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EFFECT OF AMYTAL ON THE PERMEABILITY OF THE MITOCHONDRIAL MEMBRANE IN RAT LIVER MITOCHONDRIA

SALVATORE PASSARELLA, PAOLO RICCIO, ERSILIA MARRA and ERNESTO QUAGLIARIELLO
CNR Unit for the Study of Mitochondria and Bioenergetics, and Department of Biochemistry, University of Bari, 70126 Bari, Italy

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Abstract—The effect of 5-ethyl, 5-isoamyl barbituric acid (amytal) on the permeability of the mitochondrial membrane has been investigated using rat liver mitochondria. It was found that amytal inhibits the malonate–malonate exchange, mediated by the dicarboxylate carrier, in a purely non-competitive manner. Furthermore, amytal causes release of intramitochondrial Pi from the matrix in the presence of *N*-ethylmaleimide. Some features of the Pi efflux induced by amytal in rat liver mitochondria have been studied. The efflux shows saturation characteristics, pH and temperature dependence according to a carrier-mediated process. It is suggested that the dicarboxylate carrier caused the efflux of Pi induced by amytal. The existence of subunits in the molecule of the dicarboxylate carrier is tentatively proposed.

For a long time barbiturates have been known to inhibit mitochondrial respiration. 5-Ethyl, 5-isoamyl barbituric acid (amytal) has been used widely in addition to the fish poison rotenone [1] as a specific agent for blocking the aerobic oxidation of substrates linked to pyridine nucleotides. Moreover, amytal, unlike rotenone, has been found to influence some energy transfer reactions such as the inorganic orthophosphate–ATP exchange [2] and the dinitrophenol-induced adenosine triphosphatase [3]. Inhibition of succinate oxidation by amytal has been mentioned by several authors [4–6] and more recently the inhibition of the metabolite permeation by barbiturates has been proposed in rat liver mitochondria, and amytal found to be a competitive inhibitor of succinate oxidation and transport [7]. On the other hand, a purely non-competitive inhibition by amytal of the oxoglutarate–oxoglutarate exchange in rat liver mitochondria has been reported [8]. However, knowledge about the molecular effect of this drug on the permeability properties of the mitochondrial membrane is far from being clarified at the moment. In view of the importance of the transport processes in mitochondrial bioenergetics and in cellular activity, we decided to investigate the effect of barbiturates, namely amytal, on the activity of the dicarboxylate carrier and generally on the permeability of the mitochondrial membrane in rat liver mitochondria.

This study aims to increase the general knowledge of the influence shown by different drugs on the carrier-mediated transport of substrates in mitochondria. With regard to this, the effect of the diuretic ethacrynic acid on the permeability of the mitochondrial membrane in rat liver mitochondria has been recently reported [9]. The reported results demonstrate that amytal does inhibit the dicarboxylate carrier and that the mechanism of the inhibition depends on the nature of the translocated anions. Moreover, amytal causes release of inorganic phosphate from the mitochondrial matrix.

METHODS

Preparation of rat liver mitochondria. All studies utilized 200–250 g male Wistar rats. Mitochondria were isolated in 0.25 M sucrose, 20 mM Tris–HCl, (pH 7.25) and 1 mM EGTA, as previously described [10]. The final mitochondrial pellet was suspended in the same medium to give a protein concentration between 40 and 60 mg/ml.

Loading of mitochondria with labelled or unlabelled metabolites. This was achieved essentially according to [11]. The mitochondria (40–50 mg protein) were incubated at 20° in 10 ml of medium consisting of 100 mM KCl, 1 mM EGTA, 20 mM Tris–HCl (pH 7.0), in one case with Pi and in the other with malonate (2 mM). After 2 min the mitochondria loaded with malonate were washed in the medium without the metabolites and then suspended (40–50 mg of mitochondrial protein/ml). To the mitochondria loaded with Pi, on the other hand, 1.5 mM *N*-ethylmaleimide (NEM) was added after 2 min, and 1 min later the mitochondria were washed and suspended as described. In the presence of Pi, 5 μg/ml of oligomycin was added to inhibit the incorporation of Pi into ATP. In some experiments the mitochondria were labelled with ^{32}Pi. This was achieved by loading the mitochondria with Pi as described above and by introducing into the suspension carrier-free ^{32}Pi in amounts of 1 μCi/ml each. Equilibration of the radioactive isotope between the extramitochondrial and intramitochondrial pools of Pi is obtained after 10 min at 0°. Oligomycin was present throughout the entire procedure. The mitochondria loaded in this way and suspended for 2 min at 8° in 1 ml of reaction mixture in the absence of counteranion contained metabolites at a concentration ranging between 14 and 36 mM, whereas the extramitochondrial concentration was less than 10 μm.

Measurement of the rate of oxygen uptake. Mitochondrial oxygen uptake was measured polarograph-

ically at 26° with a Clark electrode using an incubation medium containing 0.2 M sucrose, 20 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonate (HEPES)–Tris (pH 7.0), 1 mM $MgCl_2$, 10 mM KCl and 3 μg/ml rotenone. Mitochondria (1–2 mg protein) were added and the assay was started by the addition of 5 mM succinate, followed by the addition of carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (final concentration 1 μM). At the time indicated, phenyl succinate, butylmalonate, mersalyl, bathophenanthroline and amytal at the indicated concentrations were added.

Measurement of the Pi efflux. Mitochondria labelled with ^{32}Pi (about 2 mg of protein) were incubated at 8° in 1.0 ml of medium containing 0.2 M sucrose, 20 mM HEPES–Tris (pH 7.0), 1 mM $MgCl_2$, 10 mM KCl, 1 mM NEM and 1 μg rotenone. In some reactions the medium included one of the inhibitors mersalyl, bathophenanyhroline, phenylsuccinate or butylmalonate. After 1 min the reaction was started by the addition of amytal, sulphate or Pi and terminated 30 sec later either by centrifugation in an Eppendorf microcentrifuge for amytal-induced efflux, or by addition of 20 mM butylmalonate when the efflux of Pi was induced by sulphate or Pi.

The percentage of efflux was calculated according to the equation: percentage of efflux = 100 $(Ca - Cp)/Ca$, where Cp represents the radioactivity (c.p.m.) measured in the presence of amytal or other substrates and Ca represents the radioactivity measured in the absence of any substrate. The results were corrected for small time-dependent spontaneous leakages of radioactivity.

Measurement of uptake of substrate. The kinetics of the uptake were studied by using the inhibitor stop method essentially according to the procedure described previously [11]. Mitochondria loaded with the metabolite indicated were incubated at 8° in 1 ml of medium consisting of 0.2 M sucrose, 20 mM HEPES–Tris (pH 7.0), 1 mM $MgCl_2$, 10 mM KCl, 1 μg rotenone and 3 μg oligomycin. After 1 min the assay was started by the addition of labelled substrate and terminated at the time indicated by rapid addition of 20 mM butylmalonate. During the first 4–6 sec the rate of uptake was constant within the limits of experimental error.

Other methods. After rapidly centrifuging the mitochondria in an Eppendorf microcentrifuge for 1 min at 0°, the radioactivity in the pellet and supernatants was measured as previously described [11, 12]. The mitochondrial protein was determined by a modified biuret method [13]. Inorganic phosphate was estimated by the method of Beremblum and Chain [14]. To measure the amount of metabolites in the matrix, the space available to ^3H_2O and ^{14}C-sucrose was determined in parallel experiments as previously described [11, 12].

RESULTS

The effect of amytal on the activity of the dicarboxylate carrier was investigated by measuring the possible inhibition by the barbiturate on the rate of uptake of malonate and Pi measured according to the stop inhibitor method. Figure 1 shows the dependence of the rate of malonate–malonate exchange on increasing concentrations of amytal, investigated according to Dixon. The concentrations of malonate were 0.2 and 0.06 mM. Amytal gives a non-competitive inhibition, for which the value of K_i was 6.5 mM. The inset, in which the reciprocal plots of the fractional inhibition (i) against inhibitor concentrations are reported as advised (see ref. 15), shows that linear functions are obtained which intersect the ordinate at unit, indicating that amytal is a pure non-competitive inhibitor with respect to malonate entry into rat liver mitochondria. It should be noted that under the conditions used the exchange is catalysed entirely by the dicarboxylate carrier, given that the affinity of malonate for the oxoglutarate carrier is much lower [16].

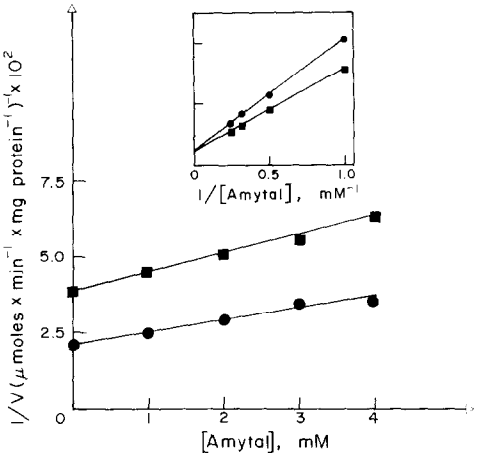

Fig. 1. Kinetic analysis of the inhibition of the malonate–malonate exchange by amytal using the Dixon plot. Malonate-loaded mitochondria (1.7 mg protein) were incubated for 1 min at 8° in 1.0 ml of standard medium consisting of 0.2 M sucrose, 20 mM HEPES–Tris (pH 7.0), 1 mM $MgCl_2$, 10 mM KCl, 1 mM *N*-ethylmaleimide and 3 μg rotenone. The reaction was started by the addition of 0.2 (●) or 0.06 mM (■) malonate added in the presence or absence of increasing concentrations of amytal. The exchange was stopped 6 sec later by rapid addition of 20 mM butylmalonate and the rate of uptake calculated as described in Methods. The inset is a replot of the data where $i = 1 - v_i/v; v_i$ and v are the rates of malonate uptake in the presence or in the absence of amytal, respectively.

It is known that the dicarboxylate carrier can catalyse the uptake of dicarboxylates and Pi in exchange with intramitochondrial Pi [8]; thus the effect of amytal on the rate of malonate–Pi and Pi–Pi exchange was investigated by using Pi-loaded mitochondria. Amytal inhibits both these exchanges; however, the Dixon plots do not show straight lines when the malonate and Pi concentrations are 0.2 mM and 0.5 mM, respectively, so the results shown report the per cent of the control increasing concentrations of amytal. It appears that amytal strongly inhibits both the reactions and 50 per cent of the control is obtained at about 2.5 mM amytal (Fig. 2, panels a and b).

The amount of the intramitochondrial Pi was measured in the pellet of the same mitochondrial preparation after stopping the uptake of the substrates in the presence or in the absence of amytal. The dependence of the intramitochondrial content

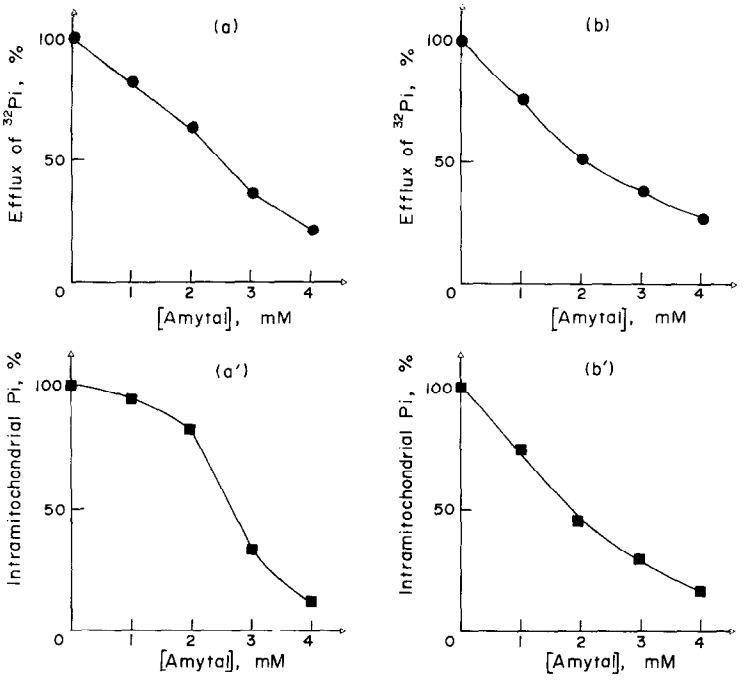

Fig. 2. Inhibition by amytal of the rate of malonate–Pi exchange and Pi–Pi exchange and dependence of the intramitochondrial content of Pi on increasing concentrations of amytal. In Panels a and b, Pi loaded mitochondria (1.4 mg protein in a and 1.55 in b), were incubated under experimental conditions as in Fig. 1. The rate of uptake of 0.2 mM malonate (a) and 0.5 Pi (b) was measured as previously described in the absence or presence of increasing concentrations of amytal. The intramitochondrial inorganic phosphate content was measured in aliquots or perchloric extract of the mitochondria obtained in the experiment reported in panels a and b and plotted in panels a' and b', respectively, as a function of the amytal concentration. In a' the amount of Pi in the absence of amytal was 36 nmoles/mg protein, in b' 14 nmoles/mg protein.

of Pi on the concentration of amytal is described in Fig. 2, panels a' and b'. Surprisingly, in the presence of amytal, which inhibits the malonate/Pi and Pi/Pi exchange, the amount of the intramitochondrial Pi strongly decreases with respect to that present in the mitochondria after the reaction of uptake in the absence of inhibitor. It should be noted that, under the experimental conditions used (reaction stopped after 6 sec uptake), only 3 nmoles of internal Pi could be extruded in exchange for the malonate taken up, according to a malonate/Pi exchange. Moreover, no change in the intramitochondrial Pi content occurs in the case of Pi–Pi exchange. Thus the presence of amytal in the reaction medium causes the release of Pi from the mitochondrial matrix.

It has been reported that amytal does not inhibit mitochondrial swelling in ammonium phosphate and that the uptake of Pi is inhibited by amytal only if this compound is preincubated with mitochondria [7]. We tested the effect of amytal on the exchange arsenate–Pi catalysed by the Pi carrier. Inhibition was found. This datum, together with [7], is not consistent with the possibility of efflux of Pi induced by amytal via Pi carrier. The possibility of a reaction of efflux mediated by the dicarboxylate carrier was examined.

Firstly, the sensitivity of the efflux of Pi to known inhibitors of the dicarboxylate carrier caused by amytal was examined in Pi-loaded mitochondria. Phenylsuccinate and butylmalonate, compounds which bind to the dicarboxylate binding site of the carrier [17], were tested with respect to their ability to inhibit Pi efflux induced by amytal in the presence of NEM. Phenylsuccinate, up to a concentration of 9 mM, does not inhibit the efflux of Pi in 30 sec as well as does butylmalonate. No inhibition was found even if the dicarboxylates were added to the mitochondrial before amytal.

The mercurial mersalyl and the metal-complexing agent bathophenanthroline, both used in concentrations up to 150 μM, were also tested with respect to their ability to inhibit the amytal-induced efflux of Pi. Both these compounds are competitive inhibitors of the dicarboxylate uptake, but non-competitive inhibitors of the Pi uptake via the dicarboxylate carrier [18, 19]. The efflux of Pi appears to be insensitive to these compounds, even if used at high concentration or if preincubated with the mitochondria.

In order to investigate further whether simple diffusion or carrier-mediated efflux of Pi occurs, some features of the reaction have been investigated. The time course of the efflux of Pi caused by the addition of amytal to the mitochondrial suspension was studied. As a comparison in the same experiment, the sulphate–Pi exchange was measured (Fig. 3). It is known that sulphate is a specific substrate of the dicarboxylate carrier [20, 21]. After 30 sec incubation with 1 mM amytal, roughly maximum exchange was achieved; the amount of Pi effluxed at equilibrium appeared to be rather lower with

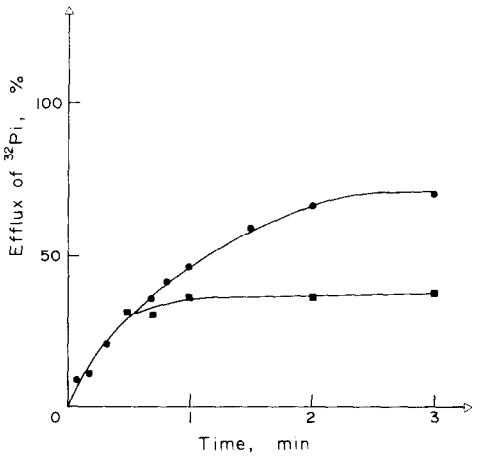

Fig. 3. Time course of the efflux of Pi induced by amytal or sulphate. Pi-loaded mitochondria (1.8 mg protein) were incubated under the experimental conditions described in Fig. 1. The efflux was started by the addition of 1 mM amytal (■) or 1 mM sulphate (●) and stopped at the time indicated by rapid centrifugation for amytal or by the addition of 20 mM phenylsuccinate for sulphate. For further details see Methods.

respect to that obtained when 1 mM sulphate was added; however, in this case the equilibrium was reached after 3 min incubation. The dependence of the efflux of Pi on the concentration of amytal was also investigated. The results of a typical experiment are reported in Fig. 4 as a double reciprocal plot. Saturation characteristics have been found. The maximum efflux occurring in the time allowed for the measurement (30 sec) was about 90 per cent, corresponding to 57 nmoles/min/mg protein. It should be noted that this value is fairly consistent with the value of V_{max} for the dicarboxylate carrier [8]. The apparent K_m, i.e. the concentration of amytal which gives half maximum efflux, is 4 mM in this experiment. In four other experiments it was found that the K_m value ranged from 2.5 to 4 mM, whereas the maximum exchange corresponded to 55–110 nmoles/min/mg protein. It should be noted that the different values of K_m very likely reflect the different intramitochondrial content of Pi.

The pH dependence of the efflux of Pi induced on the one hand by 2 mM amytal and on the other by 0.5 mM Pi was compared in the same experiment. Similar patterns are obtained; the optimum pH for both the reactions is 6.75 (Fig. 5). The activation energy for the Pi efflux induced by amytal or Pi was also measured by means of the Arrhenius plot between 4 and 25°. For both the reactions, the Ea value was 29 Kcal/mole.

The saturation characteristics, the high temperature and the characteristic pH dependence suggest the possibility that the efflux of Pi may be a carrier-mediated process. The dicarboxylate carrier seem to be the best candidate for this reaction, as suggested by the similar pH dependence and by the same value for the activation energy for both Pi- and amytal-stimulated efflux of Pi.

This conclusion, however, is not consistent with the insensitivity shown by the amytal-stimulated efflux to known inhibitors of the dicarboxylate carrier such as dicarboxylate analogues, mersalyl and bathophenanthroline. The possibility that amytal could in some manner influence the inhibition by these compounds on the dicarboxylate carrier activity was tested; the succinate oxidation stimulated by the umcoupler FCCP was examined. It was reported in fact that the succinate oxidation is controlled by the activity of the dicarboxylate carrier [22]. The effects of phenylsuccinate, butylmalonate, mersalyl and bathophenanthroline on the respiration of succinate were investigated both in the presence and in the absence of amytal. Typical experiments are summarized in Table 1. In all cases it was found that the

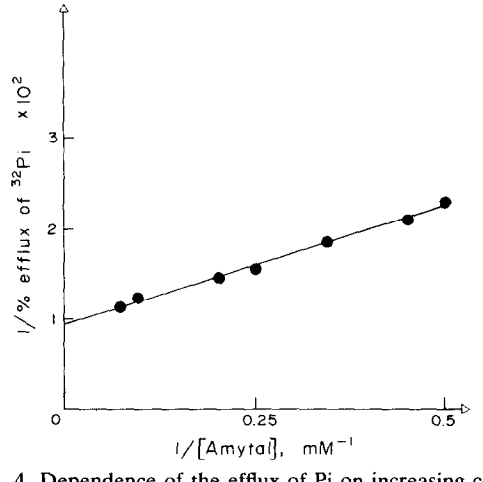

Fig. 4. Dependence of the efflux of Pi on increasing concentrations of amytal using the double reciprocal plot. ^{32}Pi-loaded mitochondria (2 mg of protein) were incubated under the experimental conditions described in Fig. 1. The efflux was started by the addition of amytal at the indicated concentration and stopped 30 sec later by rapid centrifugation as described in Methods.

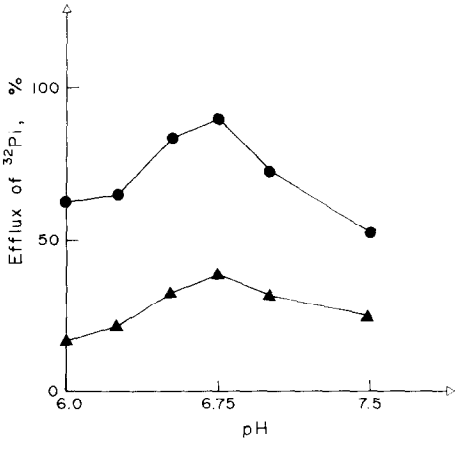

Fig. 5. pH dependence of the efflux of Pi induced by amytal or Pi. Pi-loaded mitochondria (2.1 mg of protein) were incubated under the experimental conditions described in Fig. 1, except that the pH of the medium was as indicated. The efflux was started by the addition of 2 mM Amytal (▲) or 0.5 mM Pi (●) and stopped, respectively, after 30 sec by rapid centrifugation or after 6 sec by addition of 20 mM butylmalonate. For further details see Methods.

rate of oxidation is strongly decreased by the combination of inhibitors and amytal. If amytal is added 1 min after the inhibitor, the resulting inhibition is significantly similar to the sum of the inhibitions given by amytal and inhibitors added, in different experiments. But if amytal is added before the inhibitor, the resulting inhibition is less than this sum. This finding could be interpreted in view of a possible interaction between the carrier and the inhibitor, the carrier and amytal, or between all three in such a way that the affinity of the inhibitor and/or amytal to the carrier decreases.

This result was investigated further by testing the effect of amytal in influencing the ability of 20 mM phenylsuccinate to inhibit the rate of exchange malonate/malonate and Pi/Pi in malonate or Pi-loaded mitochondria. The reaction was started by addition of the substrate in the presence of 20 mM phenylsuccinate, alone or plus amytal. The mitochondria were collected and the radioactivity measured as described in Methods. The data reported reflect the aspecific binding of the substrate and the amount of substrate taken up in the presence of the inhibitor. Amytal slightly decreases the amount of malonate taken up in malonate-loaded mitochondria

Table 1. Effect of amytal on the inhibition by phenylsuccinate, butylmalonate, mersalyl and bathophenanthroline of succinate oxidation stimulated by FCCP*

Time of addition	Additions	V	Inhibition (%)
Expt. I	Succinate FCCP	171	—
1 min	BP	97	45
2 min	Amy	43	75
Expt. II	Succinate FCCP	166	
1 min	Amy	113	32
2 min	BP	67	60
Expt. III	Succinate FCCP	126	
1 min	BuMa	49	61
2 min	Amy	9	93
Expt. IV	Succinate FCCP	142	
1 min	Amy	79	45
2 min	BuMa	43	70
Expt. V	Succinate FCCP	162	
1 min	Mersalyl	103	36
2 min	Amy	0	100
Expt. VI	Succinate FCCP	160	
1 min	Amy	97	40
2 min	Mers	59	73
Expt. VII	Succinate FCCP	171	
1 min	PheSu	62.5	54
2 min	Amy	22	94
Expt. VIII	Succinate FCCP	136	
1 min	Amy	83	40
2 min	PheSu	33	86

* Mitochondria (1.6 mg of protein) were incubated in 1 ml of medium consisting of 0.2 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES–Tris (pH 7.0) at 26°. Succinate (5 mM) was added, followed 30 sec later by 1 μM FCCP. The following additions were made after 1 min and 2 min as indicated in the table at the following concentrations: phenylsuccinate (PheSu) 3 mM, butylmalonate (BuMa) 1 mM, mersalyl (Mers) 70 μM and bathophenanthroline (BP) 50 μM. The concentration of amytal (Amy) was 1 mM (Expts. I–II) or 2 mM (Expts. III–VIII). The rate of oxygen uptake V was expressed as natoms O_2/min/mg protein.

Table 2. Effect of amytal on the inhibition of [^{14}C] malonate and [^{32}Pi] uptake by phenylsuccinate in malonate or Pi-loaded mitochondria*

Additions	Substrate associated with the mitochondria (nmoles/mg protein)	
Expt. I		
0.2 mM malonate	2.1	2.5
0.2 mM malonate + 2 mM amytal	1.4	1.4
0.2 mM malonate + 4 mM amytal	0.85	1.0
Expt. II		
0.2 mM malonate	0.65	0.7
0.2 mM malonate + 2 mM amytal	0.7	0.7
0.2 mM malonate + 4 mM amytal	0.4	0.35
Expt. III		
0.04 mM malonate	0.4	0.4
0.04 mM malonate + 2 mM amytal	0.25	0.3
0.04 mM malonate + 4 mM amytal	0.25	0.3
Expt. IV		
0.5 mM Pi	3.00	3.1
0.5 mM Pi + 0.5 mM amytal	3.8	4.25
0.5 mM Pi + 2 mM amytal	4.8	4.0
0.5 mM Pi + 3 mM amytal	5.1	6.5

* Malonate-loaded mitochondria (Expts. I–III) or Pi-loaded mitochondria (Expt. IV) were incubated for 1 min under the experimental conditions as in Fig. 1 in the presence of 20 mM phenylsuccinate. The reaction was started by the addition of [^{14}C]malonate (Expts. I–III) or [^{32}Pi](Expt. IV) in the absence or in the presence of amytal at the indicated concentrations. After 5 sec the mitochondria were centrifuged in an Eppendorf microcentrifuge and the nmoles of labelled substrate associated to the mitochondria were measured as reported in Methods. Mitochondrial protein was 1.2 (Expt. I–III) or 1.3 mg (Expt. IV). The data are reported in duplicate.

in the presence of phenylsuccinate; in contrast, when the Pi–Pi exchange is studied, amytal strongly decreases the ability of phenylsuccinate to inhibit the uptake of Pi (Table 2). Thus amytal is a poor inhibitor of the exchange malonate–malonate (K_i = 6.5 mM) and its inhibition is added to that of phenylsuccinate. But, on the contrary, if Pi is involved in the exchange, amytal is a strong inhibitor and prevents inhibition by phenylsuccinate.

DISCUSSION

The results reported in this paper give further insight into the effect of barbiturates on the structure and function of the mitochondrial membrane. It is confirmed that amytal inhibits metabolite anion transport. An additional effect, moreover, has to be considered: efflux of Pi from the matrix caused by amytal in the presence of *N*-ethylmaleimide. Amytal inhibits the transport of citric cycle intermediates in rat liver mitochondria [7]. As regards the dicarboxylate carrier, non-competitive inhibition of the malonate–malonate exchange has been found with a high value of K_i. The dicarboxylate carrier also catalyses dicarboxylate–Pi or Pi–Pi exchange. The mechanism of inhibition, however, is different: it is stronger and no linear functions can be obtained in the Dixon plot. Given that amytal promotes Pi efflux and that

the intramitochondrial content of Pi influences the activity of the dicarboxylate carrier, it is worth considering that the inhibition by amytal could be due to the decreased intramitochondrial content of Pi. The succinate oxidation and transport was inhibited in a competitive manner with a low value of K_i [7]. However, these indirect measurements were carried out in the presence of 5 mM Pi in such a way that the experimental conditions appear to be rather different with respect to the uptake measured by the stop inhibitor method. The presence of Pi in the external medium could possibly determine an active state of the carrier causing high affinity for amytal and competitive inhibition. Besides the malonate–malonate exchange, the oxoglutarate–oxoglutarate exchange via the oxoglutarate carrier is inhibited also, in a purely non-competitive way [8]. Thus the proposal of an electrostatic interaction between the anions and the membrane negatively charged by amytal in such a way that a negative surface potential would repel negatively-charged metabolites [7] is not consistent with the nature of the inhibition of the dicarboxylate and oxoglutarate carrier which was found. It should be noted that amytal causes a slight efflux of substrate from substrate-loaded mitochondria: oxoglutarate and malate (in small amounts) and Pi (in large amounts) can be extruded from the mitochondria. Thus the inhibition of metabolite transport could be due to an interaction of the mitochondrial membrane with amytal which reduces the activity of the carrier, possibly by allowing release of intramitochondrial or substrates which have just entered. The efflux of Pi from the mitochondria induced by amytal appears to proceed according to a carrier-mediated process. The saturation characteristics, the high dependence on temperature and the sensitivity to pH are in favour of this conclusion. The dependence on temperature and pH of the efflux of Pi induced by Pi, is the same as the dependence on temperature and pH of the efflux of Pi induced by amytal. This, together with the maximum efflux induced by amytal, similar to the V_{max} value of the dicarboxylate carrier [8], suggests that the efflux of Pi induced by amytal is catalysed by this carrier However, the mechanism of the efflux of Pi via the dicarboxylate carrier appears not to be consistent with the evidence that the dicarboxylates phenylsuccinate and butylmalonate, as well as the mercurial mersalyl and the metal-complexing agent bathophenanthroline, well-known inhibitors of the dicarboxylate carrier, do not inhibit Pi efflux induced by amytal. But this discrepancy can be overcome by considering that it has been found that amytal by itself decreases the affinity of these inhibitors to the carrier as revealed by the measurements of succinate oxidation stimulated by FCCP and by the results reported in Table 2. Thus the possible hypothesis arises that amytal 'uncouples' the transport of dicarboxylates from the transport of Pi, probably by inducing a conformational change and/or a bound leakage in the carrier molecule. This phenomenon could cause the Pi release from the matrix. Therefore the amytal inhibits in addition to butylmalonate when malonate–malonate exchange occurs. However, when Pi–Pi exchange occurs or succinate uptake in exchange with Pi is tested, the addition of amytal decreases the power of butylmalonate and the other inhibitors to inhibit Pi or succinate uptake, even if real uptake is inhibited by amytal. Thus two subunits can be proposed tentatively in the carrier molecule, probably sensitive to amytal. In the absence of amytal dicarboxylate analogues and the other inhibitors inhibit the transport of Pi. In the presence of amytal, however, the linkage of the two hypothetical subunits should change so that the (non-competitive) inhibition of the Pi uptake disappears. Furthermore, it is assumed that the Pi subunit translocates Pi from the mitochondria according to a concentration gradient. The dicarboxylate carrier, independent of the intramitochondrial Pi content, could be influenced by amytal in such a way that the activity decreases. It is clear that other experimental data are required to substantiate this proposal and more attention should be given to the role of *N*-ethylmaleimide. However, the suggested possibility of different subunits in the carrier molecule offer the possibility of speculation mainly in the mechanism of regulation of the carrier activity which could occur *in vivo* by means of subunit–substrate interactions. It should be noted that two different subunits have been described in the ADP–ATP carrier [23].

The efflux of Pi induced by amytal from fresh or Pi-loaded mitochondria could be utilized in order to study the dependence of uptake of substrate on the intramitochondrial concentration of Pi in addition to the possibility of washing out amytal from mitochondria maintaining the same rate of respiration [1]. Similar studies were carried out by Sluse *et al.* in dicarboxylate-loaded rat heart mitochondria (see ref. 8). The findings reported in this paper offer further explanations of many results obtained some years ago when the carrier-mediated processes were not known and no possibility could be imagined concerning the possible efflux of substrates induced by amytal. The inhibitory effect shown by amytal on Pi–ATP exchange (e.g. [2]) could be explained considering the decrease of the intramitochondrial content of Pi due to the presence of amytal. The extension of these studies to other barbiturates, such as seconal, dial, veronal, etc., should be interesting. We found that barbituric acid neither inhibits oxoglutarate–oxoglutarate exchange nor causes efflux of Pi, so the presence of the aliphatic chain in the barbiturate molecule appears to be relevant for the possibility of the drug interacting with the mitochondria. It is not possible to know what is the effect of barbiturates on the mitochondrial metabolism *in vivo*; drug administration to animals and studies on isolated cells and mitochondria should be done. Studies in this field are in progress.

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