mM-ammonium malate (*a*), sulphite (*b*) or sulphate (*c*), 10mM-ammonium phosphate, 100mM-Tris–HCl, 0.5mM-EDTA, 1mM-KCN, 1mM-sodium arsenite and 2.2mg of mitochondrial protein; volume, 2.5ml. At the arrows, 100$\mu$l of 0.5M-ammonium oxoglutarate (OG) was added (final concn., 19mM). Temperature, 24°C; pH7.4. The initial absorbance was 1.14.

maximum exchange obtainable under the experimental conditions used, and to enable a comparison to be made between the effects of inhibitors on the control exchange and the effects on exchanges with other external anions.

*Efflux of intramitochondrial, dicarboxylate anions.* Table 1 reports the exchange between intramitochondrial dicarboxylate anions and external sulphite and sulphate, together with appropriate control experiments. Extramitochondrial sulphite and sulphate both exchange with internal malonate, succinate and malate, and in each case the degree of exchange after 1min is similar to that observed in the control experiments (i.e. with externally added malonate, succinate or malate).

It is possible to calculate a minimum value for the rate of exchange between sulphite and sulphate and internal dicarboxylates: the procedure for loading the

Table 1. *Exchange between intramitochondrial dicarboxylate anions and extramitochondrial anions*

Mitochondria were loaded with either malonate, succinate, or malate and incubated in the presence of different external anions (potassium salts; 2mM) and 2mM-*N*-ethylmaleimide for 1 or 2min at 8°C (see the Experimental section). Additions: butylmalonate (20mM) phenylsuccinate (30mM) and mersalyl (0.1mM). Duplicate values are reported. A negative value means that the efflux in the presence of external anions was less than in the control.

| Intramitochondrial anion | External anion | Inhibitor | % exchange after: 1min | 2min |
|---|---|---|---|---|
| Malonate | Malonate | — | 83, 84 | 83, 85 |
| Malonate | Malonate | Phenylsuccinate | 44, 46 | 61, 65 |
| Malonate | Malonate | Butylmalonate | −6, −2 | 28, 36 |
| Malonate | Malonate | Mersalyl | 38, 32 | 56, 61 |
| Malonate | Sulphite | — | 80, 86 | 85, 85 |
| Malonate | Sulphite | Phenylsuccinate | 44, 50, | 62 65 |
| Malonate | Sulphite | Butylmalonate | −5, −1 | 13, 15 |
| Malonate | Sulphite | Mersalyl | 3, −1 | 4, −6 |
| Malonate | Sulphate | — | 73, 77 | 87, 89 |
| Malonate | Sulphate | Phenylsuccinate | 35, 36 | 44, 48 |
| Malonate | Sulphate | Butylmalonate | 0, −4 | 3, 3 |
| Malonate | Sulphate | Mersalyl | 1, −1 | −5, 1 |
| Succinate | Succinate | — | 85, 85 | |
| Succinate | Succinate | Phenylsuccinate | 23, 21 | |
| Succinate | Succinate | Butylmalonate | 6, 10 | |
| Succinate | Succinate | Mersalyl | 58, 61 | |
| Succinate | Sulphite | — | 79, 80 | |
| Succinate | Sulphite | Phenylsuccinate | 20, 20 | |
| Succinate | Sulphite | Butylmalonate | 3, 0 | |
| Succinate | Sulphite | Mersalyl | 0, 2 | |
| Succinate | Sulphate | — | 78, 79 | |
| Succinate | Sulphate | Phenylsuccinate | 19, 21 | |
| Succinate | Sulphate | Butylmalonate | 6, 5 | |
| Succinate | Sulphate | Mersalyl | 1, 1 | |
| Malate | Malate | — | 84, 80 | |
| Malate | Malate | Phenylsuccinate | 81, 80 | |
| Malate | Malate | Butylmalonate | 79, 80 | |
| Malate | Malate | Mersalyl | 80, 82 | |
| Malate | Sulphite | — | 76, 77 | |
| Malate | Sulphite | Phenylsuccinate | 29, 23 | |
| Malate | Sulphite | Butylmalonate | −5, −3 | |
| Malate | Sulphite | Mersalyl | 3, 0 | |
| Malate | Sulphate | — | 85, 87 | |
| Malate | Sulphate | Phenylsuccinate | 39, 33 | |
| Malate | Sulphate | Butylmalonate | 0, 1 | |
| Malate | Sulphate | Mersalyl | −4, 4 | |

mitochondria with dicarboxylate anions produced an internal dicarboxylate concentration of at least 7nmol/mg of mitochondrial protein, and approx. 85% of the internal dicarboxylate exchanged within 1min. It follows that the rates of sulphite–dicarboxylate and sulphate–dicarboxylate exchange are not less than 6nmol/mg of mitochondrial protein per min at 8°C.

Malonate, succinate and malate are all transported by both the dicarboxylate and oxoglutarate carriers whereas malate, but not succinate or malonate, is also a substrate for the tricarboxylate carrier (Meijer & Tager, 1966; Chappell & Haarhoff, 1967; Robinson & Chappell, 1967; Palmieri *et al.*, 1971, 1972*a*,*b*). An attempt was made, by using the inhibitor mersalyl, to clarify the contribution of these carriers to the exchange reactions between internal dicarboxylate anions and external sulphite and sulphate. Mersalyl was used at a concentration (0.1mM; 40–50nmol/mg of mitochondrial protein) in excess of that required for complete inhibition of the dicarboxylate carrier [20–25nmol/mg (Meijer *et al.*, 1970); confirmed in our laboratory], but which inhibits only slightly the oxoglutarate carrier (Quagliariello & Palmieri, 1971), and the tricarboxylate carrier (Palmieri *et al.*, 1972*a*). In accordance with these considerations, Table 1 shows that the malonate–malonate and succinate–succinate exchanges occur in the presence of 0.1mM-mersalyl, albeit at a lower rate, which can be attributed to the activity of the oxogluratate carrier; malate–malate exchange is not inhibited by 0.1mM-mersalyl, since under these conditions exchange is catalysed by the tricarboxylate carrier, in addition to the oxoglutarate carrier. However, there is no significant exchange with external sulphite or sulphate in the presence of 0.1mM-mersalyl, which suggests that the exchanges between sulphite and sulphate and internal malonate, succinate and malate are catalysed by the dicarboxylate carrier, and not by the oxoglutarate or citrate carriers.

The malonate–malonate and succinate–succinate exchanges are inhibited by phenylsuccinate and butylmalonate, whereas the malate–malate exchange is not affected (Table 1). This is understandable, since these compounds are good inhibitors of both the dicarboxylate and oxoglutarate carriers (Robinson & Chappell, 1967; Palmieri *et al.*, 1971, 1972*b*), but are much less effective against (i.e. have much lower affinities for) the tricarboxylate carrier (Palmieri *et al.*, 1972*a*). The inhibition by phenylsuccinate and butylmalonate of sulphite and sulphate exchange with all three dicarboxylate anions provides additional evidence against the involvement of the tricarboxylate carrier in sulphite and sulphate transport.

*Efflux of intramitochondrial citrate and oxoglutarate.* The ability of the tricarboxylate and oxoglutarate carriers to transport sulphite and sulphate was tested directly. Table 2 shows that the exchange between intramitochondrial citrate and external sulphite and sulphate is extremely small in comparison with the degree of exchange in the control experiments using external citrate and malate. In the other experiments reported in Table 2, intramitochondrial oxoglutarate exchanges to a considerable degree with both oxoglutarate and malonate, two known substrates of the oxoglutarate carrier. In contrast, the efflux of

Table 2. *Exchange between intramitochondrial citrate and oxoglutarate and extramitochondrial anions*

Mitochondria were loaded with either citrate or oxoglutarate and incubated in the presence of different external anions (potassium salts; 2mM) for 1 or 2min at 8°C (see the Experimental section). Duplicate values are reported.

| Intramitochondrial anion | External anion | % exchange after: 1min | 2min |
|---|---|---|---|
| Citrate | Citrate | 56, 50 | 71, 73 |
| Citrate | Malate | 54, 55 | |
| Citrate | Sulphite | 0, 1 | 2, 3 |
| Citrate | Sulphate | 2, 2 | 3, 4 |
| Oxoglutarate | Oxoglutarate | 83, 82 | 81, 85 |
| Oxoglutarate | Malonate | 69, 70 | |
| Oxoglutarate | Sulphite | 2, 2 | 9, 15 |
| Oxoglutarate | Sulphate | 6, 8 | 10, 14 |

Table 3. *Exchange between intramitochondrial phosphate and extramitochondrial anions*

Mitochondria were loaded with phosphate and incubated in the presence of different external anions (potassium salts; 2mM) for 1 min at 8°C (see the Experimental section). Additions: *N*-ethylmaleimide (2mM), butylmalonate (20mM), phenylsuccinate (30mM), and mersalyl (0.1mM). Duplicate values are reported.

| External anion | Inhibitor | % exchange after 1min |
|---|---|---|
| Phosphate | — | 75, 74 |
| Phosphate | Phenylsuccinate | 72, 73 |
| Phosphate | Butylmalonate | 65, 66 |
| Phosphate | Mersalyl | 7, 5 |
| Phosphate | *N*-ethylmaleimide | 68, 74 |
| Phosphate | *N*-ethylmaleimide + butylmalonate | 8, 10 |
| Sulphite | — | 70, 66 |
| Sulphite | Phenylsuccinate | 65, 68 |
| Sulphite | Butylmalonate | 63, 64 |
| Sulphite | Mersalyl | 9, 12 |
| Sulphite | *N*-ethylmaleimide | 61, 63 |
| Sulphite | *N*-ethylmaleimide + butylmalonate | 6, 7 |
| Sulphate | — | 70, 70 |
| Sulphate | Phenylsuccinate | 26, 28 |
| Sulphate | Butylmalonate | 6, 9 |
| Sulphate | Mersalyl | 5, 9 |
| Sulphate | *N*-ethylmaleimide | 70, 79 |
| Sulphate | *N*-ethylmaleimide + butylmalonate | 5, 1 |

internal oxoglutarate was very slow when external sulphite and sulphate were used.

*Efflux of intramitochondrial phosphate.* Table 3 reports data about the exchange between intramitochondrial phosphate and external sulphite and sulphate. Two carriers, i.e. the phosphate and dicarboxylate carriers (Chappell, 1968), are known to catalyse the transport of phosphate in rat liver mitochondria; this may be resolved by the use of specific inhibitors. The transport of phosphate by the phosphate carrier, but not by the dicarboxylate carrier is inhibited by 2mM-*N*-ethylmaleimide (Meijer *et al.*, 1970), whereas phenylsuccinate and butylmalonate inhibit only the phosphate transport catalysed by the dicarboxylate carrier (Robinson & Chappell, 1967; Meijer & Tager, 1969; Palmieri *et al.*, 1971). Mersalyl, however, inhibits the transport of phosphate by both carriers (Meijer *et al.*, 1970). Thus Table 3 shows that phosphate–phosphate exchange is inhibited by mersalyl and by *N*-ethylmaleimide plus butylmalonate, but is not inhibited by either *N*-ethylmaleimide, butylmalonate or phenylsuccinate when these compounds are present singly.

Both sulphate and sulphite exchange with intramitochondrial phosphate (Table 3). In the case of sulphate, the exchange is inhibited by butylmalonate, phenylsuccinate and mersalyl, but not by *N*-ethylmaleimide, which indicates that sulphate–phosphate exchange is catalysed only by the dicarboxylate carrier. In contrast, sulphite–phosphate exchange is inhibited only slightly by butylmalonate and phenylsuccinate, although strong inhibition is observed with mersalyl and when butylmalonate and *N*-ethylmaleimide are included together. From this it appears that both the phosphate and dicarboxylate carriers must be inhibited in order to inhibit sulphite–phosphate exchange, which indicates an involvement of both carriers in this exchange. However, the suggestion that sulphite is transported by the phosphate carrier during sulphite–phosphate exchange may be only specious, since sulphite–phosphate exchange, sensitive to inhibition by *N*-ethylmaleimide, would be predicted if sulphite permeation was in exchange for hydroxyl ions (either directly or effectively, e.g. permeation of sulphurous acid or $SO_2$), but not catalysed by the phosphate carrier. In this case, a countermovement of phosphate and sulphite would occur, mediated by the movement of hydroxyl ions or protons across the mitochondrial membrane, and the inhibition of the phosphate–sulphite exchange by *N*-ethylmaleimide would be conferred by the known sensitivity of the phosphate–hydroxyl exchange to this inhibitor.

*Efflux of intramitochondrial sulphate.* The ability of intramitochondrial sulphate to exchange with sulphite and certain known substrates of the phosphate, dicarboxylate, oxoglutarate and citrate carriers of rat liver mitochondria is presented in Table 4. In the control experiment, 75% of the intramitochondrial sulphate exchanged with external sulphate within 1min. A similar exchange was observed with external phosphate, malate, malonate, succinate and sulphite.

Table 4. *Exchange between intramitochondrial sulphate and extramitochondrial anions*

Mitochondria were loaded with sulphate and incubated in the presence of different external anions (potassium salts; 2mM) for 1 or 2min at 8°C (see the Experimental section). Additions: *N*-ethylmaleimide (2mM), butylmalonate (20mM), phenylsuccinate (30mM) and mersalyl (0.1mM). Duplicate values are reported. A negative value indicates that the efflux in the presence of the external anion was less than in the control.

| External anion | Inhibitor | % exchange after: 1min | 2min |
|---|---|---|---|
| Sulphate | — | 75, 75 | |
| Sulphate | Phenylsuccinate | 19, 25 | |
| Sulphate | Butylmalonate | 0, −2 | |
| Sulphate | Mersalyl | 5, 7 | |
| Sulphate | *N*-ethylmaleimide | 73, 70 | |
| Sulphite | — | 76, 73 | |
| Sulphite | Phenylsuccinate | 22, 23 | |
| Sulphite | Butylmalonate | −1, −2 | |
| Sulphite | Mersalyl | 8, 4 | |
| Sulphite | *N*-ethylmaleimide | 65, 67 | |
| Phosphate | — | 79, 84 | 88, 89 |
| Phosphate | Phenylsuccinate | 33, 30 | 54, 50 |
| Phosphate | Butylmalonate | 0, 2 | 2, 6 |
| Phosphate | Mersalyl | 8, 7 | 7, 10 |
| Phosphate | *N*-ethylmaleimide | 81, 79 | 90, 89 |
| Malonate | — | 80, 82 | 89, 88 |
| Malonate | Phenylsuccinate | 57, 54 | 72, 75 |
| Malonate | Butylmalonate | 2, 3 | 5, 13 |
| Malonate | Mersalyl | 12, 6 | 10, 15 |
| Succinate | — | 74, 78 | 89, 90 |
| Succinate | Phenylsuccinate | 47, 43 | 65, 60 |
| Succinate | Butylmalonate | 3, 1 | 5, 0 |
| Succinate | Mersalyl | 1, 9 | 3, 8 |
| Malate | — | 82, 83 | 87, 87 |
| Malate | Phenylsuccinate | 50, 44 | 75, 69 |
| Malate | Butylmalonate | 2, 3 | 12, 8 |
| Malate | Mersalyl | 8, 5 | 13, 16 |
| Oxoglutarate | — | 6, 8 | 3, 7 |
| Citrate | — | 4, 6 | 0, 7 |

The simplest interpretation of these data is that the exchange of sulphate for external sulphate, phosphate and dicarboxylate anions is catalysed by the dicarboxylate carrier; this view receives support from the observed inhibition of the exchange by butylmalonate, phenylsuccinate and mersalyl, all of which are known inhibitors of the dicarboxylate carrier. Since the exchange of sulphate for external sulphite is also sensitive to these three inhibitors, an additional capacity of the dicarboxylate carrier to transport sulphite is implied.

It should be emphasized that the inhibition of sulphate efflux in exchange for external phosphate and sulphate by butylmalonate and phenylsuccinate rules against the involvement of the phosphate carrier in sulphate transport.

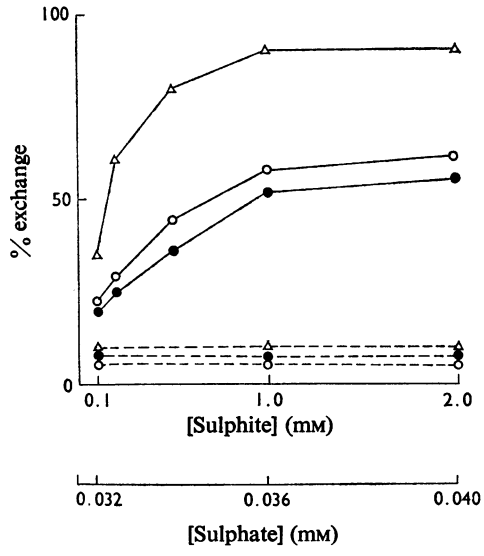



Fig. 5. *Contribution of sulphate, formed from sulphite, to the exchange between external sulphite and intramitochondrial anions*

Mitochondria were loaded with either malonate, phosphate or sulphate (see the Experimental section) and incubated with different concentrations of sulphite and sulphate at 10°C (see the text). Each concentration of sulphate used represents the maximum amount of sulphate formed (during exchange) from the corresponding concentration of sulphite stated on the abscissa. Symbols: △, intramitochondrial malonate; ○, intramitochondrial phosphate; ●, intramitochondrial sulphate. The solid lines refer to experiments using external sulphite and the dotted lines to experiments with external sulphate.

Very little exchange was detected between sulphate and external oxoglutarate and citrate, which indicates that the oxoglutarate and citrate carriers are not able to transport sulphate.

*Formation of sulphate during exchanges involving external sulphite.* The observed efflux of intramitochondrial dicarboxylate anions, phosphate and sulphate (Tables 1, 3 and 4) produced by addition of sulphite and sulphate has been interpreted to indicate that both sulphite and sulphate are substrates of the dicarboxylate carrier. However, during the exchange reactions involving sulphite, some formation of extramitochondrial sulphate would occur, owing to the activity of sulphite oxidase (Wattiaux-De Coninck & Wattiaux, 1971; Cohen *et al.*, 1972). Hence it is not clear from these experiments whether the efflux of the loaded metabolite occurs in exchange for the sulphite added, or for the sulphate produced by oxidation of sulphite. This problem was approached as follows.

Measurements were made of the rate of sulphite oxidation by rat liver mitochondria suspended in the same medium as used for the exchange experiments and at 10°C. The amounts of sulphite oxidized during a period of 70s immediately after the commencement of the reaction by addition of sulphite were: 32nmol/2mg of mitochondrial protein with 0.1mM-sulphite, 36nmol/2mg with 1mM-sulphite, and 40nmol/2mg with 2mM-sulphite.

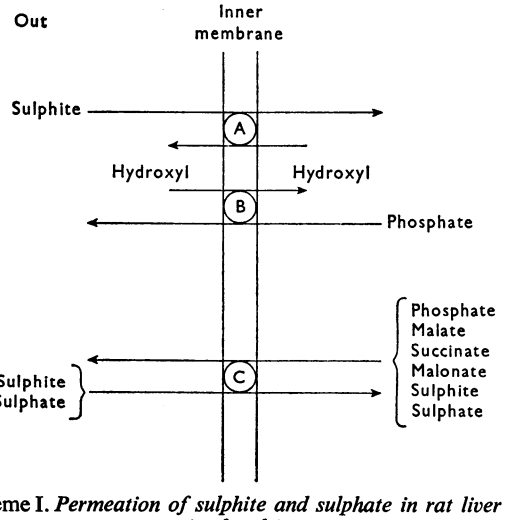
The degree of exchange was determined between intramitochondrial sulphate, phosphate and malonate, and 0.1–2.0mM-external sulphite.

In these experiments the exchange reactions were allowed to continue for 10s (instead of the usual 1 or 2min), after which the mitochondria (2mg) were sedimented by centrifugation for 1min (see the Experimental section). The maximum possible amounts of sulphate formed during the exchanges were 32–40nmol, and other exchange experiments were performed in this range of external sulphate concentrations.

The results (Fig. 5) show that the degrees of exchange between external sulphite and internal sulphate, phosphate and malonate are considerably greater than those with external sulphate. This strongly suggests that the exchanges observed with externally added sulphite can be due only very slightly to the sulphate formed during the exchange reactions.

## Discussion

The proposed mechanisms of sulphite and sulphate permeation into rat liver mitochondria are presented in Scheme 1; this is arranged to show sulphite and sulphate influx, although the reverse processes may also occur. The evidence for these processes is summarized below.




Scheme I. *Permeation of sulphite and sulphate in rat liver mitochondria*

Symbols: A, sulphite–hydroxyl exchange; B, the phosphate carrier; C, the dicarboxylate carrier.

Swelling experiments (Figs. 1*a*, 2*a*–2*c*) indicate that sulphite is able to permeate the inner membrane in exchange for hydroxyl ion(s) or by a process equivalent to this, i.e. sulphurous acid or $SO_2$ permeation (in Scheme 1, the permeation of sulphite is represented as an exchange for hydroxyl for the sake of convenience and does not imply that the mechanism involves a direct exchange between sulphite and hydroxyl ions). This conclusion is supported by the observation that addition of sulphite causes phosphate efflux from mitochondria in the presence of butylmalonate (Table 3). Since under these conditions phosphate transport is catalysed only by the phosphate carrier it may be assumed that sulphite exchanges either with phosphate directly or with hydroxyl ions (or the equivalent of this; see above) which then re-enter in exchange for phosphate. The latter possibility seems to be more likely, since the influx of $(NH_4)_2SO_3$ is not inhibited by *N*-ethylmaleimide (Figs. 1*a*, 2*a*–2*c*), a recognized inhibitor of the phosphate carrier. This implies that sulphite permeation does not involve the phosphate carrier. In fact, it must be emphasized that the present results provide no evidence that a carrier is involved in penetration of $(NH_4)_2SO_3$ into rat liver mitochondria; further work is needed to answer this.

However, sulphite and also sulphate, are able to exchange with phosphate (in the presence of *N*-ethylmaleimide) and the dicarboxylate anions which are known substrates of the dicarboxylate carrier (for sulphite, Figs. 2*a*–2*d*, Tables 1 and 3; for sulphate, Fig. 2*e*, Tables 1, 3 and 4), and all these exchanges are inhibited by known inhibitors of the dicarboxylate carrier, i.e. mersalyl, butylmalonate and phenylsuccinate. These inhibitors are also effective against the exchange of sulphate for sulphate and sulphite (Table 4). This suggests a previously unknown capacity of the dicarboxylate carrier to transport both sulphite and sulphate.

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