# THE EFFECT OF *p*-HYDROXYMERCURIBENZOATE ON MITOCHONDRIAL ANION EXCHANGE REACTIONS INVOLVING L-MALATE

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## 1. Introduction

It has been shown by Meijer, Groot and Tager [1] that strong sulphydryl-blocking reagents such as *p*-hydroxymercuribenzoate (pHMB) and mersalyl can inhibit not only the phosphate transporting system [2, 3] but also the dicarboxylate transporting system of rat liver mitochondria. Weaker –SH blocking reagents such as *N*-ethylmaleimide and 5′,5′-dithio-bis-(2-nitrobenzoate) on the other hand inhibit the phosphate transporting system, [1, 4, 5], but not the dicarboxylate transporting system.

Since methods are now available [6–8] for studying the exchange reactions of the dicarboxylate, tricarboxylate and 2-oxoglutarate transporting systems in rat liver mitochondria, it was decided to test the sensitivity of these exchange reactions to *p*-hydroxymercuribenzoate.

## 2. Methods and materials

Rat liver mitochondria were prepared as described by Robinson and Chappell [9] and were loaded with either $^{14}$C-citrate [7] or $^{14}$C-L-malate [6] as described previously.

L-Malate was measured by the method of Hohorst [10] and citrate by the method of Moellering and Grueber [11]. Further experimental details are given either in the text or in the legends to figures.

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## 3. Results

Rat liver mitochondria which had been loaded with $^{14}$C-L-malate were added to 1 ml incubations of a buffer containing 125 mM KCl, 20 mM tris HCl, pH 7.4 at 10°. In a series of incubations increasing concentrations of pHMB were present. One minute after addition of the mitochondria, 1 mM of either citrate, $P_i$, malonate or 2-oxoglutarate were added to the incubation. After standing for 2 min, the mitochondria were then separated by centrifugation at 18,000 *g* for 4 min and samples of the supernatant and pellet deproteinised and prepared for counting and assay of L-malate as described previously [6]. Unseparated samples of mitochondria were also prepared for counting and assay of L-malate to give the total amount of L-malate in the system.

Fig. 1. shows the effect of increasing concentration of pHMB on the observed exchanges of labelled material, while table 1 shows the results at 100 μM pHMB obtained by enzymatic assay. The table shows that pHMB added to the mitochondria in the absence of an exchanging anion gave a higher level of intramitochondrial L-malate than the control incubation showing that a small leak of labelled material occurred in the control during the time of incubation. For this reason, the situation found in this incubation (pHMB only added) rather than the control with no additions was taken as the reference point for both the radioactive and enzymatic assay data i.e. the point of zero exchange. pHMB inhibited both the $P_i$/$^{14}$C-L-malate exchange and the citrate $^{14}$C-L-malate exchange, the concentrations required for half maximal inhibition being 30 μM and 60 μM respectively. On the other

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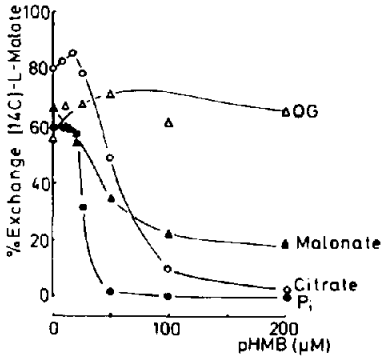


Fig. 1. The effect of increasing concentrations of *p*-hydroxymercuribenzoate on the exchanges of phosphate, citrate, malonate and 2-oxoglutarate with $^{14}$C-L-malate. The experiment was carried out as described in the text. Protein was 19.6 mg per incubation. Additions: 1 mM 2-oxoglutarate (△—△), 1 mM malonate (▲—▲), 1 mM citrate (○—○), 1 mM phosphate (●—●).

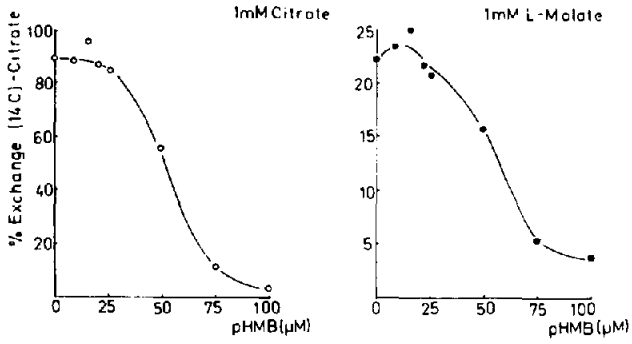


Fig. 2. The effect of increasing concentration of *p*-hydroxymercuribenzoate on citrate /$^{14}$C-citrate and L-malate/$^{14}$C-citrate exchanges. The experiment was carried out described in the text. Protein was 16.3 mg per incubation. Additions: 1 mM citrate (○—○) 1 mM L-malate (●—●).

hand 2-oxoglutarate/$^{14}$C-L-malate exchange did not seem to be affected by the –SH blocking reagent over the concentration range used while malonate/$^{14}$C-L-malate exchange was inhibited, but not to the same extent as the $P_i$ or citrate driven exchanges. In general, the results found by enzymatic assay of L-malate agree very well with those obtained by the monitoring of label exchange, the presence of 100 μM pHMB preventing the transfer of L-malate from the mitochondria to the incubation medium in the presence of citrate, phosphate and malonate but not in the presence of 2-oxoglutarate.

In order to test further the effect of pHMB on the citrate transporting system, rat liver mitochondria were loaded with $^{14}$C-citrate and added to 1 ml incubations of KCl/tris HCl medium, as described for

Table 1

| Addition | L-Malate (nmoles) | | Exchange (%) |
|---|---|---|---|
| | Intramitochondrial | Extramitochondrial | |
| None | 29.0 | 14.2 | 12.7 |
| pHMB | 33.1 | 10.1 | 0 |
| 2-Oxoglutarate (1 mM) | 15.2 | 28.4 | 55.6 |
| 2-Oxoglutarate (1 mM) + pHMB 100 μM | 18.5 | 25.4 | 46.5 |
| $P_i$ (1 mM) | 15.9 | 27.2 | 51.5 |
| $P_i$ (1 mM) + pHMB (100 μM) | 32.8 | 10.1 | 0 |
| Citrate (1 mM) | 10.1 | 33.4 | 70.6 |
| Citrate (1 mM) + pHMB (100 μM) | 31.0 | 13.3 | 10.0 |
| Malonate (1 mM) | 15.6 | 28.4 | 56.4 |
| Malonate (1 mM) + pHMB (100 μM) | 29.3 | 14.2 | 12.6 |

The effect of *p*-hydroxymercuribenzoate on the efflux of L-malate resulting from the addition of 2-oxoglutarate, phosphate, citrate and malonate to rat liver mitochondria. The experiment was carried out as described in fig. 1.

Table 2

| Additions | Citrate (nmoles) | | Exchange (%) |
|---|---|---|---|
| | Intramitochondrial | Extramitochondrial | |
| None | 61.1 | 5.8 | 0 |
| pHMB | 62.0 | 5.1 | −1.1 |
| L-Malate (1 mM) | 48.8 | 17.9 | 21.1 |
| L-Malate (1 mM + pHMB + (100 μM) | 58.6 | 7.5 | 2.8 |

The effects of pHMB on the efflux of citrate resulting from the addition of L-malate to rat liver mitochondria. The experiment was performed as described for fig. 2. in the text and samples from the incubations were assayed for citrate.

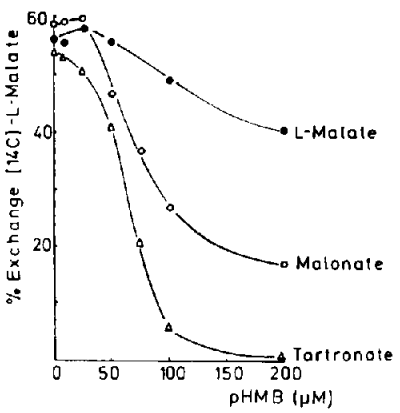


Fig. 3. The effect of increasing concentrations of *p*-hydroxymercuribenzoate on the exchanges of L-malate, tartronate and malonate with $^{14}$C-L-malate. Rat liver mitochondria (17.1 mg protein per incubation) were added to 1 ml incubations of a medium containing 125 mM KCl, 20 mM tris HCl, pH 7.4 at 10°. Increasing concentrations of *p*-hydroxymercuribenzoate were present in the incubations. After 1 min, 1 mM of either L-malate (●——●), malonate (○——○) or tartronate (△——△) was added and after a further 2 min the mitochondria were separated by centrifugation and samples of supernatant and pellet were prepared as described, previously [6]. An incubation with *p*-hydroxymercuribenzoate only added was taken as the reference point for calculating the percentage exchange, i.e. the point of zero exchange.

fig. 1, containing increasing concentrations of pHMB. After 1 min either 1 mM citrate or 1 mM L-malate was added to the incubations and the mitochondria separated, after a further 2 min, by centrifugation at 18,000 *g* for 4 min. Samples of the supernatant and pellet were deproteinised and prepared for counting and assay of citrate as described by Robinson et al. [7]. Fig. 2. shows the effect of increasing concentrations of pHMB on the observed exchanges. Both citrate/$^{14}$C-citrate and L-malate/$^{14}$C-citrate exchange were inhibited by pHMB, the concentrations for half-minimal inhibition being around 55 μM. Table 2 shows that the transfer of citrate from mitochondria to incubation medium brought about by the addition of L-malate is blocked by pHMB, confirming the results of the experiment as determinated by following the movement of labelled material. The effects of pHMB on the exchange of 1 mM L-malate, tartronate (2-hydroxymalonate) or malonate with $^{14}$C-L-malate in rat liver mitochondria were then investigated (fig. 3). L-Malate/$^{14}$C L-malate exchange was not greatly inhibited by pHMB but tartronate/$^{14}$C-L-malate exchange was completely inhibited by 200 μM pHMB, the concentration for half maximal inhibition being around 60 μM. As was seen in fig. 1, malonate/$^{14}$C-L-malate exchange was inhibited but not completely by pHMB.

*N*-Ethylmaleimide at concentrations of up to 2 mM had no affect on any of the exchanges reported in this communication.

## 4. Discussion

Meijer and Tager [12] reported that mersalyl had no effect on either 2-oxoglutarate/dicarboxylate exchange or tricarboxylate/malate exchange in rat liver mitochondria. However it is obvious from the results presented here that the somewhat stronger –SH blocking reagent *p*-hydroxymercuribenzoate is able

to block almost completely the exchange of citrate with $^{14}$C-L-malate as viewed both by measurement of label exchange and by enzymatic assay of L-malate. By the same criteria it seems that pHMB is unable to block the exchange of 2-oxoglutarate for $^{14}$C-L-malate. The inhibition of citrate/$^{14}$C-citrate and L-malate/$^{14}$C-citrate exchanges by pHMB confirmed that the exchange reactions of the citrate transporting system were indeed sensitive to inhibition by –SH blocking reagents. The inhibition of $P_i$/$^{14}$C-L-malate exchange by the –SH reagent observed agreed with results obtained previously [1, 12] but Meijer and Tager found no inhibition of malonate/L-malate exchange at 20° by mersalyl, in contrast to the results presented here, which show that malonate/$^{14}$C-L-malate exchange is markedly though not completely sensitive to inhibition by pHMB at 10°. The fact that the concentration of pHMB required for half maximal inhibition of citrate transport was double that required for half maximal inhibition of dicarboxylate ($P_i$/L-malate) transport is interesting, since it partly explains why the weaker reagent, mesalyl, gave inhibition of dicarboxylate but not tricarboxylate/transport [12]. The difference is presumably due to the relative accessibility of the –SH group or groups on the two transporting systems to added –SH blocking reagents. Robinson and Williams [6] reported that L-malate/$^{14}$C-L-malate exchange at 0° could not be inhibited completely by 2-butylmalonate, a specific inhibitor of the dicarboxylate transporting system, but could be inhibited completely by *p*-iodobenzylmalonate, a compound, which has subsequently been shown to inhibit the exchange reactions of the tricaboxylate, dicarboxylate and 2-oxoglutarate transporting systems [8]. pHMB appeared to be like 2-butylmalonate in being able to block phosphate/$^{14}$C-L-malate exchange but not L-malate/$^{14}$C-L-malate exchange. The possibility emerges then, as suggested by Meijer and Tager [12], that a system other than the dicarboxylate transporting system can catalyse dicarboxylate/dicarboxylate exchanges. The most likely candidates for this role are the 2-oxoglutarate and citrate transporting system, both of which have affinities for L-malate and other dicarboxylate anions [13, 14].

If we assume that these two systems can catalyse dicarboxylate/dicarboxylate exchanges we might expect that tartronate, since it cannot acitivate 2-oxoglutarate transport [14], could exchange for L-malate on either the dicarboxylate or tricarboxylate transporting systems but not on the 2-oxoglutarate transporting system. Conversely malonate, since it is unable to activate tricarboxylate transport [13, 14], could exchange for L-malate on either the dicarboxylate or 2-oxoglutarate but not the 2-oxoglutarate transporting system. Since pHMB inhibits both the dicarboxylate and tricarboxylate transporting systems but not the 2-oxoglutarate transporting system we would expect tartronate/$^{14}$C-L-malate exchange to be fully inhibited by pHMB, which was found to be the case (fig. 3). On the other hand both L-malate/$^{14}$C-L-malate and malonate/$^{14}$C-L-malate exchanges could still be catalysed by the 2-oxoglutarate carrier when both the dicarboxylate and tricarboxylate carriers were blocked by pHMB. The fact the malonate/$^{14}$C-L-malate exchange was inhibited much more than L-malate/$^{14}$C-L-malate exchange by pHMB may indicate that the 2-oxoglutarate transporting system is able to catalyse an L-malate/$^{14}$C-L-malate exchange more rapidly than a malonate/$^{14}$C-L-malate exchange, which is not surprising since L-malate is a natural substrate for the system.

The evidence then indicates that at 10°, under the conditions described, a dicarboxylate/dicarboxylate exchange may be catalysed by not only the dicarboxylate transporting system but also the tricarboxylate and 2-oxoglutarate transporting systems.

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**References**

[1] A.J. Meijer, G.S.P. Groot and J.M. Tager, FEBS Letters 8 (1970) 41.
[2] A. Fonyo and S.P. Bessman, Biochem. Med. 2 (1968) 145.
[3] D.D. Tyler, Biochem. J. 111 (1969) 665.
[4] J.B. Chappell, in: Inhibitors – Tools in Cell Research, 20th Mosbach Colloquum (Springer, Heidelberg, 1969) p. 335.
[5] R.N. Johnson and J.B. Chappell, Biochem. J. 116 (1970) 37 P.

[6] B.H. Robinson, and G.R. Williams, Biochim. Biophys. Acta 216 (1970) 63.
[7] B.H. Robinson, G.R. Williams, M.L. Halperin and C.C. Leznoff, European J. Biochem. 15 (1970) 263.
[8] B.H. Robinson, G.R. Williams, M.L. Halperin and C.C. Leznoff, in press.
[9] B.H. Robinson and J.B. Chappell, Biochem. Biophys. Res. Commun. 28 (1967) 249.
[10] H.J. Hohorst in: Methods in Enzymatic Analysis, ed. H.U. Bergemeyer (Academic Press, New York, London, 1963) p. 318.
[11] H. Mollering and W. Gruber, Anal. Biochem. 17 (1966) 369.
[12] A.J. Meijer and J.M. Tager, Biochim. Biophys. Acta 189 (1969) 136.
[13] J.B. Chappell, P.J.F.Henderson, J.D.McGivan and B.H. Robinson, in: The Interaction of Drugs and Subcellular Components in Animal Cells, ed. P.N. Campbell (Churchill, London, 1968) p. 71.
[14] J.B. Chappell and B.H. Robinson, Biochem. Soc. Symp. 27 (1968) 123.