Biochimica et Biophysica Acta, 333 (1974) 195–208

BBA 46687

MECHANISM OF INHIBITION OF THE DICARBOXYLATE CARRIER OF MITOCHONDRIA BY THIOL REAGENTS

FERDINANDO PALMIERI, SALVATORE PASSARELLA, ITALO STIPANI and ERNESTO QUAGLIARIELLO

Department of Biochemistry and Centro di Studio sui Mitocondri e Metabolismo Energetico del CNR, University of Bari (Italy)

(Received August 21st, 1973)

SUMMARY

The nature of the inhibition of the dicarboxylate carrier by compounds reacting with SH groups has been investigated.

1. Mersalyl and *p*-hydroxymercuribenzoate increase the K_m without changing the V of malonate/P_i exchange, when they are added simultaneously with the dicarboxylate. If, on the other hand, the mitochondria are preincubated with SH reagents prior to the addition of malonate, the mersalyl inhibition of malonate/P_i exchange becomes predominantly non-competitive with respect to malonate.

2. In the case of P_i/P_i exchange, catalyzed by the dicarboxylate carrier, the mersalyl inhibition is competitive with respect to P_i (as indicated by Lineweaver–Burk plots), even when mersalyl is added before the substrate. Dixon plots of the rate of P_i uptake against mersalyl concentration are, however, non-linear, suggesting that the inhibition is partially competitive.

3. Dicarboxylates and dicarboxylate analogous protect against SH reagent inhibition of both dicarboxylate and P_i uptake via the dicarboxylate carrier. The protectors are effective when added before, or together with the SH reagents, but do not reverse the inhibition once it has been established. Protection by substrate analogues progressively decreases, as the time of incubation with the SH reagent increases.

4. The presence of P_i does not protect against the SH reagent inhibition of the P_i uptake.

5. The rate of SH reagent inhibition of the dicarboxylate carrier is competively inhibited by dicarboxylates.

6. It is concluded that SH reagents bind at or near the dicarboxylate specific binding site and distant from the P_i binding site. As a result of this reaction these inhibitors prevent dicarboxylate binding directly and decrease the affinity for P_i by an indirect conformational change.

Abbreviations: PHMB, *p*-hydroxymercuribenzoate; FCCP, carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate.

INTRODUCTION

Among the mitochondrial transport systems for anionic substrates, the P_i and the dicarboxylate carriers have been shown to be inhibited by low concentrations of SH-blocking reagents e.g. mersalyl and *p*-hydroxymercuribenzoate (PHMB) [1–7]. These results provide good evidence that these transport processes are mediated by polypeptide components of the membrane, containing SH groups essential for their activity. However, nothing is known about the role of these SH groups in the catalytic mechanism. One experimental approach to this question is to examine the relationship between the binding of the SH reagents and the substrates to the carrier molecules.

This paper investigates the nature of the inhibition of the dicarboxylate carrier by the SH reagents mersalyl and PHMB and the degree of protection against this inhibition, which is shown to be afforded by substrate analogues. The results are discussed in terms of the possible location of carrier SH groups relative to the substrate-binding sites. Preliminary accounts of part of this work have been communicated [8, 9].

MATERIALS AND METHODS

Materials

[1-^{14}C]Malonic acid (sodium salt), [^{32}P]phosphoric acid, [U-^{14}C]sucrose and ^3H_2O were obtained from the Radiochemical Center (Amersham, Great Britain), rotenone from F. P. Penick and Co. (New York); antimycin, oligomycin, *N*-ethylmaleimide and mersalyl [*o*-(3-hydroxymercuri-2-methoxypropyl)-carbamoylphenoxyacetate] from Sigma, *p*-hydroxymercuribenzoate (PHMB) from E. G. A. (Albuch, Germany), 2-phenylsuccinic acid from K and K Laboratories Inc. (Plainview, New York). 2-Butylmalonate was kindly supplied by Dr J. D. McGivan.

Preloading of the mitochondria

Rat liver mitochondria were isolated as previously described [10], using a medium consisting of 0.25 M sucrose, 1 mM EGTA and 20 mM Tris–HCl, pH 7.2. The technique for loading the mitochondria with P_i was similar to that described for malate [11], except that the mitochondria were preincubated in the presence of 0.5 mM P_i plus 5 μg/ml oligomycin, instead of malate. After equilibrium was reached (1 min) 1.5 mM *N*-ethylmaleimide was added in order to inhibit the P_i carrier without affecting the dicarboxylate transporting system [6, 7, 12].

In some experiments the mitochondria were loaded with malonate by incubating 40–50 mg (protein) mitochondria at 20 °C in 10 ml medium consisting of 100 mM KCl, 20 mM Tris–HCl, 1 mM EGTA, pH 6.4, in the presence of 1 μg/ml rotenone and 2 mM malonate. After 2 min, the volume was made up to 50 ml with ice-cold KCl–EGTA–Tris buffer, pH 6.4, and the mitochondria separated by centrifugation at 8000 × *g* for 10 min at 0 °C. The adherent supernatant was decanted off as much as possible and the mitochondria suspended in KCl–EGTA–Tris buffer, pH 6.8. The above loading procedures produce the following intramitochondrial concentrations: about 15 mM malonate and about 20 mM P_i.

Measurement of the rate of substrate uptake

The kinetics of substrate uptake were studied by using the inhibitor stop

method, essentially according to the procedure described previously [12–14]. Mitochondria were incubated in 1.0 ml medium under the conditions specified in the legends. The assay was started by addition of the labelled substrate, and terminated 4 s later, by rapid addition of an inhibitor. 20 mM butylmalonate or phenylsuccinate were used to inhibit the dicarboxylate carrier [15] ([^{14}C]malonate or inorganic $^{32}P_i$ transport in the presence of *N*-ethylmaleimide) and 1 mM mersalyl was used to inhibit the P_i carrier (inorganic $^{32}P_i$ transport in the presence of 10 mM phenylsuccinate) (Loebell, A., McGivan, J. D. and Klingenberg, M., unpublished results). The rates of substrates uptake were evaluated from the amount of labelled substrate taken up in 4 s. During this period the rate of uptake was constant within the limits of experimental error.

The SH reagents mersalyl and PHMB were added to the mitochondrial suspension simultaneously or before the labelled substrate. In the first condition, the inhibitors were let to incubate with the mitochondria only for the short interval of time in which the assay was performed and, consequently, higher concentrations of inhibitor were required, i.e. higher than those normally used to inhibit fully the dicarboxylate or P_i transport [1–4]. The addition of the SH reagents before the substrate allowed to use lower concentrations of inhibitors and to modulate the degree of inhibition by changing the time of interaction of mersalyl and PHMB with the mitochondria. In the legends to the figures and tables it is specified at what time after the addition of the SH reagents the assay was performed. In some cases the time period of the assay (4 s) during which mersalyl is able to interact further with the carrier represented a significant fraction of the total period over which the mitochondria were exposed to mersalyl (e.g. 14 s and 24 s in the experiment of Fig. 2). However, the difficulties in interpreting the data imposed by this problem would appear to be minimal, since the character of the inhibition was the same in assays using either a 10-s or 20-s preincubation period with mersalyl in the absence of substrate.

Other methods

Following termination of substrate uptake, the mitochondria were immediately centrifuged in an Eppendorf microcentrifuge for 1 min at 0 °C and ^{14}C and ^{3}H radioactivity in the pellets and supernatants was measured as described previously [16]. The O_2 uptake was measured polarographically with a Clark electrode. The mitochondrial protein was determined by a modified biuret method [17].

RESULTS

Dependence on substrate concentration of the SH reagent inhibition of the dicarboxylate carrier

In Fig. 1 the mersalyl inhibition of the rate of both malonate/P_i and P_i/P_i exchange via the dicarboxylate carrier was analysed as a Lineweaver–Burk plot. In these experiments *N*-ethylmaleimide was present to inhibit P_i exchange via the P_i carrier specifically [6, 7, 12]. With both substrates, the degree of mersalyl inhibition decreases with increasing the substrate concentration, i.e. mersalyl increases the K_m without changing the V of both malonate and P_i uptake. Similar results were also found using PHMB instead of mersalyl (not shown). In these experiments, mersalyl and PHMB were added simultaneously with the labelled substrate and left to incubate

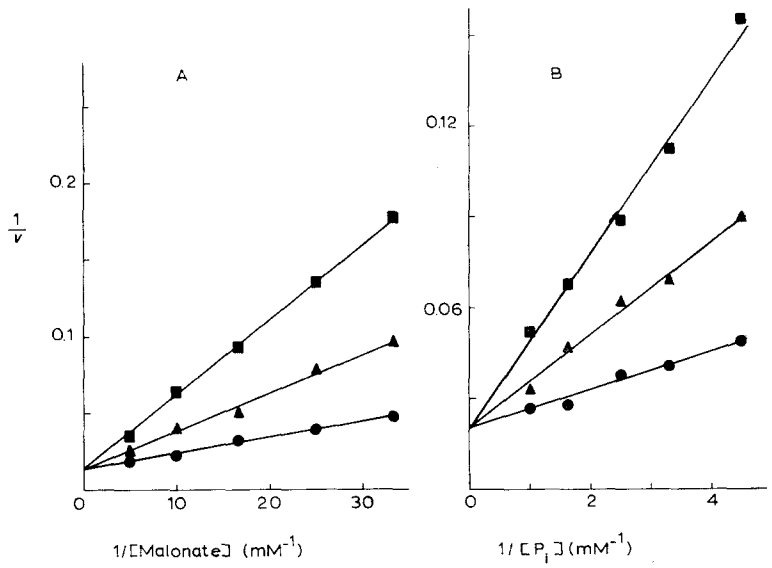

Fig. 1. Kinetic analysis of the inhibition of malonate/P_i and P_i/P_i exchanges by mersalyl, added simultaneously with the substrate. P_i-loaded mitochondria (2.0 mg protein in A and 2.5 mg in B) were incubated in a medium containing 0.2 M sucrose, 20 mM HEPES–Tris, pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, 1 μg rotenone, 3 μg oligomycin and 1 mM *N*-ethylmaleimide. After 1 min incubation the assay was started with [^{14}C]malonate in A (●) or $^{32}P_i$ in B (●) at the concentrations indicated. Where present, 100 nmoles (▲) or 300 nmoles (■) mersalyl in A, and 50 nmoles (▲) or 100 nmoles (■) mersalyl in B were added simultaneously with the labelled substrate. The assay was stopped after 4 s by the addition of 20 mM butylmalonate. *V* is expressed as μmoles · min^{-1} · g protein^{-1}. Temperature was 9 °C. Other conditions as indicated in Materials and Methods.

with the mitochondria only for the short interval of time (4 s) in which the kinetics of substrate uptake were measured.

As shown in Fig. 2, different results were obtained when mersalyl was added to the mitochondrial suspension before the addition of the substrate. In this case, the mersalyl inhibition of [^{14}C]malonate/P_i exchange is not reversed by raising the substrate concentration. The inhibition of the $^{32}P_i/P_i$ exchange, however, is decreased with increasing P_i concentration and is zero at infinite concentration. Similar effects were observed with PHMB (not shown).

The Lineweaver–Burk plots presented in Figs 1 and 2 show that the SH reagent inhibition of the uptake of P_i on the dicarboxylate carrier is competitive with the substrate. The marked differences between the Lineweaver–Burk plots in completely competitive and non-competitive types of inhibition make then easy to distinguish. However, a problem arises if the inhibition of P_i uptake by SH reagents is partially competitive, i.e. the inhibitor does not compete directly with the substrate for the same binding site, but rather decreases the affinity of P_i for the carrier as a result of binding at a different site. One can approach this problem by performing experiments in which the SH reagent concentration is varied at fixed substrate concentration and plotting the results according to the method of Dixon [18]. The plots obtained with either mersalyl added before (Fig. 3A) or simultaneously (Fig. 3B) with P_i are non-linear (in contrast to the linear relationship expected in the completely

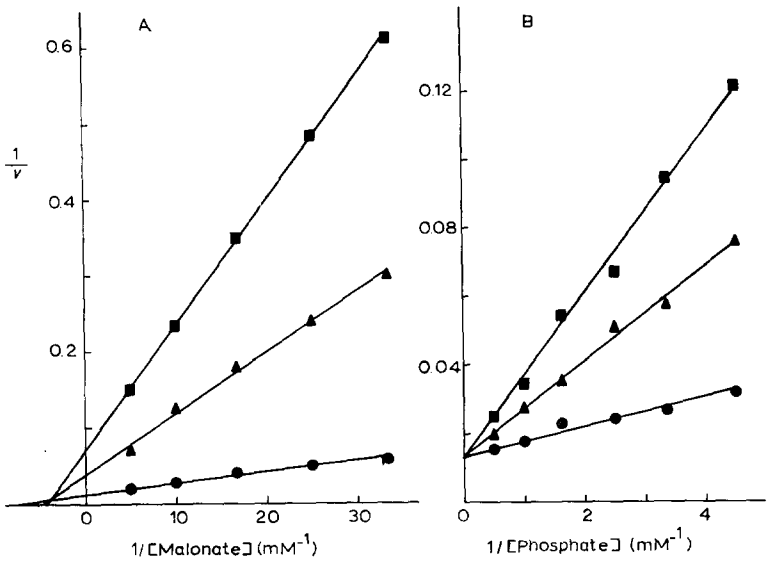

Fig. 2. Kinetic analysis of the inhibition of malonate/P_i and P_i/P_i exchanges by mersalyl, added before the substrate. (A) The reaction mixture contained 0.2 M sucrose, 20 mM HEPES–Tris, pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, P_i-loaded mitochondria (2.0 mg protein), 1 μg rotenone, 1 μg antimycin, 3 μg oligomycin and 1 mM *N*-ethylmaleimide. Mersalyl (20 nmoles/mg protein) was added after 1 min incubation and [^{14}C]malonate, at the concentrations indicated, was added to initiate the assay after a further 10 s (▲) or 20 s (■). The assay was stopped after 4 s by the addition of 20 mM butylmalonate. In the control (●) [^{14}C]malonate was added in the absence of mersalyl after 75 s from the addition of mitochondria to the incubation mixture. V is expressed as μmoles · min^{-1} · g protein^{-1}. Temperature 9 °C. Other conditions as indicated in Materials and Methods. (B) Experimental conditions as in A. Mitochondrial protein was 2.1 mg. Mersalyl (25 nmoles) was added after 1 min incubation and $^{32}P_i$, at the concentrations indicated, was added to initiate the assay after a further 10 s (▲) or 20 s (■). The assay was stopped after 4 s by the addition of 20 mM butylmalonate. In the control (●) $^{32}P_i$ was added in the absence of mersalyl after 75 s from the addition of mitochondria to the incubation mixture.

competitive type of inhibition). Similar results were also found with respect to the P_i uptake via the P_i carrier and using PHMB instead of mersalyl. There are several possible explanations for the non-linearity observed, viz., the free mersalyl is significantly depleted at low mersalyl concentrations (although this would appear unlikely with the range of concentration used), more than one molecule of mersalyl is needed to inactivate each site, or that the inhibitor is partially competitive with respect to P_i [19].

Protection by ligands of the carrier substrate-binding sites

Since the rate of succinate oxidation is quantitatively correlated to succinate uptake [20], measurement of succinate oxidation in the presence of rotenone is a simple way of studying possible interferences between the interaction of SH reagents and ligands of the substrate-binding sites with the dicarboxylate carrier.

Fig. 4A shows that when mersalyl is added 1 min before the substrate, succinate oxidation is markedly inhibited. The further addition of cysteine restores O_2 consumption to approximately the rate of the control, indicating that no permanent alteration

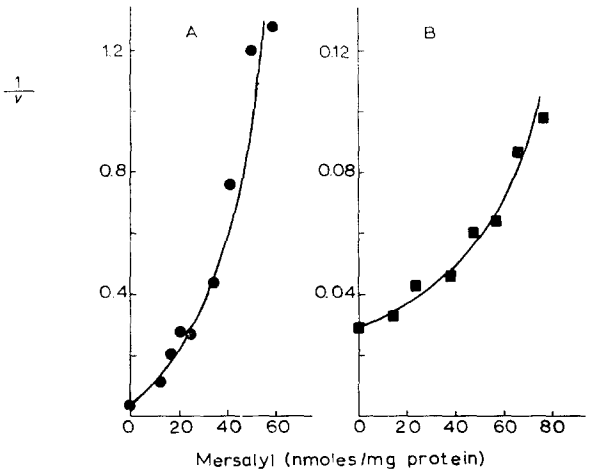

Fig. 3. Dixon plot of the inhibition of P_i/P_i exchange by mersalyl added before (A) or simultaneously (B) with the substrate. P_i-loaded mitochondria (2.4 mg protein in A and 2.1 mg in B) were incubated for 1 min in a medium containing 0.2 M sucrose, 20 mM HEPES–Tris, pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, 1 μg rotenone, 5 μg oligomycin and 1 mM *N*-ethylmaleimide. Following this incubation, mersalyl, at the concentrations indicated, was added in (A) 15 s before the addition of 0.5 mM $^{32}P_i$, and in (B) simultaneously with 0.5 mM $^{32}P_i$. The assay was started with $^{32}P_i$ and stopped after 4 s by the addition of 20 mM phenylsuccinate. *V* is expressed as μmoles · min^{-1} · g $protein^{-1}$. Temperature was 9 °C. Other conditions as indicated in Materials and Methods.

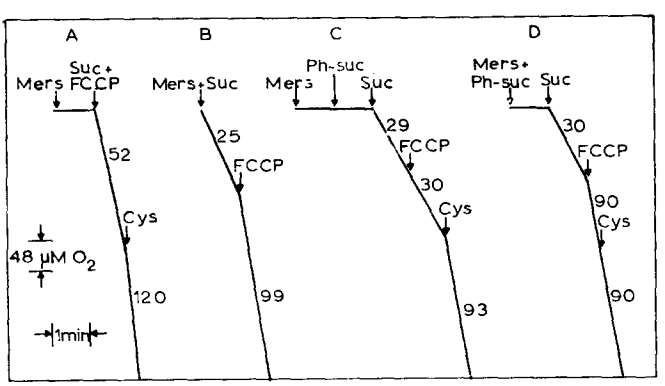

Fig. 4. Protection by succinate and phenylsuccinate against the mersalyl inhibition of the FCCP-stimulated succinate oxidation. The reaction mixture contained 0.25 M sucrose, 20 mM Tris–HCl, 1 mM EGTA, pH 7.2, 2 μg rotenone, 5.5 mg/ml mitochondrial protein in A and B and 3.9 mg/ml in C and D. Final volume 2.2 ml. Temperature 26 °C. The other additions, indicated in the figure, were made at the following concentrations: mersalyl (Mers) 45 nmoles/mg in A and B and 50 nmoles/mg in C and D; 4.5 mM succinate (Suc); 1.8 μM FCCP; 0.9 mM phenylsuccinate (Ph-suc); 4.5 mM cysteine (Cys). The rate of respiration in the control assays (succinate plus FCCP alone) ranged from 115 to 132 μatoms O/min per g protein.

has been introduced by the SH reagent. Fig. 4B shows the same experiment except that succinate is added simultaneously with the inhibitor. In this case the extent of mersalyl inhibition is significantly lowered. It should be noted that the time of contact of mersalyl with the mitochondria is equal in both experiments.

Protection against the mersalyl inhibition of succinate oxidation is also brought about by dicarboxylate analogues, such as phenylsuccinate, which block the dicarboxylate carrier by attaching to the substrate-binding site without being transported [12]. Thus, when 0.9 mM phenylsuccinate, which alone inhibits succinate oxidation only by 30% (not shown), is added after mersalyl, the inhibition of succinate oxidation is slightly increased (Fig. 4C). When, on the other hand, phenylsuccinate is added simultaneously with the inhibitor (Fig. 4D) or (not shown) before the inhibitor, succinate oxidation is little affected by the SH reagent. In this case the further addition of cysteine has no effect, suggesting that phenylsuccinate prevents the SH reagent reacting with the transport system. Essentially the same results, as those illustrated in Fig. 4, were obtained using PHMB instead of mersalyl, and butylmalonate, phthalate and maleate instead of phenylsuccinate. In control experiments, fumarate, pyruvate, β-hydroxybutyrate and citrate were found not to influence the inhibition of succinate oxidation by mersalyl, in agreement with their inability to react with the dicarboxylate carrier [8, 12].

Direct evidence that the presence of substrate analogues protects the dicarboxylate carrier against inhibition by mersalyl is provided by the data reported in Table I. Thus, despite causing some inhibition themselves, phenylsuccinate and phthalate decrease the extent of mersalyl inhibition of [^{14}C]malonate uptake, when added simultaneously with the SH reagent (Expts 1 and 2). Dicarboxylate analogues also protect

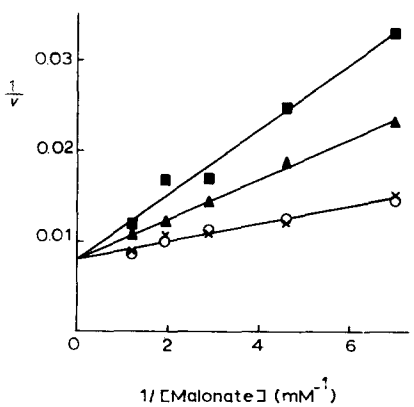

Fig. 5. Kinetic analysis of the protection by malonate of the SH reagent inhibition of the [^{14}C]-malonate/malonate exchange. The reaction mixture contained 0.2 M sucrose, 20 mM HEPES–Tris (pH 7.0), 10 mM KCl, 1 mM $MgCl_2$, 2 μg rotenone and malonate-loaded mitochondria. Mitochondrial protein was 1.6 mg. (○), unlabelled malonate, at the concentrations indicated, was added after 1 min followed after a further 20 s by the addition of carrier-free [^{14}C]malonate; (×), [^{14}C]malonate, at the concentrations indicated, was added after 80 s; (▲) and (■), 40 nmoles mersalyl was added simultaneously with unlabelled malonate after 1 min followed after a further 10 s (▲) or 20 s (■) by the addition of carrier-free [^{14}C]malonate. The assay was started with [^{14}C]malonate and terminated after 4 s by the addition of 20 mM butylmalonate. V is expressed as μmoles · min^{-1} · g protein^{-1}. Temperature was 9 °C. Other conditions as indicated in Materials and Methods.

TABLE I

PROTECTION BY DICARBOXYLATE ANALOGUES AGAINST THE MERSALYL INHIBITION OF MALONATE UPTAKE AND OF P_i UPTAKE VIA THE DICARBOXYLATE CARRIER

In Expts 1–3 unloaded mitochondria (2.8–3.3 mg protein) were incubated for 20 s in a medium containing 0.2 M sucrose, 20 mM HEPES–Tris, pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, 1 μg rotenone, 1 μg antimycin and 3 μg oligomycin. In Expt 4 P_i-loaded mitochondria (1.8 mg protein) were incubated for 1 min in a medium (as above) plus 1 mM *N*-ethylmaleimide. Following these incubations, additions were made as indicated in the table at the following concentrations: 0.5 mM [^{14}C]malonate, 20–24 nmoles mersalyl/mg protein, 1 mM phenylsuccinate, 0.2 mM phthalate and 5 mM cysteine (Expts 1–3); 0.5 mM $^{32}P_i$, 20 nmoles mersalyl, 0.2 mM phenylsuccinate and 0.2 mM butylmalonate (Expt 4). The assay was started with [^{14}C]malonate or $^{32}P_i$ and terminated after 4 s by the addition of 20 mM phenylsuccinate. Without added mersalyl the rate of malonate uptake ranged from 24 to 30 μmoles · min^{-1} · g^{-1} protein and that of P_i uptake was 43 μmoles · min^{-1} · g^{-1} protein. Temperature 9 °C. Other conditions as indicated in Materials and Methods.

Additions		Inhibition of malonate or P_i (Expt 4) uptake (%)
After 0 time	After 60 s or 15 s (Expt 4)	
Expt 1		
Mersalyl	[^{14}C]Malonate	84
Phenylsuccinate	[^{14}C]Malonate	29
Mersalyl + phenylsuccinate	[^{14}C]Malonate	49
Mersalyl	[^{14}C]Malonate + phenylsuccinate	95
Expt 2		
Mersalyl	[^{14}C]Malonate	69
Phthalate	[^{14}C]Malonate	39
Mersalyl + phthalate	[^{14}C]Malonate	55
Mersalyl	[^{14}C]Malonate + phthalate	85
Expt 3		
Mersalyl	[^{14}C]Malonate	69
Cysteine	[^{14}C]Malonate	0
Mersalyl + cysteine	[^{14}C]Malonate	0
Mersalyl	[^{14}C]Malonate + cysteine	10
Expt 4		
Mersalyl	$^{32}P_i$	84
Phenylsuccinate	$^{32}P_i$	35
Mersalyl + phenylsuccinate	$^{32}P_i$	67
Mersalyl	$^{32}P_i$ + phenylsuccinate	89
Butylmalonate	$^{32}P_i$	42
Mersalyl + butylmalonate	$^{32}P_i$	62
Mersalyl	$^{32}P_i$ + butylmalonate	90

the uptake of P_i via the dicarboxylate carrier against mersalyl inhibition (Expt 4). Thus, the mersalyl inhibition of $^{32}P_i$ uptake is decreased by the presence of phenylsuccinate and butylmalonate during the interaction of the SH reagent with the mitochondria. However, as seen in Expts 1, 2 and 4, once the SH reagent has reacted with the carrier, the inhibition is not removed by the subsequent addition of dicarboxylate analogues together with [^{14}C]malonate or $^{32}P_i$. In fact, further experiments have shown that the dicarboxylate analogues phenylsuccinate and butylmalonate do not reverse the mersalyl inhibition of malonate uptake, even when they are allowed to incubate

TABLE II

LACK OF REVERSAL BY SUBSTRATE-LIKE LIGANDS OF MERSALYL INHIBITION OF MALONATE UPTAKE

P_i-loaded mitochondria (2.8 mg protein) were incubated for 1 min at 20 °C in a medium consisting of 0.2 M sucrose, 20 mM HEPES–Tris (pH 7.0), 10 mM KCl, 1 mM $MgCl_2$, 2 μg rotenone, 5 μg oligomycin and 1 mM *N*-ethylmaleimide. Following this incubation (0 time), mersalyl was added where indicated. After further 60 s the mitochondria were cooled to 9 °C. Additions were made as indicated in the table at the following concentrations: 40 nmoles mersalyl, 1 mM phenylsuccinate, 1 mM butylmalonate, 1 mM P_i, 5 mM cysteine and 0.5 mM [^{14}C]malonate. The assay was started with [^{14}C]malonate and terminated after 4 s by the addition of 20 mM phenylsuccinate. Other conditions as indicated in Materials and Methods.

Additions			Inhibition (%)
After 0 time	After 2.5 min	After 3 min	
–	[^{14}C]Malonate	–	0
–	–	[^{14}C]Malonate	0
Mersalyl	[^{14}C]Malonate	–	84
Mersalyl	–	[^{14}C]Malonate	88
–	Phenylsuccinate	[^{14}C]Malonate	34
Mersalyl	Phenylsuccinate	[^{14}C]Malonate	95
–	Butylmalonate	[^{14}C]Malonate	36
Mersalyl	Butylmalonate	[^{14}C]Malonate	94
–	P_i	[^{14}C]Malonate	30
Mersalyl	P_i	[^{14}C]Malonate	85
–	Cysteine	[^{14}C]Malonate	3
Mersalyl	Cysteine	[^{14}C]Malonate	0

TABLE III

EFFECT OF P_i ON THE MERSALYL INHIBITION OF MALONATE UPTAKE

P_i-loaded mitochondria (1.8 mg protein) were incubated for 1 min in a medium as in Table I, Expt 4. The additions indicated in the table were made at the following concentrations: 0.5 mM [^{14}C]malonate, 25 nmoles mersalyl and 0.3–1 mM P_i. The assay was started with [^{14}C]malonate and terminated after 4 s by the addition of 20 mM phenylsuccinate. Other conditions as indicated in Materials and Methods.

Additions		V (μmoles/ min per g protein)	Inhibition (%)
After 0 time	After 15 s		
–	[^{14}C]Malonate	56.8	0
Mersalyl	[^{14}C]Malonate	31.1	45
–	[^{14}C]Malonate+P_i, 0.3 mM	49.3	13
Mersalyl	[^{14}C]Malonate+P_i, 0.3 mM	25.3	55
–	[^{14}C]Malonate+P_i, 0.5 mM	45.9	19
Mersalyl	[^{14}C]Malonate+P_i, 0.5 mM	24.9	56
–	[^{14}C]Malonate+P_i, 1 mM	42.3	25
Mersalyl	[^{14}C]Malonate+P_i, 1 mM	21.7	62

with the SH reagent-treated mitochondria for 30 s before the addition of the labelled substrate (Table II).

Figs 1 and 2 have shown that the inhibition by mersalyl of P_i uptake on the dicarboxylate carrier is decreased by P_i. Therefore, it was interesting to investigate whether the mersalyl inhibition of the uptake of [^{14}C]malonate by the dicarboxylate carrier is also reversed by P_i. Table III shows that the presence of P_i does not decrease the extent of mersalyl inhibition of malonate uptake (in contrast to the reversal of the inhibition of P_i uptake); in fact P_i is similarly ineffective in reversing the established inhibition when added to SH reagent-treated mitochondria 30 s before the addition of [^{14}C]malonate (Table II). Thus, once the mersalyl inhibition of dicarboxylate uptake is established, this inhibition cannot be decreased by either dicarboxylate analogues or P_i. The inhibition by mersalyl, when established, is, however, removed by cysteine (Tables I and II).

Comparison between the protection by substrates of the SH reagent inhibition of the P_i and malonate uptake on the dicarboxylate carrier

Table IV shows the effect of the presence of P_i and malonate on the establishment of the inhibition by SH reagents of the $^{32}P_i/P_i$ and of the [^{14}C]malonate/P_i exchange, respectively. *N*-Ethylmaleimide was present to inhibit the P_i carrier so that both these exchanges were catalyzed by the dicarboxylate carrier. Mersalyl and PHMB were added to the mitochondrial suspension, either alone or in the presence of unlabelled P_i and malonate, and allowed to react for a period of 15 s before the activity of

TABLE IV

PROTECTION BY SUBSTRATES AGAINST THE SH REAGENT INHIBITION OF P_i AND MALONATE UPTAKE ON THE DICARBOXYLATE CARRIER

P_i-loaded mitochondria (1.8 mg protein) were incubated for 1 min in a medium containing 0.2 M sucrose, 20 mM HEPES–Tris (pH 7.0), 10 mM KCl, 1 mM $MgCl_2$, 2 μg rotenone, 5 μg oligomycin and 2 mM *N*-ethylmaleimide. Following this incubation, additions were made as indicated in the table at the following concentrations: 0.5 mM P_i or $^{32}P_i$, 0.2 mM malonate or [^{14}C]malonate, 25 μM mersalyl and 15 μM PHMB. Temperature was 9 °C. The assay was started with $^{32}P_i$ or [^{14}C]-malonate and terminated after 4 s by the addition of 20 mM butylmalonate. Other conditions as indicated in Materials and Methods.

Additions		V (μmoles · min^{-1} · g protein^{-1})
After 0 time	After 15 s	
–	$^{32}P_i$	34
P_i	Carrier-free $^{32}P_i$	31
Mersalyl	$^{32}P_i$	9
Mersalyl+P_i	Carrier-free $^{32}P_i$	8
PHMB	$^{32}P_i$	8
PHMB+P_i	Carrier-free $^{32}P_i$	11
–	[^{14}C]Malonate	54
Malonate	Carrier-free [^{14}C]malonate	61
Mersalyl	[^{14}C]Malonate	20
Mersalyl+malonate	Carrier-free [^{14}C]malonate	41
PHMB	[^{14}C]Malonate	21
PHMB+malonate	Carrier-free [^{14}C]malonate	39

the two exchanges was measured. The data show that the inhibition of the $^{32}P_i/P_i$ exchange by both mersalyl and PHMB is not significantly affected by the presence of P_i during the incubation of the mitochondria with the SH reagents. In contrast, the inhibition by mersalyl and PHMB of the malonate/P_i exchange is reduced by the presence of malonate during the interaction of the SH reagents with the mitochondria.

Competition between dicarboxylates and SH reagents for the dicarboxylate carrier

In a series of experiments of the type reported in Fig. 4, it was observed that the degree of protection by phenylsuccinate against the mersalyl inhibition of succinate oxidation decreased as the time increased between the addition of mersalyl plus the substrate analogue and the addition of succinate plus carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Thus, in a typical experiment, the rate of succinate (10 mM) oxidation was decreased 66% by mersalyl (34 nmoles/mg mitochondrial protein), but only 33% by mersalyl plus phenylsuccinate (0.5 mM) added simultaneously 1 min before the respiratory substrate. When, on the other hand, the mitochondrial suspension was allowed to incubate 4 min with mersalyl, the degree of inhibition was decreased only slightly (from 66 to 58%) by the addition of phenylsuccinate together with the SH reagent. These results suggest that the substrates (or analogues) exert their protective effect by reducing the rate of interaction between the SH reagent and the dicarboxylate carrier, rather than by decreasing the degree of inhibition ultimately established.

The nature of the protection against SH reagent inhibition by substrate (or analogue) was further investigated by varying the concentration of malonate present during the interactions of mersalyl with the mitochondria. The method used was as follows: malonate-loaded mitochondria were incubated with mersalyl and different external concentrations of unlabelled malonate. After an incubation of 10 or 20 s the rate of malonate/malonate exchange was measured by adding carrier-free [^{14}C]-malonate. There are two observations that warrant comment. Firstly, during the incubation period any uptake of unlabelled malonate occurs in exchange with the internal malonate so that the internal and the external concentrations do not change. This was checked by measuring the rate of [^{14}C]malonate uptake under two conditions: (a) after a preincubation period of 20 s with unlabelled malonate (following which, the [^{14}C]malonate was added), and (b) by adding the unlabelled malonate together with [^{14}C]malonate. The lower curves of Fig. 5 are the results of these control experiments and demonstrate that any changes in the intramitochondrial concentration of malonate which possibly may occur during the preincubation period are not sufficient to significantly change the subsequent rate of [^{14}C]malonate uptake. Secondly, the malonate transport in rat liver mitochondria is catalyzed by both the dicarboxylate and the oxoglutarate carriers [21, 22]. However, the low mersalyl concentrations used in these experiments are insufficient to inhibit the oxoglutarate carrier [5, 23–25], i.e. the inhibition of malonate uptake by the SH reagent is due solely to an inhibition of the dicarboxylate carrier. Furthermore, in spite of the fact that two distinct exchanges are occurring, there is no obvious distorsion of the reciprocal plots from linearity. It is likely that the contribution by the oxoglutarate carrier to the total rate of malonate exchange is small, since the V for the dicarboxylate carrier is approximately twice the V for the oxoglutarate carrier [8, 12, 25] and also the K_m of the oxoglutarate carrier for malonate is about 400 μM (unpublished results)

compared to about 50 μM for the dicarboxylate carrier in the conditions used in these experiments. Fig. 5 reveals that the degree of mersalyl inhibition decreases when the concentration of malonate (present together with the SH reagent) is increased, and is zero at infinite concentration, i.e. the protection by substrate is competitive with the inhibitor. Furthermore, since the degree of inhibition by mersalyl approximately doubles on increasing the time of incubation from 10 to 20 s, these results indicate that the rate of inactivation of the dicarboxylate carrier by mersalyl is competitively inhibited by the dicarboxylate. Similar results, as those illustrated in Fig. 5, were obtained using PHMB instead of mersalyl (not shown).

DISCUSSION

Previous work [12] has suggested that the dicarboxylate carrier may have two separate binding sites, one specific for P_i and the other specific for the dicarboxylates.

The SH reagents establish a covalent bond with thiols [26] and their interaction with the carrier has to be regarded as practically irreversible. Hence, if mitochondria are preincubated with mersalyl in the absence of added malonate or P_i, one can study the affect of bound mersalyl on the kinetics of dicarboxylate and P_i transport. The results presented in Fig. 2 show that under these conditions, i.e. mersalyl added before the substrate, the inhibition of dicarboxylate uptake is not reversed by raising the substrate concentration, whereas the inhibition of P_i uptake is decreased by increasing P_i concentration. A possible interpretation is that the reaction between mersalyl and the dicarboxylate carrier occurs at or near the dicarboxylate binding site and, being irreversible, prevents dicarboxylate binding in a non-competitive manner. This binding of mersalyl at or near the dicarboxylate binding site, i.e. away from the P_i binding site, may be considered to change the K_m of P_i binding by an indirect conformational change.

The rate of binding of mersalyl with the dicarboxylate carrier is slow; this is evident since the establishment of full inhibition by mersalyl requires an incubation of at least 1 min [1–4]. Since the rate of binding of the substrates to the carrier, as deduced by the rate of their transport, is much faster [13], the simultaneous addition of substrates and mersalyl provides a suitable condition to establish whether the presence of the substrates interferes with the binding of the SH reagents to the dicarboxylate carrier. Fig. 1 shows that, when mersalyl is added simultaneously with malonate and let to incubate with the mitochondria only for 4 s, the mersalyl inhibition of the dicarboxylate carrier is decreased by malonate in a manner which is competitive with mersalyl (in contrast to the non-competitive inhibition by mersalyl when added before the substrate). This result is in agreement with the proposal that mersalyl binds at or near the dicarboxylate binding site and that the dicarboxylates may decrease the rate at which mersalyl gains access to the carrier. This interpretation would explain why dicarboxylates added simultaneously with the SH reagents decrease the extent of inhibition, but do not release the inhibition if added after the SH reagent, i.e. when the inhibition has been established (Fig. 4, Tables I and II). Direct evidence that dicarboxylates protect against mersalyl inhibition by depressing the rate of binding of SH reagents to the carrier was obtained by measuring the inhibition by mersalyl which occurred during fixed time periods in the presence of different concentrations of

malonate (Fig. 5). These data further indicate that dicarboxylates decrease the rate of binding of SH reagents in a manner competitive with mersalyl.

Our experiments, however, do not necessarily imply that dicarboxylates bind directly with SH groups. It has been earlier proposed that a metal ion is an essential component of the dicarboxylate carrier [27, 28] and that the substrate carboxyl groups bind to the metal [8]. It is possible that the SH groups hold the metal ion located at the dicarboxylate-binding site (see ref. 29 for references).

The following observations suggest that the P_i-binding sites of the dicarboxylate carrier are not occupied by the SH reagent: (a) The inhibition of P_i uptake by SH reagents is abolished at infinite P_i concentration, even when the inhibitor is added before the substrate (Figs 2B); (b) The presence of P_i does not protect the P_i uptake from being inhibited by SH reagents (Table IV); (c) Dixon plots of the rate of P_i uptake against SH reagent concentration are non-linear (Fig. 3), suggesting partial competitivity.

As a simple explanation it may be assumed that, although the SH reagent and P_i combine to the carrier at different sites, the interaction energy of the reaction between P_i and P_i-binding site is changed when SH groups are occupied by the SH reagent, presumably through a conformational change of the carrier. In this case the carrier forms a complex between both the SH reagent and P_i which translocates at the same rate as the carrier–P_i complex. Consequently the V is not affected, but the dissociation constant for P_i in the presence of the SH reagent is increased. A decrease in the affinity of the dicarboxylate carrier for P_i has also been observed as a consequence of malate binding to the dicarboxylate carrier [12]. Thus, the interaction energy of the reaction between P_i and the P_i-binding site may be influenced by the binding of compounds at or near the dicarboxylate-binding site. Evidence of a different type has suggested that binding of ADP and ATP to the adenine nucleotide carrier induces a conformational change [30]. Conformational changes of the mitochondrial carriers as a result of substrate binding may be a general phenomenon.

REFERENCES

1 Fonyo, A. (1968) Biochem. Biophys. Res. Commun. 32, 624
2 Tyler, D. D. (1968) Biochem. J. 107, 121
3 Tyler, D. D. (1969) Biochem. J. 111, 665
4 Meijer, A. J. and Tager, J. M. (1969) Biochim. Biophys. Acta 189, 136
5 Robinson, B. H. (1970) FEBS Lett. 11, 200
6 Meijer, A. J., Groot, G. S. P. and Tager, J. M. (1970) FEBS Lett. 8, 41
7 McGivan, J. D. and Klingenberg, M. (1971) Eur. J. Biochem. 20, 392
8 Quagliariello, E. and Palmieri, F. (1972) Symposium on the Biochemistry and Biophysics of Mitochondrial Membranes (Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E. and Siliprandi, N., eds) p. 659, Academic Press, New York
9 Quagliariello, E., Passarella, S. and Palmieri, F. (1972) Abstr. Commun. Meet. Fed. Eur. Biochem. Soc. 8, North-Holland, Amsterdam 171
10 Klingenberg, M. and Slenczka, N. (1959) Biochem. Z. 331, 486
11 Palmieri, F., Stipani, I., Quagliariello, E. and Klingenberg, M. (1972) Eur. J. Biochem. 26, 587
12 Palmieri, F., Prezioso, G., Quagliariello, E. and Klingenberg, M. (1971) Eur. J. Biochem. 22, 66
13 Quagliariello, E., Palmieri, F., Prezioso, G. and Klingenberg, M. (1969) FEBS Lett. 4, 251
14 Meisner, H., Palmieri, F. and Quagliariello, E. (1972) Biochemistry 11, 949
15 Chappell, J. B. and Robinson, B. H. (1968) in Metabolic Roles of Citrate (Goodwin, T. W., ed.), p. 123, Academic Press, New York

16 Palmieri, F., Quagliariello, E. and Klingenberg, M. (1970) Eur. J. Biochem. 17, 230
17 Kroger, A. and Klingenberg, M. (1966) Biochem. Z. 344, 317
18 Dixon, M. (1953) Biochem. J. 55, 170
19 Dixon, M. and Webb, E. C. Enzymes, 2nd edn, p. 320, Longmans Green, London
20 Quagliariello, E. and Palmieri, F. (1968) Eur. J. Biochem. 4, 20
21 Chappell, J. B. (1968) Br. Med. Bull. 24, 150
22 Klingenberg, M. (1970) in Essays in Biochemistry (Campbell, P. N. and Dickens, F., eds), Vol. 5, p.119, Academic Press, London and New York
23 Sluse, F. E., Meijer, A. J. and Tager, J. M. (1971) FEBS Lett. 18, 149
24 Sluse, F. E., Ranson, M. and Liébecq, C. (1972) Eur. J. Biochem. 25, 207
25 Palmieri, F., Quagliariello, E. and Klingenberg, M. (1972) Eur. J. Biochem. 29, 408
26 Dixon, J. L. (1966) in Enzyme and Metabolic Inhibitors (Webb, J. L., ed.), Vol. 3, Chapter 4, Academic Press, London and New Work
27 Tyler, D. D. and Newton, J. (1970) FEBS Lett. 8, 325
28 Luciani, S. (1971) FEBS Lett. 12, 213
29 Vallee, B. L. and Wacker, W. E. C. (1970) in The Proteins, Vol. 5, Metalloproteins (Neurath, H., ed.) Chapter 4, Academic Press, New York and London
30 Klingenberg, M., Grebe, K. and Scherer, B. (1971) FEBS Lett. 16, 253