# The Sensitivity of the Exchange Reactions of Tricarboxylate, 2-Oxoglutarate and Dicarboxylate Transporting Systems of Rat Liver Mitochondria to Inhibition by 2-Pentylmalonate, *p*-Iodobenzylmalonate, and Benzene 1,2,3-tricarboxylate

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A new specific inhibitor, benzene 1,2,3-tricarboxylate is described for the tricarboxylate transporting system in rat liver mitochondria. This compound, together with the inhibitors 2-pentylmalonate and 2-*p*-iodobenzylmalonate, has been used to elucidate the exchange reactions of the citrate, 2-oxoglutarate and malate transporting system in rat liver mitochondria. It is proposed that both the 2-oxoglutarate and citrate transporting systems are able to catalyse L-malate/L-malate exchanges in addition to the L-malate/L-malate exchange catalysed by the dicarboxylate transporting system. *p*-Iodobenzylmalonate was found to inhibit all three transporting systems investigated. The implications of these findings are discussed.

The exchange reactions of $^{14}C$-labelled intramitochondrial succinate, L-malate and malonate with inorganic phosphate and unlabelled dicarboxylate anions in rat liver mitochondria at 0° C were recently investigated [1]. It was found that unlike the other exchanges tested malate/malate exchange was not completely sensitive to inhibition by 2-*n*-butylmalonate and 2-*n*-pentylmalonate but was inhibited fully by *p*-iodobenzylmalonate. At higher temperatures, 20° C, Meijer and Tager [2] found malonate/malate exchange to be butylmalonate insensitive under the conditions used and proposed that a separate carrier distinct from the dicarboxylate carrier could catalyse this exchange. A system for investigating the exchange reactions of the citrate transporting system has recently been described [3] and an inhibitor of mitochondrial citrate transport, 2-ethylcitrate characterised. With the available techniques and the newly described inhibitor of mitochondrial citrate transport, benzene 1,2,3-tricarboxylate, it was decided to investigate the exchange reactions of the citrate, 2-oxoglutarate and dicarboxylate transporting systems in relation to their inhibitor sensitivity.

A preliminary report of this work was presented at the 8th International Congress of Biochemistry, Lucerne, Switzerland, September 1970.

*Unusual Abbreviations.* FCCP, carbonyl-cyanide-*p*-trifluoromethoxy-phenylhydrazone.

*Enzymes.* Aconitate hydratase (EC 4.2.1.3); NAD-isocitrate dehydrogenase (EC 1.1.1.41); NADP-isocitrate dehydrogenase (EC 1.1.1.42).

## METHODS AND MATERIALS

Mitochondria were prepared as described previously [4]. Experimental methods for the loading of mitochondria with L-[$^{14}C$]malate and [$^{14}C$]citrate (plus isocitrate) measurement of isotope exchange and pyridine nucleotide fluorescence were as described previously [1,3]. Reagents, chemicals and enzymes were obtained as described [1,3]. Benzene 1,2,3-tricarboxylic acid was obtained from the Aldrich Chemical Co. (Milwaukee, Wisc.).

### *Choice of Inhibitors*

2-*n*-Pentylmalonate was used throughout the course of these investigations as an inhibitor of the dicarboxylate transporting system in preference to

2-*n*-butylmalonate [1]. This was done because the addition of butylmalonate at high concentrations (15—25 mM) to mitochondria loaded with L-[$^{14}$C]-malate caused a small but significant exchange of L-[$^{14}$C]malate in the absence of other anions, when compared with control incubations. Recrystallisation of the butylmalonate, three times from benzene, reduced this exchange slightly. The exchange of label thus observed was thought to be due either to impurities in the butylmalonate or to a small amount of butylmalonate/L-[$^{14}$C]malate exchange taking place. In contrast, 2-*n*-pentylmalonate gave no exchange with L-[$^{14}$C]malate, neither did *p*-iodobenzylmalonate or benzene 1,2,3-tricarboxylate. Neither benzene 1,2,3-tricarboxylate, 2-*n*-pentylmalonate nor *p*-iodobenzylmalonate gave any exchange with [$^{14}$C]citrate when added to [$^{14}$C]citrate loaded rat liver mitochondria.

### *Standardisation of Experiments*

All incubations unless stated otherwise were carried out over a standard time period of 2 min followed by a 4 min centrifugation at $18000 \times g$ to separate the mitochondria [3]. After this time the supernatants were removed and prepared for counting as described in [3]. This standardisation was made so that the action of an inhibitor on one transporting system could be compared with its action on another under the same conditions of time, pH and temperature.

## RESULTS

When the oxidation of *threo*-D$_s$-isocitrate by rat liver mitochondria was monitored by fluorimetric measurement of intramitochondrial NAD(P) reduction it was found that the presence of L-malate was necessary to facilitate the entry of the tricarboxylate anion [4]. The addition of benzene 1,2,3-tricarboxylate before isocitrate or L-malate resulted in a much slower rate of observed oxidation (Fig. 1) which could be increased by the addition of higher concentrations of isocitrate. When a Dixon plot [5] for this inhibitor was constructed from fluorimetric measurement of rates of pyridine nucleotide reduction it was found that the inhibition was competitive with isocitrate rather than L-malate (Fig. 2), the $K_1$ being 0.16 mM.

Similar observations were made for citrate and *cis*-aconitate oxidation, while benzene 1,2,3-tricarboxylate at 5 mM did not inhibit the oxidation of either 1 mM glutamate or 1 mM 3-hydroxybutyrate as monitored fluorimetrically. When tested on a broken mitochondrial preparation as described previously [3], benzene 1,2,3-tricarboxylate was found to be a weak inhibitor of NADP-isocitrate dehydrogenase and aconitase but was without effect on the NAD-isocitrate dehydrogenase. The observed inhibitions of tricarboxylate oxidation were considerably

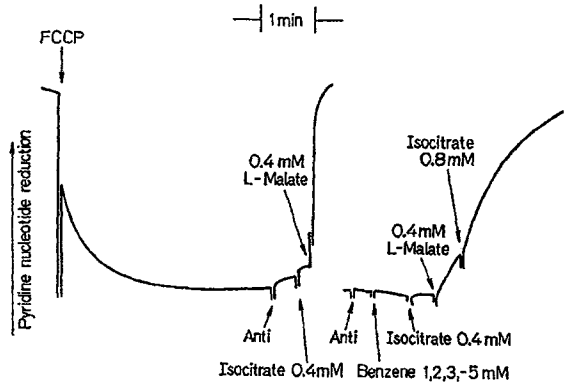


Fig. 1. *The inhibition of* threo-D$_s$-*isocitrate oxidation by benzene 1,2,3-tricarboxylate.* Rat liver mitochondria (5.0 mg protein) were suspended in 3.0 ml of a medium containing 125 mM KCl, 20 mM Tris-Cl and 2 mM inorganic phosphate, pH 7.4 at 30° C. Pyridine nucleotide oxidation/reduction changes were followed fluorimetrically. Only experiment A is shown in full since the initial oxidation of intramitochondrial NAD(P) by uncoupling agent was the same for A and B. Additions were as follows: Expt. (A) FCCP 1 μM, antimycin (anti) 0.2 μg/ml, *threo*-D$_s$-isocitrate 0.4 mM, L-malate 0.4 mM. Expt. (B) FCCP 1 μM, antimycin 0.2 μg/ml, benzene 1,2,3-tricarboxylate (benzene 1,2,3-) 5 mM, *threo*-D$_s$-isocitrate 0.4 mM, L-malate 0.4 mM; *threo*-D$_s$-isocitrate 0.8 mM

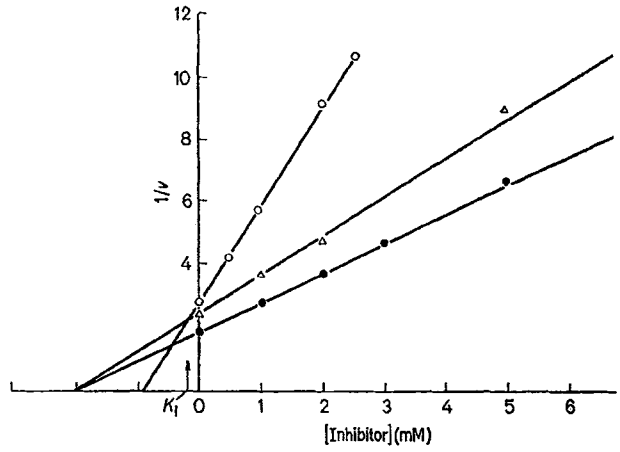


Fig. 2. *The kinetics of benzene 1,2,3-tricarboxylate inhibition of* threo-D$_s$-*isocitrate oxidation.* The reciprocal of the velocity of intramitochondrial NAD(P) reduction by *threo*-D$_s$-isocitrate, $1/v$, is plotted against inhibitor (benzene 1,2,3-tricarboxylate) concentration under three sets of conditions. Rat liver mitochondria (2.9 mg protein) were suspended in 3.0 ml of a medium containing 125 mM KCl, 20 mM Tris-Cl and 2 mM inorganic phosphate, pH 7.4 at 30° C. 1 μM FCCP was added and after 30 min, 0.2 μg/ml antimycin followed by benzene 1,2,3-tricarboxylate (final concentrations shown), *threo*-D$_s$-isocitrate and L-malate. The rate of intramitochondrial NAD(P) reduction on the addition of L-malate was monitored fluorimetrically. The three sets of conditions were; (○), 0.12 mM isocitrate, 0.12 mM L-malate; (△) 0.20 mM isocitrate, 0.12 mM L-malate; (●) 0.20 mM isocitrate, 0.40 mM L-malate

Table. *Inhibition of citrate,* cis-*aconitate and isocitrate oxidation in whole and broken mitochondria by benzene 1,2,3-tricarboxylate*
Experiments to estimate inhibition in whole mitochondria were performed as described in Fig. 1 at the concentrations of tricarboxylate anion shown in the presence of 0.4 mM L-malate. For the experiments to estimate the inhibition in broken mitochondria, sonicated mitochondria (2 mg protein) were prepared and rate of tricarboxylate oxidation was monitored spectrophotometrically as described previously [6] in the presence and absence of 5 mM benzene 1,2,3-tricarboxylate

| Enzyme | Substrate | Substrate concn. | Inhibition | |
|---|---|---|---|---|
| | | | Broken mitochondria | Whole mitochondria |
| | | µM | % | % |
| Aconitate hydratase | Citrate | 800 | 22 | 56 |
| Aconitate hydratase | *cis*-Aconitate | 600 | 9.8 | 58 |
| NADP-Isocitrate dehydrogenase | *threo*-$D_s$-Isocitrate | 250 | 15.0 | 73 |
| NAD-Isocitrate dehydrogenase | *threo*-$D_s$-Isocitrate | 250 | 0 | |

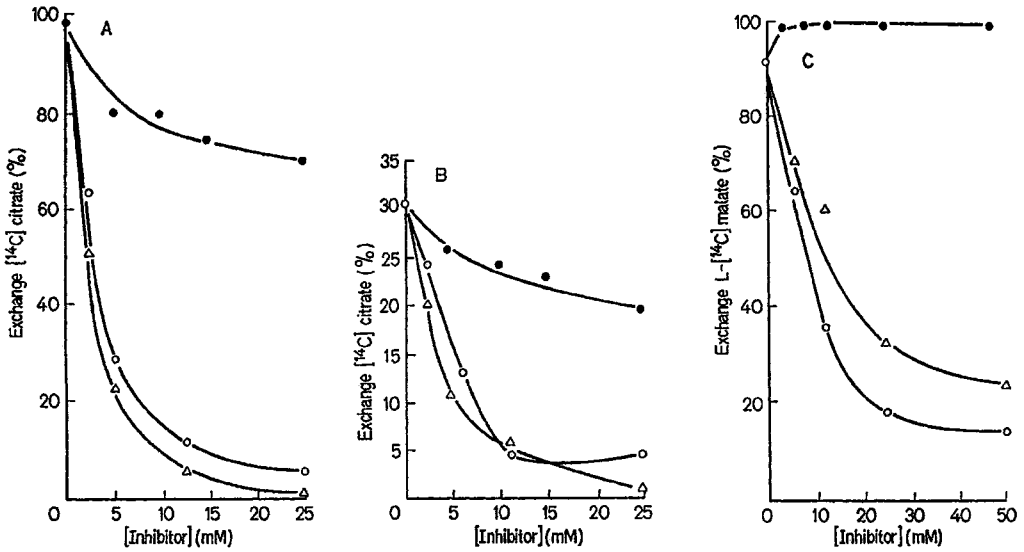


Fig. 3. *The sensitivity of (A) citrate/[$^{14}$C]citrate, (B) malate/[$^{14}$C]citrate and (C) citrate/[$^{14}$C]malate exchanges in rat liver mitochondria to the inhibitors benzene 1,2,3-tricarboxylate, 2-pentylmalonate and* p-*iodobenzylmalonate.* Rat liver mitochondria (8 mg protein aliquots) loaded with [$^{14}$C]citrate or [$^{14}$C]malate were added to 1 ml incubations of a medium containing 125 mM KCl, 20 mM Tris-Cl and 1 mM citrate or 1 mM malate pH 7.4 at 10° C. Control incubations were carried out in the same medium in the absence of the 1 mM citrate or 1 mM malate. Inhibitors were included at the concentrations shown; benzene 1,2,3-tricarboxylate (△) 2-pentylmalonate (●), *p*-iodobenzylmalonate (○). After addition of the mitochondria, the incubations were allowed to stand for 2 min, after which the mitochondria were separated by centrifugation and samples of the supernatant were prepared for counting as described previously [3]. An unseparated sample was also prepared for counting to allow calculation of the total [$^{14}$C]citrate or [$^{14}$C]malate in each incubation. The extent of the exchange of label over the time period was expressed as percentage exchange. This is defined as: 100 (radioactivity of incubation supernatent-radioactivity of control supernatent)/radioactivity of control incubation pellet

less in broken than in whole mitochondria when the same concentrations of substrates and inhibitors were used (Table).

When the exchange of either 1 mM L-malate or citrate with intramitochondrial [$^{14}$C]citrate or intramitochondrial L-[$^{14}$C]malate with 1 mM citrate was monitored as described by Robinson *et al.* [3] but at 10° C rather than 0° C it was found that increasing concentrations of benzene 1,2,3-tricarboxylate reduced the extent of these exchanges (Fig. 3), half maximal inhibition occurring at about 3 mM inhibitor. 2-Ethylcitrate, which at 0° C was shown to be a good inhibitor of citrate/[$^{14}$C]citrate exchange [3] was not as effective as benzene 1,2,3-tricarboxylate in reducing the observed exchange when used at 10° C, this probably being due to the tendency of 2-ethylcitrate to act as a substrate, and thus an exchanger, for this transporting system [3]. 2-Pentylmalonate which is a potent inhibitor of the dicarboxylate transporting system had only a weak inhibitory effect on these exchanges but *p*-iodobenzylmalonate which was previously thought to be specific only for the di-

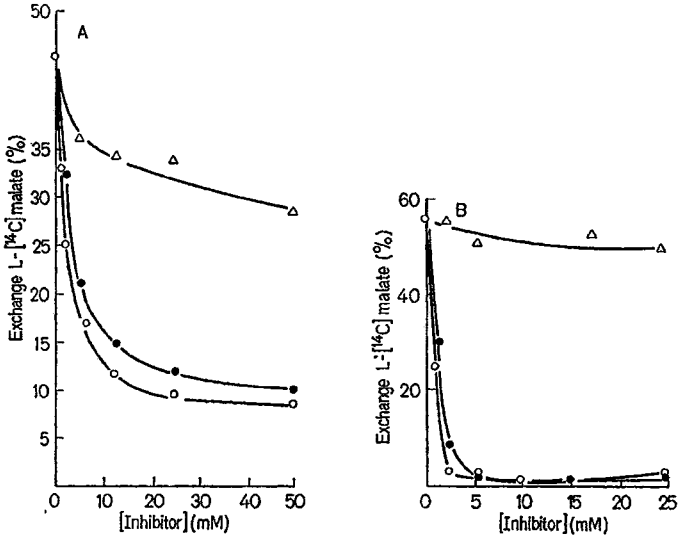


Fig. 4. *The sensitivity of (A) malonate/L-[$^{14}$C]malate and (B) inorganic phosphate/L-[$^{14}$C]malate exchanges in rat liver mitochondria to the inhibitors benzene 1,2,3-tricarboxylate, 2-pentylmalonate and* p-*iodobenzylmalonate.* The experiments were carried out as described for Fig. 3 except that 1 mM malonate or 1 mM inorganic phosphate were used as exchangers instead of 1 mM citrate with L-[$^{14}$C]malate loaded mitochondria. Inhibitors: benzene 1,2,3-tricarboxylate (△); 2-pentylmalonate (●); *p*-iodobenzylmalonate (○)

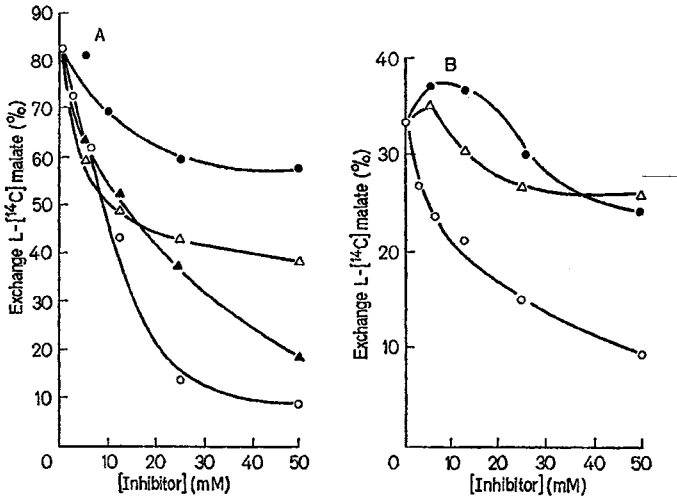


Fig. 5. *The sensitivity of (A) L-malate/L-[$^{14}$C]malate and (B) 2-oxoglutarate/L-[$^{14}$C]malate exchanges in rat liver mitochondria to the inhibitors benzene 1,2,3-tricarboxylate, 2-pentylmalonate and* p-*iodobenzylmalonate.* The experiments were carried out as described for Fig. 3 except that 1 mM L-malate or 1 mM 2-oxoglutarate were used as exchangers instead of 1 mM citrate with L-[$^{14}$C]malate loaded mitochondria. Inhibitors: benzene 1,2,3-tricarboxylate (△); 2-pentylmalonate (●); *p*-iodobenzylmalonate (○); benzene 1,2,3-tricarboxylate plus 2-pentylmalonate (▲)

carboxylate anion transporting system [1] was almost as effective as benzene 1,2,3-tricarboxylate in blocking the exchanges. Interestingly, these inhibitors were not as effective against the [$^{14}$C]malate/citrate exchange as they were in blocking malate/[$^{14}$C]citrate and citrate/[$^{14}$C]citrate exchanges.

When the exchange of 1 mM malonate with L-[$^{14}$C]malate at 10° C was monitored it was found that both *p*-iodobenzylmalonate and 2-*n*-pentylmalonate were effective inhibitors but benzene 1,2,3-tricarboxylate was without much effect (Fig. 4A). The exchange of 1 mM inorganic phosphate with

L-[$^{14}$C]malate at 10° C was also relatively insensitive to benzene 1,2,3-tricarboxylate inhibition while being very strongly inhibited by *p*-iodobenzylmalonate and 2-*n*-pentylmalonate. When the exchange of 1 mM L-malate with L-[$^{14}$C]malate at 10° C (Fig. 5A) was measured it was found that *p*-iodobenzylmalonate was an effective inhibitor compared with either benzene 1,2,3-tricarboxylate or 2-*n*-pentylmalonate. A similar situation was observed when the exchange of 1 mM succinate with L-[$^{14}$C]malate was monitored with these inhibitors at 10° C. When benzene 1,2,3-tricarboxylate and 2-*n*-pentylmalonate were used in combination the inhibition was additive at higher concentrations of inhibitor. At 0° C, malate/L-[$^{14}$C]-malate was inhibited fully by 50 mM *p*-iodobenzylmalonate [1], but not by 50 mM benzene 1,2,3-tricarboxylate or 2-pentylmalonate.

It has been demonstrated by De Haan and Tager that malonate/2-oxoglutarate or L-malate/2-oxoglutarate exchange is mediated by the 2-oxoglutarate transporting system in rat liver mitochondria [6]. It was thus decided to use the exchange of 2-oxoglutarate with L-[$^{14}$C]malate to test the sensitivity of this transporting system to the inhibitors used for the dicarboxylate and tricarboxylate transporting systems. The exchange was monitored over the same period as the previous exchanges with 1 mM 2-oxoglutarate added to the incubations at 10° C. 2-Pentylmalonate and benzene 1,2,3-tricarboxylate inhibited the observed exchange weakly at high concentrations (Fig. 5B) while *p*-iodobenzylmalonate appeared to be an inhibitor for this exchange giving 70% inhibition at 50 mM. When 2-oxoglutarate/L-[$^{14}$C]malate exchange was monitored at 0° instead of 10° C, 50mM *p*-iodobenzylmalonate was found to give complete inhibition, while benzene 1,2,3-tricarboxylate and pentylmalonate inhibited again only weakly. It was found impossible to test whether aspartate was an inhibitor of 2-oxoglutarate/L-[$^{14}$C]malate exchange as proposed by McGivan *et al.* [7] since L-aspartate gave an appreciable exchange with L-[$^{14}$C]malate at 10° C when added on its own to mitochondria loaded with L-[$^{14}$C]malate. This exchange was sensitive to inhibition by either 2-pentylmalonate or *p*-iodobenzylmalonate but not benzene 1,2,3-tricarboxylate.

## DISCUSSION

The inhibition of pyridine nucleotide reduction in rat liver mitochondria by benzene 1,2,3-tricarboxylate was shown to be competitive with tricarboxylate anion rather than L-malate by the use of the Dixon kinetic plot. The $K_i$ of 0.16 mM found for this compound is lower than that for other citrate analogues found to inhibit the liver mitochondrial isocitrate oxidation system [3]. As citrate, *cis*-aconitate and isocitrate oxidation were all effectively inhibited by this compound, it was most probable that a common site of inhibition was involved. This could be either at the level of the membrane or at the isocitrate dehydrogenases. Since, however, the inhibition of oxidation observed in broken mitochondria was substantially lower than that observed in whole mitochondria, the site of inhibition may be placed at the level of the membrane.

The measurement of citrate/citrate and malate/citrate exchange over fixed time intervals confirmed that benzene 1,2,3-tricarboxylate was indeed an inhibitor of the transporting system. The effectiveness of benzene 1,2,3-tricarboxylate in inhibiting the exchange both of citrate and malate with the labelled material gave further support to the proposition that citrate and L-malate attach to a common site on the carrier at the extramitochondrial facet [6] so that citrate analogues such as 2-ethylcitrate, 2-propylcitrate and benzene 1,2,3-tricarboxylate block both exchanges.

The fact that benzene 1,2,3-tricarboxylate significantly inhibited only the citrate/[$^{14}$C]citrate and malate/[$^{14}$C]citrate and citrate/[L-[$^{14}$C]malate exchanges as opposed to the phosphate/[$^{14}$C]malate exchange and the 2-oxoglutarate/[$^{14}$C]malate exchange is most important. Since the latter two exchanges involved the dicarboxylate anion transporter and the 2-oxoglutarate transporter respectively we may deduce that benzene 1,2,3-tricarboxylate is relatively specific with respect to the tricarboxylate transporting system. This compound was found to be especially useful since at 10° C the citrate transporting system was not as sensitive to inhibition by 2-ethylcitrate, which was the best of the compounds previously demonstrated to inhibit this transporting system [3]. This specific inhibition by benzene 1,2,3-tricarboxylate also strengthens the case for the separate identity of the citrate and 2-oxoglutarate transporting systems [8]. 2-Pentylmalonate likewise showed a singular specificity, in this case for the dicarboxylate anion carrier as had been thought previously [4, 8—11]. Thus phosphate/malate exchange was strongly inhibited while citrate/malate and oxoglutarate/malate exchanges were relatively unaffected by this compound. *p*-Iodobenzylmalonate, which was introduced as an inhibitor for the dicarboxylate anion carrier with greater potency than butylmalonate [10], was found to inhibit all three transporting systems. Thus, although being most effective as an inhibitor of phosphate/malate exchange, it was inhibitory both to citrate/citrate and 2-oxoglutarate/malate exchanges. Despite the low specificity of the compound, its potency as an inhibitor may be most useful for example in the case of the 2-oxoglutarate transporting system for which no inhibitor has been described, other than L-aspartate [11].

*Dicarboxylate Exchanges*

Since none of the dicarboxylate/dicarboxylate exchanges investigated at 10° C appeared to be completely inhibited by 2-pentylmalonate it was thought possible that the 2-oxoglutarate and tricarboxylate transporting systems might also be able to catalyse such exchanges by virtue of their affinity for such anions as malonate, succinate and L-malate [6]. Malonate/malate exchange might be expected to occur *via* the 2-oxoglutarate transporter and dicarboxylate transporter only, since malonate does not exchange for citrate [8]. Thus benzene 1,2,3-tricarboxylate had no effect on malonate/[$^{14}$C]malate exchange while 2-pentylmalonate and *p*-iodobenzylmalonate were partially successful as inhibitors of this exchange. Since *p*-iodobenzylmalonate inhibits both the dicarboxylate and 2-oxoglutarate transporters it might be expected that *p*-iodobenzylmalonate would be the best inhibitor for malonate/[$^{14}$C]malate exchange, which was the case. In the case of malate/[$^{14}$C]malate exchange it might be expected that all three transporting systems would be able to catalyse such a dismutation. Thus at 10° C this exchange is partially blocked by benzene 1,2,3-tricarboxylate and 2-pentylmalonate used individually, the extent of inhibition being increased when used in combination. Even in the presence of these two inhibitors however, the 2-oxoglutarate system is still free to catalyse the exchange so that *p*-iodobenzylmalonate is the best inhibitor of malate/L[$^{14}$C]malate exchange by virtue of its ability to inhibit all three transporting systems. In addition, since 50 mM *p*-iodobenzylmalonate inhibits both malate/malate exchange [1] as well as 2-oxoglutarate/malate and malate/citrate exchanges completely at 10° C the argument that malate/malate exchange can be catalysed by all three systems is strengthened. The reason for malonate/L[$^{14}$C]malate and succinate/L-[$^{14}$C]malate exchange at 0° C being almost completely sensitive to inhibition by specific dicarboxylate carrier inhibitors [1] is thought to be that at 0° C contribution of 2-oxoglutarate and citrate carrier to the dicarboxylate exchange reactions is small when one of the dicarboxylates is either succinate or malonate. It seems then, that Meijer and Tager [2] were correct in proposing that a carrier other than the dicarboxylate transporter could catalyse dicarboxylate/dicarboxylate exchanges. The case of succinate/[$^{14}$C]malate exchange is very similar to malate/[$^{14}$C]malate exchange in being best inhibited by *p*-iodobenzylmalonate so that again it seems that all three transporting systems can catalyse this exchange.

The possible exchange reactions catalysed by the three transporting systems and the points of action of the inhibition used are summarized in Fig. 6. Firstly the exchange of phosphate for either phosphate [10] or a dicarboxylate anion as well as the

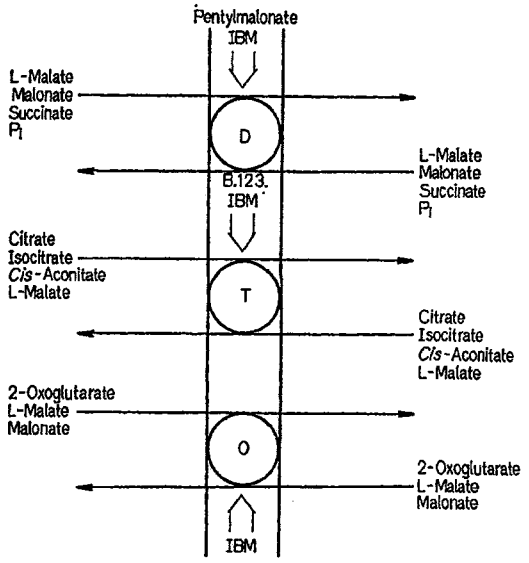


Fig. 6. *Diagram to show the exchange reactions catalysed by the three transporting systems and the site of action of inhibitors.* D = dicarboxylate transporting system; T = tricarboxylate transporting system; O = 2-oxoglutarate transporting system. IBM = *p*-iodobenzylmalonate; B 123 = benzene 1,2,3-tricarboxylate

exchange of dicarboxylate anion for dicarboxylate anion may be catalysed by the dicarboxylate transporting system. This system may be inhibited by 2-pentylmalonate or *p*-iodobenzylmalonate. Secondly the exchange of tricarboxylate anion for tricarboxylate anion or L-malate (or succinate) and the exchange of L-malate or succinate for L-malate or succinate may be catalysed by the tricarboxylate anion transporting system. This system may be inhibited by benzene 1,2,3-tricarboxylate or *p*-iodobenzylmalonate. Thirdly, the exchange of 2-oxoglutarate for 2-oxoglutarate (unpublished observations) or L-malate (or malonate) and exchange of L-malate (or malonate or succinate) for L-malate (or malonate or succinate) may be catalysed by the 2-oxoglutarate transporter. This system may be inhibited only by *p*-iodobenzylmalonate at high concentrations.

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