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INHIBITION OF ANION TRANSPORT ACROSS THE MITOCHONDRIAL MEMBRANE BY AMYTAL

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SUMMARY

It has been found that amytal competitively inhibits succinate (+ rotenone) oxidation by intact uncoupled mitochondria. Similar results were obtained in metabolic state 3, the K_i value being 0.45 mM. Amytal did not affect succinate oxidation by broken mitochondria and submitochondrial particles (at a concentration which inhibited succinate oxidation by intact mitochondria). Amytal inhibited the swelling of mitochondria suspended in ammonium succinate or ammonium malate but was without effect on the swelling of mitochondria in ammonium phosphate and potassium phosphate in the presence of valinomycin+carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

Using [^{14}C]succinate and [^{14}C]citrate it has been shown that amytal inhibited the succinate/succinate, succinate/P_i, succinate/malate, and citrate/citrate and citrate/malate exchanges. Amytal inhibited P_i transport across mitochondrial membrane only if preincubated with mitochondria. Other barbiturates: phenobarbital, dial, veronal were found to inhibit [^{14}C]succinate/anion (P_i, succinate, malonate, malate) exchange reactions in a manner similar to amytal. It is concluded that barbiturates non-specifically inhibit the dicarboxylate carrier system, tricarboxylate carrier and P_i translocator. It is postulated that the inhibition of succinate oxidation by barbiturates is caused mainly by the inhibition of succinate and P_i translocation across the mitochondrial membrane.

INTRODUCTION

For a long time barbiturates have been known to inhibit mitochondrial respiration. Amytal has been the barbiturate widely used as a specific agent for blocking electron transport in the region of the NAD-linked flavoprotein. However, it is now known that barbiturates can also influence mitochondrial reactions other than NADH oxidation. Brody and Bain [1] showed that barbiturates inhibit phosphorylation to a greater extent than oxidation. Siekevitz et al. [2] found that amytal inhibits 2,4-dini-

Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; EGTA, ethyleneglycolbis-(β-aminoethylene)-*N,N'*-tetraacetic acid.

trophenol-stimulated ATPase activity in rat liver mitochondria. Löw et al. [3] reported that amytal inhibited the ATP/P_i exchange reaction of liver mitochondria. Chance and Hollunger[4] postulated that amytal affected the energy transfer reactions at the first site. An inhibition of succinate oxidation by amytal was also mentioned by Chance and Hollunger [5] and by Pumphrey and Redfearn [6, 7]. According to Pumphrey and Redfearn[7] barbiturates inhibit succinate oxidation by interfering with the energy transfer reaction of oxidative phosphorylation. However, it is difficult to explain why the inhibition by amytal of succinate oxidation was not reversed by 2,4-dinitrophenol. On the other hand the amytal inhibition of succinate oxidation may be prevented by disruption of the mitochondria or by the addition of Ca^{2+} which is known to cause large swelling and damage of the mitochondria structure. These results suggested that inhibition of succinate oxidation by amytal might be caused by inhibition of succinate transport into the mitochondria. The results presented below provide evidence which indicates that barbiturates might affect the permeability of the mitochondrial membrane for the metabolite anion.

METHODS

Rat liver mitochondria were prepared as described previously[8]. Mitochondrial protein was determined by ultraviolet absorption [9, 10].

Measurement of mitochondrial respiration

Respiration was measured with a Clark oxygen electrode at room temperature in 2.4 ml of medium (pH 7.4) containing: 15 mM KCl, 50 mM Tris–HCl, 5 mM $MgSO_4$, 5 mM potassium phosphate, 2 mM EDTA, 4 μg rotenone, 1 mM ADP or 1 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and succinate–Tris as indicated in the figures. The reaction was started by the addition of 50 μl of mitochondrial suspension. Other additions were as indicated in the legend to the figures. Amytal was dissolved in ethanol and added in a volume of 10 μl.

Measurement of mitochondrial swelling

Mitochondrial swelling was recorded at 546 nm on a Unicam SP-800 spectrophotometer. For experimental conditions see the legends to the corresponding figures.

The variation of extramitochondrial pH corresponding to the uptake of malate was monitored as described by Michejda et al. [11].

Isotopic assays of anion translocation

Methods used for the determination of the [^{14}C]succinate and [^{14}C]citrate exchange reaction as well as for [^{14}C]succinate and [^{14}C]citrate loading were the same as those used by Robinson and Williams [12] and Halperin et al. [13], respectively. The measurement of the kinetics of $^{32}P_i$ uptake was the same as that applied by Meisner [14]. For radioisotopic experiments all the barbiturates were used as potassium salts. For the particular conditions, see the legends of the tables.

Chemicals

Mersalyl, rotenone, antimycin A, oligomycin, succinate, phenazine methosulphate, Triton X-100, amytal (5-ethyl,5-isoamylbarbituric acid) were purchased from

Sigma Chem. Co.; malate, malonate from Koch–Light; citrate from P.O.Ch., Gliwice, Poland; 1,2,3-benzenetricarboxylic acid from Merck–Schuchardt; [1,5-$^{14}C_2$] citrate from the Radiochemical Centre, Amersham; [1,4-$^{14}C_2$]succinate from Isocommerz GmbH (G.D.R.); $^{32}P_i$ from I. B. J., Świerk (Poland). Butylmalonate was a generous gift from Dr Popinigis. Other barbiturates examined: phenobarbital (5-ethyl,5-phenylbarbituric acid), dial (5,5-diallylbarbituric acid), and veronal (5,5-diethylbarbituric acid) were kindly provided by Polfa, Warszawa, Poland.

RESULTS

Effect of amytal on mitochondrial respiration with succinate as substrate

The effect of amytal on succinate oxidation in the uncoupled state is shown in Fig. 1. Amytal at a concentration of 4 mM caused an immediate inhibition of succinate oxidation. This inhibition was reversed by 4 mM succinate but not by other anions examined such as citrate, isocitrate, malate, glutamate or pyruvate. The inhibitory effect of amytal on succinate oxidation in the uncoupled state was compared with the effect of a known inhibitor of the dicarboxylic acid transporter butylmalonate[12]. Data presented on Fig. 1 show that 4 mM amytal inhibited succinate oxidation in a similar way to 2 mM butylmalonate. The same concentration of amytal produced only a slight inhibition of succinate oxidation by broken mitochondria (Fig. 1C) or submitochondrial particles (not shown). The inhibitory effect of amytal on succinate oxidation was also observed in metabolic state 3. Both ADP- and FCCP-stimulated succinate oxidation was found to be inhibited by amytal, the inhibition being reversible by higher concentrations of succinate. The results obtained in these experiments are presented as Lineweaver–Burk plots (Fig. 2), and reveal an apparent competition between succinate and amytal. The results, presented in the form of a Dixon plot (Fig. 3), showed that the K_i value obtained in these conditions for amytal is about 0.45 mM.

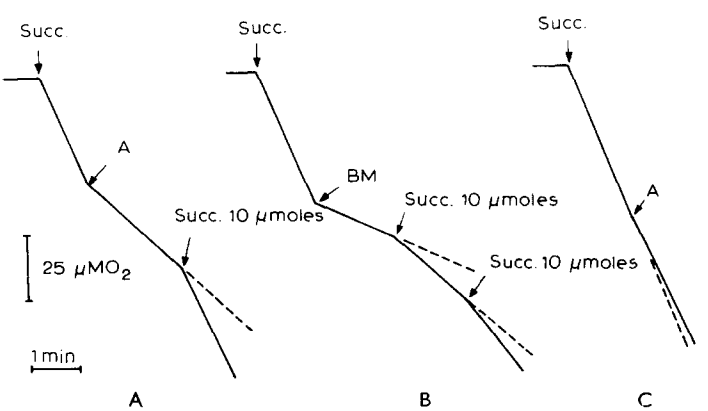

Fig. 1. Effect of amytal (A and C) and butylmalonate (B) on succinate oxidation. The basis experimental conditions were as described under Methods. Succ, 2 mM succinate; A, 4 mM amytal; BM, 2 mM butylmalonate. The concentration of mitochondrial protein was 2.6 mg. In Expt C (broken mitochondria) 0.06 % Triton X-100 was added to the incubation medium and 0.1 mg/ml phenazine methosulphate as an autooxidizable electron acceptor from succinate dehydrogenase.

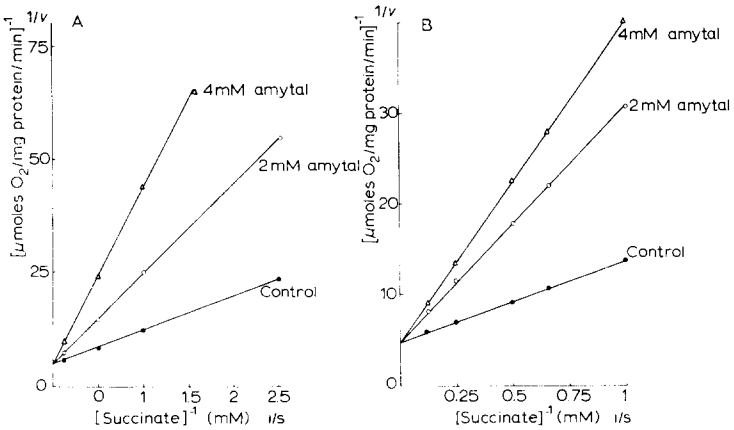

Fig. 2. Double-reciprocal plots of the rate of succinate oxidation against succinate concentration in the presence of various concentrations of amytal as indicated. (A) Succinate oxidation, ADP stimulated. (B) Succinate oxidation, FCCP stimulated. Experimental conditions were as described under Methods. Protein concentration, 2.4 mg.

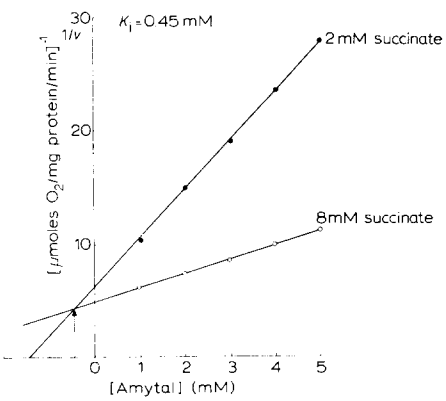

Fig. 3. Inhibition by amytal of succinate oxidation stimulated by ADP presented in the form of a Dixon plot. Experimental conditions were as described under Methods. Protein concentration, 2.2 mg.

Effect of amytal on the swelling of mitochondria in ammonium succinate, malate and phosphate

The competitive inhibition of succinate (+rotenone) oxidation in intact mitochondria and the insensitivity of succinate oxidation by broken mitochondria or submitochondrial particles to the same concentration of amytal suggested that the action of the inhibitor was on the entry of substrate via the dicarboxylic acid carrier system. Succinate and malate transport into mitochondria was, therefore, examined by studying the swelling of mitochondria in solutions of ammonium succinate (Fig. 4A) and ammonium malate (Fig. 4B) containing ammonium phosphate at a final concentration of 3 mM. Mitochondrial swelling in these conditions was inhibited by amytal. The results of experiments presented on Figs. 4A and 4B suggest that amytal is an inhibitor of the dicarboxylic acid transporter. However, it is known that inhi-

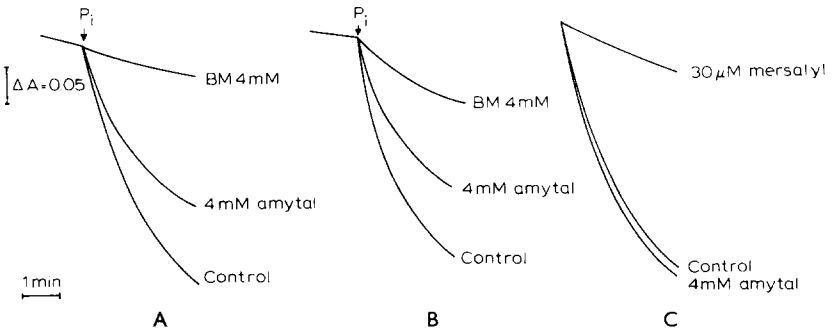

Fig. 4. Effect of amytal on mitochondrial swelling in ammonium succinate (A), ammonium malate (B), and ammonium phosphate (C). The swelling was measured as described under Methods. The incubation medium had the following composition: 100 mM ammonium salts of either succinate, malate or phosphate, 5 mM Tris–HCl (pH 7.4), 0.1 mM ethyleneglycolbis-(β-aminoethylene)-*N,N'*-tetraacetic acid (EGTA), 2 μg rotenone, 2 μg antimycin A in a final volume 2.5 ml. The swelling in Expts A and B was initiated by the addition of 3 mM ammonium phosphate. Mitochondrial protein was 1.5 mg (A and B) and 0.9 mg (C). Other additions as indicated on the figure. Temperature in A and B was 30 °C, in C 20 °C.

bition of mitochondrial swelling suspended in ammonium succinate or in ammonium malate might be inhibited by an agent which inhibits phosphate translocation across the mitochondrial membrane [15]. Therefore, we decided to investigate the effect of amytal on mitochondrial swelling in ammonium phosphate. Data presented in Fig. 4C show that amytal was without effect on mitochondrial swelling in ammonium phosphate. Lack of the effect of amytal on the penetration of phosphate was also observed in the presence of potassium phosphate plus valinomycin and FCCP, a system known to induce mitochondrial swelling, as shown by Meijer [16].

Effect of amytal on pH change accompanying malate transport into mitochondria

Papa et al. [17] showed that the malate/citrate exchange diffusion is accompanied by translocation of protons across the mitochondrial membrane in the same direction as citrate; P_i/malate exchange is accompanied by proton translocation in

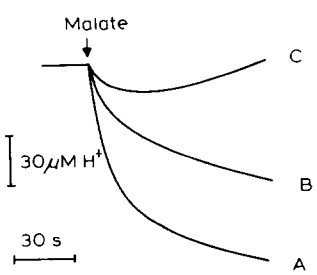

Fig. 5. Effect of amytal on proton movements accompanying the penetration of malate into mitochondria. Mitochondria (14.6 mg) were preincubated in 3.5 ml of medium containing: 125 mM KCl, 0.5 mM EGTA, 1 μg/ml antimycin A. The pH was adjusted to 6.30. Malate at concentration 0.6 mM was added after a 2-min incubation. In Expts B and C 3.7 mM amytal or 1.45 mM butylmalonate, respectively, were added at zero time. Temperature was 25 °C.

the direction of malate. We also used this technique of measuring malate transport into mitochondria. As may be seen in Fig. 5 the entry of malate was accompanied by a rapid disappearance of protons from the medium measured as an increase of external pH. This increase of pH was inhibited by amytal and butylmalonate.

Radioisotopic assay of dicarboxylic acid transport across mitochondrial membrane: effect of amytal

The effect of amytal on succinate oxidation, mitochondrial swelling in ammonium succinate and malate, and the changes of pH accompanied by malate translocation indicate that amytal might be an inhibitor of the dicarboxylic acid transporter. This assumption was verified by radioisotopic assays. Rat liver mitochondria were loaded with [^{14}C]succinate as described by Robinson and Williams [12] and the loaded mitochondria were added to the incubation medium described in Table I. The presence of anions in the incubation medium (P_i, succinate, malate, malonate) caused an efflux of labelled succinate from the mitochondria, depending on the kind of anion (Table I). All the exchange reactions investigated were reduced by 5 mM amytal. P_i/[^{14}C]succinate and succinate/[^{14}C]succinate exchange reactions were more sensitive to amytal than malate/[^{14}C]succinate and malonate/[^{14}C]succinate exchange reactions. These results indicate that amytal inhibits the dicarboxylate anion translocator.

TABLE I

EXCHANGE OF [^{14}C]SUCCINATE FROM RAT LIVER MITOCHONDRIA

Mitochondria (7.2 mg protein), prelabelled with [^{14}C]succinate according to Robinson and Williams [12] were incubated at 5 °C in 1 ml of medium containing: 125 mM KCl and 20 mM Tris–HCl (pH 7.4), and other additions as indicated in the table. The reaction was started by the addition of 0.1 ml prelabelled mitochondria (suspended in 250 mM sucrose + 5 mM Tris–HCl, pH 7.4) and stopped after 2 min by the addition of 25 mM butylmalonate. The samples were immediately centrifuged at 14 000 × *g* for 1.5 min. Then 0.8 ml of the supernatant was drawn off and added to 0.5 ml of 10 % $HClO_4$. The acidified supernatants were centrifuged at 3000 × *g* for 5 min. Mitochondrial pellets were resuspended in 0.5 ml 10 % $HClO_4$, extracted for 30 min and then centrifuged at 14 000 × *g* for 3 min. 0.1 ml of supernatant and pellet extracts were added to a glass counting vial. The radioactivity was determined in a spectrometer for liquid scintillation Isocap 300 after 10 ml of scintillator solution (which contained 4 g PPO and 0.2 g POPOP in 0.7 l toluene + 0.3 l methanol) had been added. The percentage exchange was equal to: Supernatant dpm after incubation with anion. — supernatant dpm after incubation without anion/Mitochondrial dpm after incubation without anion × 100. The percentage inhibition of the dicarboxylate carrier is calculated by taking the percentage exchange after 2 min in the absence of amytal as the baseline value.

Additions	Supernatant (dpm/sample)	Exchange		Inhibition (%)
		dpm	%	
None	8 100			
1 mM L-malate	19 400	11 300	67	–
1 mM L-malate + 5 mM amytal	16 200	8 100	48	28
1 mM malonate	17 600	9 500	56	–
1 mM malonate + 5 mM amytal	14 400	6 300	37	34
1 mM succinate	13 900	5 800	34	–
1 mM succinate + 5 mM amytal	9 500	1 400	8	76
1 mM phosphate	17 300	9 200	54	–
1 mM phosphate + 5 mM amytal	10 800	2 700	16	70

Inhibition of citrate/[^{14}C]citrate and malate/[^{14}C]citrate exchange reactions by amytal

In an attempt to determine whether the inhibitory effect of amytal was specific for the dicarboxylate transporting system, the tricarboxylate transporting system in rat liver mitochondria was examined. Using rat liver mitochondria preloaded with [^{14}C]citrate according to Halperin et al. [13] we measured the exchange of [^{14}C]citrate with citrate and [^{14}C]citrate with malate. It was found that amytal at a concentration of 5 mM inhibited both exchange reactions. The inhibition by amytal of the citrate/[^{14}C]citrate exchange was greater than the inhibition of the malate/[^{14}C]citrate exchange reaction (Table II). The results of the experiments presented above indicate that amytal also inhibits the tricarboxylic acid carrier.

TABLE II

EXCHANGE OF [^{14}C]CITRATE OUT OF RAT LIVER MITOCHONDRIA

Mitochondria (4.2 mg protein), prelabelled with [^{14}C]citrate according to Halperin et al. [13] were incubated at 6 °C in 1 ml of medium containing: 125 mM KCl and 20 mM Tris–HCl (pH 7.4), and other additions as indicated in the table. The reaction was started by the addition of 0.1 ml prelabelled mitochondria (suspended in 250 mM sucrose + 5 mM Tris–HCl, pH 7.4) and stopped after 2 min by the addition of 25 mM 1,2,3-tricarboxybenzene. The samples were immediately centrifuged at 14 000 × *g* for 1.5 min. Further procedures were as described in Table I.

Additions	Supernatant (dpm)	Exchange		Inhibition (%)
		dpm	%	
None	6 500			
1 mM citrate	17 000	10 500	42	–
1 mM citrate + 5 mM amytal	11 300	4 800	19	55
1 mM L-malate	14 000	7 500	30	–
1 mM L-malate + 5 mM amytal	11 600	5 100	20	33

TABLE III

EFFECT OF AMYTAL ON PHOSPHATE UPTAKE BY MITOCHONDRIA

In Expt A mitochondria (4.5 mg protein) were preincubated for 1 min at 6 °C in 1 ml of medium containing: 125 mM KCl, 20 mM Tris–HCl (pH 7.4), 2 μg rotenone, 0.5 μg antimycin A, 2 μg oligomycin, 5 mM butylmalonate and amytal where indicated. The reaction was initiated with 0.2 mM $^{32}P_i$ (290 000 cpm), and stopped at 10 s with 1 mM mersalyl. In Expt B no preincubation took place, the reaction was started by the addition of a mitochondrial suspension and 0.2 mM $^{32}P_i$ (140 000 cpm). Other conditions were as in Expt A. The samples were immediately centrifuged. The pellets were suspended in 0.5 ml 10 % $HClO_4$, extracted for 30 min, and centrifuged at 14 000 × *g* for 3 min. The following procedure was as described in Table I. Controls were incubated with 1 mM mersalyl added before the labelled P_i had been added, and the amount of radioactivity taken up was subtracted from the experimental samples, in order to arrive at the P_i incorporated into the matrix space.

Expt	Additions	Phosphate in matrix space	
		Total cpm	*v* (nmoles/mg per min)
A	None	16 600	15.2
	+ 5 mM amytal	5 850	5.3
B	None	6 000	11.5
	+ 5 mM amytal	6 000	11.5

Effect of amytal on P_i transport into mitochondria

Inhibition by amytal of dicarboxylate and tricarboxylate transporting systems suggested a non-specific effect of the inhibitor. However, amytal was without effect on the mitochondrial swelling in ammonium phosphate (Fig. 4). We, therefore, decided to examine the effect of amytal on P_i transport by radioisotopic assays. Table III reveals that the uptake of phosphate was inhibited by amytal only if this compound was preincubated with mitochondria. Without preincubation amytal did not affect the P_i uptake.

The lack of the inhibitory action of amytal on the monocarboxylic acids or on the so-called lipophilic anions transport

Data presented above indicate that amytal inhibits phosphate, dicarboxylate and tricarboxylate translocators. The questions arise as to whether amytal also inhibits penetration across the mitochondrial membrane of monocarboxylic acid or of the so-called lipophilic anions which are assumed to penetrate without carriers. Table IV shows that amytal inhibited succinate oxidation stimulated by the addition of Ca^{2+} when phosphate was added as the penetrant anion. When P_i was replaced by acetate only a slight inhibition of Ca^{2+}-stimulated respiration was observed. Ca^{2+}-stimulated succinate oxidation in these conditions is dependent on the transport of phosphate or acetate. Amytal was without effect on succinate oxidation in the state of slow oxidation (no additions in Table IV) as then the respiration is not limited by the entry of substrate into mitochondria. Therefore, the results presented in Table IV suggest that amytal inhibits phosphate transport (see also Table III) and is without effect on acetate transport. To test these suggestions further, the effect of amytal on mitochondrial swelling in ammonium acetate was studied. The swelling was not affected by amytal even when the mitochondria had been preincubated with amytal (not shown).

To check whether amytal inhibits penetration of the so-called lipophilic anions

TABLE IV

THE EFFECT OF AMYTAL ON THE RESPIRATION OF RAT LIVER MITOCHONDRIA

The reaction mixture (final volume 2.5 ml) contained: 15 mM KCl, 50 mM Tris–HCl (pH 7.4) and 4 μg rotenone. Rat liver mitochondria (4.15 mg protein) had been preincubated at 25 °C for 1.5 min either without or with amytal added. Then succinate was added.

Expt	Additions	Rate of oxygen uptake (natom O/min per mg mitochondrial protein)	
		5 mM succinate only	4 mM amytal and 5 mM succinate
1	None	17	17
	0.5 μmole $CaCl_2$ +3.75 μmoles phosphate	162	102
2	None	17	17
	0.5 μmole $CaCl_2$ +3.75 μmoles acetate	115	110

into mitochondria the effect of the barbiturate on mitochondrial swelling suspended in 100 mM NH_4 SCN, 2 mM Tris–HCl (pH 7.2), 4 μg rotenone and 1 μM FCCP was studied. Under these conditions amytal did not affect mitochondrial swelling (not shown). This suggests that amytal is without effect on SCN^- penetration across the mitochondrial membrane.

Effect of barbiturates on the phosphate/[^{14}C]succinate exchange reaction

The inhibitory effects of other barbiturates on the phosphate/[^{14}C]succinate exchange reaction is presented in Table V. It may be seen that amytal was the most effective inhibitor, dial and phenobarbital at the same concentration affected this exchange reaction to a smaller extent and veronal acted only slightly.

TABLE V

THE EFFECT OF SEVERAL BARBITURATES ON [^{14}C]SUCCINATE/PHOSPHATE EXCHANGE

Mitochondria (6.9 mg protein), prelabelled with [^{14}C]succinate were incubated at 7 °C in 1 ml of medium. Barbiturates were 5 mM. Experimental conditions as in Table I.

Additions	Supernatant (dpm)	Exchange		Inhibition (%)
		dpm	%	
None	8 000			
Control	14 500	6500	61	–
+ amytal	9 300	1300	12	80
+ phenobarbital	10 800	2800	26	57
+ dial	11 500	3500	33	46
+ veronal	12 800	4800	45	26

DISCUSSION

Data presented in this paper indicate that amytal inhibits the transport of metabolic anions across the mitochondrial membrane. A possible explanation concerning the mechanism of the action of amytal (and other examined barbiturates) is that they bind to phosphate, dicarboxylic and tricarboxylic acids carriers. It is worth mentioning a recent report of Meisner [14] on the effect of the permeant anion tetraphenyl boron on the transport across the mitochondrial membrane of adenine nucleotide and other anions as phosphate, succinate and malonate. Meisner [14] showed a marked inhibition of this anion transport into mitochondria caused by tetraphenyl boron. He proposed that the lipophilic tetraphenyl group binds to the hydrophobic part of the mitochondrial membrane and that the high negative charge density of the boron anion creates a negative surface potential that acts to repel the anion. The effect of barbiturates on anion transport might be explained similarly. Barbiturates contain three carbonyl oxygens which give acidic properties to the compounds. On the other hand Goldbaum and Smith [18] have noted that the binding by bovine serum albumin is dependent on the length and nature of the side chain of the barbiturates. It is probable that the lipophilic side chain of amytal can bind to the proteins of mitochondrial membrane or to the other hydrophobic part of the mem-

brane and the negative charge of the barbituric acid may create a negative surface potential that would repel negatively charged metabolites.

The data presented here prove that the inhibition of succinate oxidation by amytal is mainly caused by the inhibition of succinate and phosphate transport across the mitochondrial membrane. This contrasts with the view of Pumphrey and Redfearn [7] suggesting that barbiturates inhibit succinate oxidation by interference with the energy transfer reaction of oxidative phosphorylation. Inhibition of phosphate transport by amytal explains the observation of Siekevitz et al. [2] who found that amytal inhibits the 2,4-dinitrophenol-stimulated ATPase activity of rat liver mitochondria. It is known that agents which inhibit phosphate transport also inhibit uncoupler-stimulated ATPase activity [19].

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