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FUMARATE PERMEATION IN RAT HEART MITOCHONDRIA

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SUMMARY: The possible fumarate translocation in rat heart mitochondria is examined. This substrate, which is claimed to be a non permeant ion in rat liver mitochondria appears to cross the mitochondrial membrane in cardiac mitochondria. This conclusion was proposed on the basis of experimental results which show swelling by rat heart mitochondria in ammonium fumarate, uptake by mitochondria of fumarate, Pi efflux from the matrix induced by fumarate and appearance of malate in the reaction mixture which follows the addition of fumarate to the mitochondria and depends on the fumarase activity. The existence of a carrier unknown so far as well as a possible physiological role of this transport is proposed.

Transport of citric cycle intermediates across the mitochondrial inner membrane has been largely investigated in rat liver mitochondria (for ref. see 1). Fumarate was claimed to be a non-penetrant anion (2) and no inhibition was shown by this substance on transport by the dicarboxylate, tricarboxylate and oxoglutarate carriers (3-5). In rat heart mitochondria the oxoglutarate carrier has been extensively investigated (6-9) and recently oxaloacetate permeation has been also demonstrated (10).

In this paper investigations of fumarate translocation in rat heart mitochondria are described and a possible physiological role is proposed for this transport.

MATERIALS AND METHODS: [^{32}P]-phosphoric acid, [^{14}C]-fumaric acid, $^{3}H_2O$ and [U-^{14}C] sucrose are supplied by the Radiochemical Center (Amersham, England), rotenone by F.P. Penick and Co. (New York), oligomycin, malic enzyme, oxaloacetic, fumaric and succinic acids by Sigma, NADP$^+$ by Boehringer, n-butylmalonic acid by Aldrich, p-iodobenzylmalonic acid by K. and K. Laboratories (Plainview, New York) and sodium thiocyanate by BDH. Phthalonic acid was synthesized according to Graebe and Trümpy (11). Measurement of the Pi efflux was carried out as follows: mitochondria were isolated as previously described (12); the intramitochondrial Pi was labelled by adding to the mitochondrial suspension carrier free ^{32}Pi. Mitochondria (about 0.5 mg protein) were incubated at 20°C in 0.5 ml of standard medium containing 0.2 M sucrose, 10 mM KCl, 20 mM HEPES-Tris, pH 7.0, 1 mM $MgCl_2$, 1 µg rotenone, in the presence of 3 µg of oligomycin. After 1 min the assay was started by the addition of unlabelled substrates and stopped after a further 2 min by rapid addition of 20 mM butylmalonate.

Abbreviations: FCCP, carboxyl cyanide p-trifluoromethoxyphenylhydrazone; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate.

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After rapidly centrifuging the suspension in an Eppendorf microcentrifuge, the radioactivity in the pellet was measured as described elsewhere (12). Measurement of fumarate uptake was carried out as follows: mitochondria were incubated in 0.5 ml of standard medium under the conditions specified for the measurement of Pi efflux. The assay was started by the addition of the labelled substrate, and terminated after the time indicated by rapid addition of 20 mM butylmalonate. Following termination of substrate uptake the mitochondria were immediately centrifuged in an Eppendorf microcentrifuge for 1 min and ^{14}C and ^{3}H radioactivity in the pellet and supernatant was measured as previously described (13). For measuring the amount of radioactivity in the matrix the space available to $^{3}H_2O$ and $[^{14}C]$-sucrose was determined in parallel experiments as previously described (13). Mitochondrial swelling was monitored by recording the decrease in A_{540} with an Eppendorf photometer (Model 1001 M).
Changes in the redox state of $NADP^+$ were followed by fluorimetry using an Eppendorf photometer (Model 1001 M) equipped with the appropriate filters. Mitochondrial protein was determined by a modified biuret method (14).

RESULTS

Mitochondria suspended in 100 mM ammonium fumarate do not swell, if however 2 µM FCCP is added swelling is found; the addition of 0.8 mM inorganic phosphate to mitochondria suspended in ammonium fumarate causes swelling and the addition of the uncoupler causes a further rapid swelling (Fig.1).These results suggest the penetration of fumarate in rat heart mitochondria.

In order to substantiate this finding the uptake of fumarate by mitochondria was tested by measuring the radioactivity in the mitochondria after the addition of $[^{14}C]$-fumarate (Table I). Radioactivity in the pellet was found

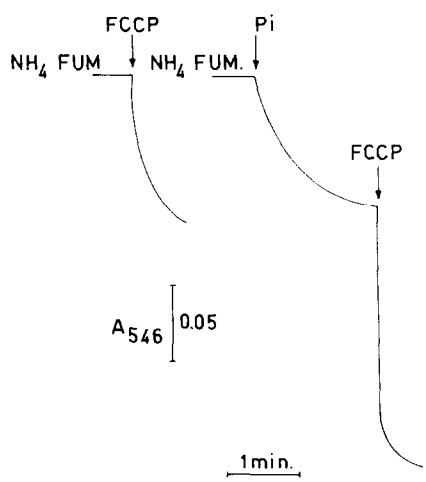

Fig.1 Swelling of heart mitochondria in ammonium fumarate

The reaction medium contained the 100 mM ammonium fumarate pH 7.0 and 0.5 mg of mitochondrial protein. At the arrows the suspension was made 2 µM or 0.8 mM with respect to FCCP and inorganic phosphate respectively.

TABLE I

Uptake of fumarate into mitochondria

Time of incubation (sec)	cpm/mg protein
10	2740
30	3318
60	3735

The uptake of [^{14}C]-fumarate (specific activity: 2500 cpm/nmole) into mitochondria (0.6 mg protein) was measured as described into the Methods section.

TABLE II

Efflux of intramitochondrial Pi induced by some added compounds

	Additions	Concentration (mM)	Radioactivity in the pellet (cpm)	Efflux (%)
Exp. I	None		10730	-
	Fumarate	0.1	7190	33
	Fumarate	0.3	6960	35
	Fumarate	0.5	6150	43
Exp.II	None		9380	-
	Pi	1	6470	31
	Oxaloacetate	0.5	9500	-1
	Oxaloacetate	4	9260	1

The experiments were carried out as described in the Methods section.Mitochondrial protein was 0.5 mg.

increasing with the time of incubation of fumarate. Owing to the activity of the intramitochondrial enzymes the radioactivity of the pellet could be due not only to fumarate, but also to malate and succinate formed in the matrix.

The ability of fumarate to promote Pi efflux from mitochondria was investigated in the experiment reported in Table II. In incubations lasting

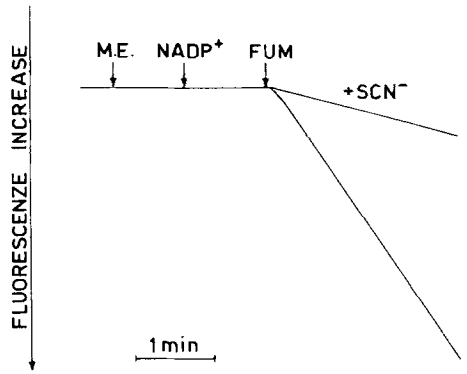

Fig.2 Appearance of malate in the incubation medium induced by the addition of fumarate to rat heart mitochondria.

Rat heart mitochondria (0.65 mg protein) were incubated at 30°C in 1 ml of standard medium containing 0.2 M sucrose 10 mM KCl, 20 mM HEPES-Tris pH 7.0, 1 mM $MgCl_2$, 1 µg rotenone. After incubation for 2 min, 0.1 unit of malic enzyme (M.E.) (previously dialyzed against 100 mM Tris-HCl pH 7.0) was added, followed 1 min later by $NADP^+$ (final concentration 250 µM). After a further min, fumarate (FUM) (final concentration 100 µM) was added in the absence or in the presence of 10 mM thiocyanate (SCN^-). $NADP^+$ reduction was followed fluorimetrically.

for 2 min, the extent of efflux of Pi was increased slightly raising the fumarate concentration. An efflux of 43% was found at 0.5 mM fumarate. In another experiment carried out with the same mitochondrial suspension possible Pi/Pi and oxaloacetate/Pi exchanges were examined. A Pi concentration of 1 mM caused a 30% efflux of internal Pi, whereas on the other hand no Pi efflux was found at concentration of oxaloacetate as high as 4 mM; this is consistent with previous results (12).

According to previously reported results (10), no malate is present in extramitochondrial phase of rat heart mitochondria suspended in an isosmotic incubation medium. Hence addition of malic enzyme to a suspension of mitochondria does not reduce externally added $NADP^+$. If, however, fumarate is added to the mitochondrial suspension after addition of malic enzyme and $NADP^+$, an extensive reduction of the pyridine nucleotide occurs (Fig.2).This result implies the appearance of malate in the phase outside the mitochondria during incubation of the mitochondria with fumarate. A possible explanation of this result is that fumarate is taken up in the mitochondria in exchange for a small amount of endogenous malate or phosphate. Inside the mitochondria fumarate would be hydrated to malate which in turn could be exchanged for further fumarate. Thiocyanate, a non-competitive inhibitor of the fumarase (15), was found to strongly inhibit the $NADP^+$ reduction at a concentration of 10 mM. No effect of thiocyanate or of any other of the

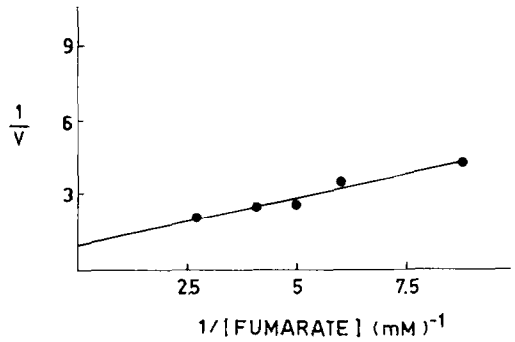

Fig.3 The dependence of the rate of appearance of malate on the external fumarate concentration.

The experiment was carried out as described in Fig.2. The reduction of $NADP^+$ was followed fluorimetrically after the additions of various concentrations of fumarate. The initial rate of reduction (V) is expressed in arbitrary units of scale divisions/min. (Mitochondrial protein was 0.55 mg).

tested inhibitors was found on the activity of the malic enzyme. The dependence of the rate of $NADP^+$ reduction on fumarate concentration was investigated and is shown in Fig.3 as a double reciprocal plot. Saturation kinetics were found, the value of the apparent Km for fumarate being about 0.37 mM in the reported experiment. It should be noted that no increase of the rate of reduction of $NADP^+$ was found on increasing the amount of malic enzyme or of $NADP^+$ at the highest substrate concentration. In order to investigate the mechanism of fumarate permeation, the ability of fumarate and of succinate to cause appearance of malate was compaired and the sensitivity of the reaction to phthalonate was investigated (Fig.4). It should be noted that succinate can cross the mitochondrial membrane via the oxoglutarate carrier (16,17). Both substrates were found to cause appearance of malate but a lag was observed in the case of succinate. Phthalonate has been reported to be a potent and relatively specific inhibitor of oxoglutarate and oxaloacetate uptake in rat liver and heart mitochondria (10,18,19). This compound did not influence the fumarate induced $NADP^+$ reduction whilst on the other hand it strongly inhibited the reaction when promoted by succinate. The appearance of malate stimulated by fumarate was found to be inhibited by dicarboxylate analogues such as phenylsuccinate and malonate derivates. In Fig.5 the nature of the inhibition by p-iodobenzylmalonate of the rate of reduction of $NADP^+$ was investigated using a Dixon plot. Competitive inhibition was found.

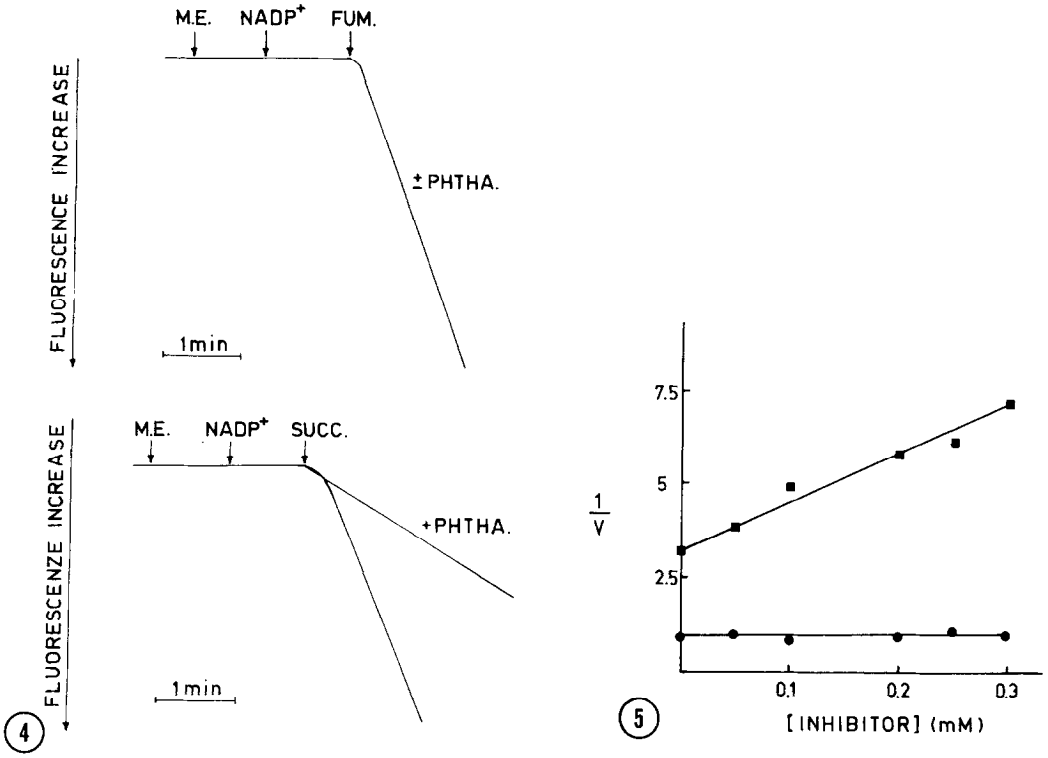

Fig.4 The effect of phthalonate on the appearance of malate induced by addition of fumarate or succinate.

The experiment was carried out as reported in Fig.2. Substances were added at the points shown at the following concentrations.Malic enzyme (M.E.) 0.1 unit, $NADP^+$ 250 μM, fumarate (FUM) 100 μM,succinate (SUCC) 100 μM, phthalonate (PHTHA) 100 μM. (Mitochondrial protein was 0.6 mg).

Fig.5 Kinetic analysis of the inhibition by p-iodobenzylmalonate of appearance of malate induced by the addition of fumarate using a Dixon Plot.

The experiment was carried out as described in Fig.2. The rate of $NADP^+$ reduction which followed the addition of 0.05 mM fumarate (■) and 1 mM fumarate (●), in the absence or presence of increasing concentration of p-iodobenzylmalonate was measured. The initial rate of $NADP^+$ reduction (V) is expressed in arbitrary units of scale divisions/min. Mitochondrial protein was 0.5 mg.

DISCUSSION

The experiments reported in this paper show that fumarate permeation occurs in rat heart mitochondria. Mitochondria swell in ammonium fumarate in the presence of an uncoupler; this suggests that the membrane is permeable to both NH_3 and the tested anion under the experimental conditions; given that the swelling could be limited by the production of OH^- in the interior, the addition of an uncoupler, which is presumed to

act as proton conductor, removes this restriction (20). The role of phosphate, which is required for swelling to occur in ammonium fumarate recalls the analogous activation of swelling in ammonium succinate in rat liver mitochondria (2) and suggests possible occurrence of fumarate/Pi exchange in rat heart mitochondria. The Pi efflux induced by fumarate supports this suggestion and is in favour of the permeation of fumarate. This conclusion is supported by another piece of evidence; malate appearance in the suspension medium following addition of fumarate. This result shows that fumarate causes efflux of substrate from the mitochondrial matrix. The sensitivity of the reduction of $NADP^+$ to thiocyanate, demonstrating the basic function of the intramitochondrial fumarase,confirms the influx of fumarate to the matrix. The demonstration of saturation kinetics argues in favour of a carrier mediated process, probably with the fumarate/malate exchange as the rate limiting step since the activity of fumarase is very high (21). The lack of inhibition by phthalonate strongly suggests that fumarate and malate across the mitochondrial membrane via a specific carrier, which is strongly inhibited by p-iodobenzylmalonate and which is different from the oxoglutarate carrier. The activity of this carrier does not seem to require a cis configuration in the substrate. The occurrence of a fumarate/malate exchange could explain the quite low radioactivity found in the mitochondrial pellet after the addition of fumarate, thus the kinetic study of the uptake requires the inhibition of the activity of fumarase, probably by means of thiocyanate. This investigation is currently in progress. Possible occurrence of transport under physiological conditions can be tentatively correlated with a translocation mechanism for fumarate derived from cytosolic amino acid catabolism in heart cells, and more importantly may also have a relevant function concerning the purine nucleotide cycle (22). It has been proposed that, in muscle, ammonia production arises from the reaction catalyzed by the cytoplasmic adenylate deaminase; the reconversion of IMP to AMP occurs by two reactions which finally produce fumarate from aspartate in the cytosol (23). Owing to the absence of extramitochondrial fumarase activity in heart (15), regeneration of aspartate may require fumarate translocation and the coupled activity of the mitochondrial malate dehydrogenase and aspartate aminotransferase.

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