Pyruvate metabolism in castor-bean mitochondria

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1. We report the isolation of mitochondria from the endosperm of castor beans (*Ricinus communis*). These mitochondria oxidized succinate, external NADH, malate and pyruvate with respiratory-control and ADP/O ratios consistent with those found previously with mitochondria from other plant sources. 2. The mitochondria exhibited considerable sensitivity to the electron-transport-chain inhibitors antimycin A and cyanide when oxidizing succinate and external NADH. 3. Pyruvate-dependent O₂ uptake was relatively insensitive to these inhibitors, although the residual O₂ uptake could be inhibited by salicylhydroxamic acid. We conclude that a cyanide-insensitive alternative terminal oxidase is functional in these mitochondria. However, electrons from the succinate dehydrogenase or external NADH dehydrogenase seem to have no access to this pathway. There is little interconnection between the salicylhydroxamic acid-sensitive and cyanide-sensitive pathways of electron transport. 4. α -Cyanocinnamate and its analogues, compound UK5099 [α -cyano- β -(1-phenylindol-3-yl)acrylate] and α -cyano-4-hydroxycinnamate, were all found to be potent non-competitive inhibitors of pyruvate oxidation in castor-bean mitochondria. 5. The accumulation of pyruvate by castor-bean mitochondria was determined by using a silicone-oil-centrifugation technique. The accumulation was shown to observe Michaelis-Menten kinetics, with a K_m for pyruvate of 0.10 mM and a V_{max} of 0.95 nmol/min per mg of mitochondrial protein. However, the observed rates of pyruvate accumulation were insufficient to account for the pyruvate oxidation rates found in the oxygen-electrode studies. We were able to demonstrate that this is due to the immediate export of the accumulated radiolabel in the form of malate and citrate. 6. Compound UK5099 inhibited the accumulation of [2-¹⁴C]pyruvate by castor-bean mitochondria at concentrations similar to those required to inhibit pyruvate oxidation.

INTRODUCTION

The movement of pyruvate from the cytosol to the mitochondria is a known feature of the energy metabolism of non-photosynthesizing plant cells. However, in some C₄ plants the movement of pyruvate between the mesophyll and bundle sheath cells, and across the membranes of chloroplasts and mitochondria, is an important feature of carbon fixation.

Pyruvate has been shown to enter rat liver and heart mitochondria by a saturable transport system which is inhibited by the α -cyanocinnamates (Halestrap & Denton, 1974; Halestrap, 1975, 1978; Paradies & Papa, 1975, 1977; Halestrap *et al.*, 1980). When mitochondria are exposed simultaneously to the α -cyanocinnamates and pyruvate, these compounds compete for the binding site of the transport protein over the first 15 s. Thus the inhibition of pyruvate accumulation by the α -cyanocinnamates shows typical competitive kinetics (Paradies & Papa, 1975, 1977). However, when mitochondria are incubated with α -cyanocinnamates for 2 min before the addition of pyruvate and the subsequent assay of transport activity, the mechanism of inhibition of uptake appears to be non-competitive (Halestrap, 1975). The transport of pyruvate is also inhibited by *N*-ethylmaleimide and *N*-phenylmaleimide (Papa & Paradies, 1974; Thomas & Halestrap, 1981), suggesting a possible role for thiol groups in the mechanism of pyruvate accumulation.

In contrast, little is known about the pyruvate translocator of plant mitochondria, except the report that the transport and oxidation of pyruvate in corn

mitochondria is inhibited in a non-competitive manner by the α -cyanocinnamates (Day & Hanson, 1977b).

In the present paper we report data on the characteristics of the uptake and the oxidation of pyruvate by mitochondria isolated from the endosperm of *Ricinus communis*, the castor bean.

EXPERIMENTAL

Materials

Except where stated below, substrates, cofactors and all other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K. Bovine serum albumin (fraction V), rotenone, salicylhydroxamic acid and antimycin A were from Sigma Chemical Co., Poole, Dorset, U.K. Percoll was from Pharmacia, Milton Keynes, Beds., U.K. α -Cyano-4-hydroxycinnamate and α -cyanocinnamate were from Aldrich Chemical Co., Gillingham, Dorset, U.K., and Lancaster Synthesis, Morecambe, Lancs, U.K., respectively. Compound UK5099 [α -cyano- β -(1-phenylindol-3-yl)acrylate] was a gift from Pfizer (U.K.), Sandwich, Kent, U.K. [2-¹⁴C]pyruvate and NCS Tissue Solubilizer were from the Radiochemical Centre, Amersham, Bucks., U.K. On receipt, the [2-¹⁴C]pyruvate was dissolved in water, and divided into 2.5 μ Ci samples in sealed tubes and frozen at -80 °C. These samples were used within 3 months. The scintillation fluid, Cocktail T 'Scintran', was obtained from BDH Chemicals, and Dow Corning silicone oils were from Hopkin and Williams, Chadwell Heath, Essex, U.K. K2-Cellulose t.l.c. plates were from Whatman Laboratory Sales, Maidstone, Kent, U.K.

Preparation of mitochondria

Castor beans (80g) were soaked in running water for 24 h, planted in moist Levington compost and germinated for 5 days at 28–30 °C in total darkness as described by Chappell & Beevers (1983). After germination, the testa were removed to yield 30–40 g of endosperms, which were chopped roughly with a razor blade in 80 ml of chilled homogenization medium [0.3 M-mannitol, 30 mM-Mops, 1 mM-EDTA, 0.05% (w/v) cysteine, 0.1% (w/v) bovine serum albumin, pH 7.4]. The chopped endosperms were homogenized with a Polytron PT35 2/M probe at speed 6 for 1 s. The homogenate was squeezed through three layers of muslin, and the mitochondria were then isolated by differential centrifugation by the method of Bonner (1967). The final pellet was resuspended in 2–3 ml of homogenization medium lacking cysteine, to give a final protein concentration of 25–30 mg/ml.

For some experiments, the mitochondria were purified further by centrifugation on Percoll gradients to remove glyoxysomes. The resuspended mitochondrial pellet obtained from the previous procedure was mixed with 22.5 ml of a solution containing 30% (v/v) Percoll, 0.25 M-mannitol and 20 mM-Mops, pH 7.4. The mixture was centrifuged at 70000 g_{av} for 30 min in a MSE Superspeed 65 ultracentrifuge with a 60Ti fixed-angle rotor. During this centrifugation the Percoll formed a continuous density gradient and the particles present separated according to their densities. The mitochondria and glyoxysomes were at densities of 1.05 and 1.07 g/ml respectively. The mitochondrial band was removed with a Pasteur pipette and diluted in 6 vol. of resuspension medium. After centrifugation for 15 min at 10000 g_{av} , the mitochondrial pellet was resuspended as above to a final protein concentration of 16–20 mg/ml.

Total protein was determined by the protein-precipitation method of Peterson (1977). Bovine serum albumin was used as a standard.

Determination of mitochondrial respiration

Mitochondrial respiration was measured polarographically with a Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.) in a closed cell at 30 °C. Castor-bean mitochondria (approx. 1 mg of protein) were suspended in 3 ml of reaction buffer, consisting of 0.25 M-mannitol, 15 mM-KCl, 5 mM-MgCl₂, 5 mM-K₂HPO₄, adjusted to pH 7.2, and the assay was started by the addition of the appropriate substrate. Substrates and inhibitors were added at the concentrations indicated in the Figure legends. Pyruvate oxidation required the presence of 'sparker' concentrations of DL-malate (1.5 mM), and the cofactors CoA (50 μ M) and thiamin pyrophosphate (100 μ M). This is in common with mitochondria isolated from some other plant tissue sources (Day & Hanson, 1977b; Douce *et al.*, 1977).

Measurement of pyruvate accumulation

Mitochondria (2–3 mg of protein) were preincubated in the above reaction buffer (2.1 ml) with thiamin pyrophosphate (100 μ M), CoA (500 μ M), DL-malate (1.5 mM) and ADP (0.7 mM), in a continuously stirred vessel at 30 °C. After 2 min [2-¹⁴C]pyruvate (0.25 μ Ci/ml) was added at the desired concentration.

Pyruvate uptake was stopped after the appropriate incubation time by the addition of 10 μ M-UK5099, and the mitochondria were separated from the suspending

medium by a silicone-oil centrifugation procedure (Vaartjes *et al.*, 1979). A 0.7 ml portion of the incubation mixture was rapidly transferred after the incubation period to a 1.5 ml conical centrifuge tube containing a 200 μ l bottom layer of 4% (w/v) HClO₄, which was overlaid with 250 μ l of silicone oil (sp.gr. 1.051). The tube was centrifuged at 12000 g for 60s to sediment the mitochondria, in a Hermle Z-230M microfuge. The upper supernatant layer was removed with a Gilson pipette, and 0.5 ml of distilled water was layered on to the silicone oil. This wash was removed with a vacuum line, and NCS tissue solubilizer (100 μ l) was added to the remaining contents of the tube, which were mixed vigorously and incubated at 45 °C for 45 min. After incubation, the contents of the tubes were added to cocktail T scintillation fluid (4 ml), and the radioactivity was determined in an Intertechnique SL30 liquid-scintillation spectrophotometer. Quench correction was by an external standard.

The uptake of pyruvate into the mitochondrial matrix was determined by making a correction for the pyruvate content of (1) the extra-matrix space and (2) the adhering suspension medium. The carry-over of suspension medium was estimated by the addition of *N*-ethylmaleimide (1 mM) to the precubation medium before the addition of [2-¹⁴C]pyruvate. At this concentration *N*-ethylmaleimide inhibits the uptake of pyruvate by castor-bean mitochondria.

Identification of the products of pyruvate metabolism

Mitochondria (2 mg) were incubated for 3 min in the reaction buffer (4.0 ml) described for the measurement of pyruvate accumulation. The control incubations also contained either 10 μ M-UK5099 or 1 μ M-antimycin A. To initiate assay, 1 μ Ci of [2-¹⁴C]pyruvate was added to give a final concentration of 0.1 mM. After the desired reaction time, 0.6 ml samples were removed and the mitochondria were sedimented by centrifugation for 60 s at 12000 g in a Hermle Z-230M microfuge. Samples (6 μ l) of the supernatants were combined with 2.5 μ l of a solution containing 100 μ M-pyruvate, 100 μ M-citrate and 100 μ M-malate. These mixtures were subjected to two-dimensional t.l.c. The 0.25 mm-thick cellulose t.l.c. plates were developed in the first dimension with diethyl ether/formic acid (90%, v/v)/water (7:2:1, by vol.), followed in the second dimension by phenol (80%, v/v)/formic acid (90%, v/v)/water (94:1:6, by vol.) as described by Myers & Huang (1969). The areas of the plates containing the resolved compounds were scraped off the t.l.c. plate and their radioactivity contents determined as described above.

RESULTS AND DISCUSSION

Preparation of mitochondria

The isolation procedure described in the Experimental section gives high yields of intact mitochondria from castor-bean endosperm in a time that is very much shorter than that described by Chappell & Beevers (1983). The final pellet contained a mixture of mitochondria and glyoxysomes. Control experiments were carried out to determine the possible interference of the glyoxysomes with those properties of the mitochondria that are reported here. Glyoxysomes were removed from the mitochondria by centrifugation on Percoll gradients

Table 1. Oxidation of succinate, NADH, malate and pyruvate by castor-bean mitochondria

Castor-bean mitochondria isolated by differential centrifugation (approx. 1 mg of protein) were incubated with the appropriate substrate in 3 ml of reaction buffer (0.25 M-mannitol, 15 mM-KCl, 5 mM-MgCl₂, 5 mM-K₂HPO₄, pH 7.2) at 30 °C in a Rank oxygen electrode. After a steady rate of O₂ consumption was established, ADP (150 μM) was added and the rate of respiration measured. When pyruvate was used as the substrate it was also necessary to add 1.5 mM-DL-malate, 50 μM-CoA and 100 μM-thiamin pyrophosphate. Results are given as the means ± s.e.m. for at least eight separate observations, and the pyruvate rates are corrected for the component corresponding to malate.

Substrate	Oxidation (nmol of O ₂ consumed/min per mg of mitochondrial protein)		ADP/O Ratio
	–ADP	+ADP	
Succinate (5 mM)	25 ± 5	72 ± 6	2.1
NADH (5 mM)	16 ± 3	51 ± 5	1.7
Malate (5 mM)	6 ± 1	22 ± 2	2.7
Pyruvate (5 mM) + DL-malate (1.5 mM)	9 ± 1	33 ± 4	2.6

as described in the Experimental section. The glyoxysome-free mitochondria behaved in exactly the same manner as those mixed with glyoxysomes. There was an increase in the respiration rates, due to the removal of the glyoxysomal protein. Most of the experiments reported here were performed with the mixture of mitochondria and glyoxysomes.

The data presented in Table 1 show that the castor-bean mitochondria oxidize malate, succinate and added NADH, and synthesize ATP, at rates comparable with those reported for mitochondria isolated from other plants (Douce *et al.*, 1977; Jackson *et al.*, 1979; Chappell & Beevers, 1983). We conclude that the mitochondria used in the experiments presented here are intact, and that they have an external NADH dehydrogenase located on the outer face of the inner membrane (Coleman & Palmer, 1972; Douce *et al.*, 1973; Day & Wiskich, 1974). In common with mitochondria isolated from a variety of plant tissue sources (Day & Hanson, 1977b; Douce *et al.*, 1977), pyruvate is only oxidized in the presence of 'sparker' concentrations of malate (1.5 mM) and the cofactors CoA (50 μM) and thiamin pyrophosphate (100 μM), which presumably exert their effects via the pyruvate dehydrogenase complex. This view is supported by data which suggest that CoA (Neuberger *et al.*, 1984) and possibly thiamin pyrophosphate (Day & Hanson, 1977a) can enter plant mitochondria by carrier-mediated transport.

Effects of antimycin A and cyanide on substrate oxidation in castor-bean mitochondria

The O₂ consumption associated with the oxidation of pyruvate in the presence of malate and the cofactors, CoA and thiamin pyrophosphate was not totally inhibited by either antimycin A or cyanide (Fig. 1, symbols ○ and □). These are inhibitors of the

Fig. 1. Effects of antimycin A and cyanide on substrate oxidation in castor-bean mitochondria

The oxidation of pyruvate (○, □) or succinate (●, ■) by castor-bean mitochondria was studied in the presence of 0.7 mM-ADP as described in the legend to Table 1. Once the State-3 rate was established, either antimycin A (○, ●) or cyanide (□, ■) was added in successively increasing concentrations until maximal inhibition was achieved.

electron-transport pathway that utilizes cytochromes *a* and *a*₃ as the terminal oxidase. Salicylhydroxamic acid (1.0 mM), an inhibitor of the cyanide-insensitive alternative terminal oxidase (Schonbaum *et al.*, 1971), similarly would not inhibit completely the pyruvate-dependent O₂ uptake. A combination of salicylhydroxamic acid (0.3 mM) and either 0.7 μM-antimycin A or 0.7 μM-cyanide totally inhibited the oxidation of pyruvate. In contrast, antimycin A and cyanide were both potent inhibitors of the oxidation of succinate (Fig. 1, symbols ● and ■) and external NADH (results not shown), giving over 80% inhibition. The addition of salicylhydroxamic acid (0.3 mM) did not give more than 10% inhibition of the respiration rate with succinate or added NADH.

These data indicate that castor-bean mitochondria have both a functional cyanide-sensitive electron-transport pathway and a salicylhydroxamic acid-sensitive cyanide-insensitive terminal oxidase. These are organized in such a manner that the electrons from the succinate dehydrogenase and external NADH dehydrogenase are channelled via the cyanide-sensitive pathway, whereas the electrons from the oxidation of pyruvate appear to be partitioned between the two terminal oxidases. There appears therefore to be little interconnection between these two systems. Control experiments were carried out to ensure that the partitioning of electrons between the two systems was not a function of the rate of electron transport. Malonate (1 mM) was used to decrease the O₂ consumption associated with the oxidation of succinate to that normally observed with pyruvate. The potency and the extents of inhibition shown by the various inhibitors was unchanged. The barrier to the entry of

Table 2. Sensitivity of pyruvate oxidation in castor-bean mitochondria to inhibition by α -cyanocinnamic acid and its analogues

The inhibition of pyruvate oxidation by these inhibitors was studied as described in the legend to Fig. 1. Rates of pyruvate oxidation were corrected for the component caused by the addition of 'sparker' concentrations of malate. The data are presented as the titre of inhibitor required to give 50% inhibition (I_{50}) of the pyruvate oxidation rate. The K_i values were obtained from the data presented in Fig. 2.

Inhibitor	I_{50} (nmol/mg of protein)	K_i (μ M)
UK5099	0.9	0.03
α -Cyanocinnamate	10.5	0.45
α -Cyano-4-hydroxycinnamate	14.7	1.20

electrons from succinate oxidation into the alternative oxidase is not therefore a function of the relatively high rate of electron input from this substrate compared with that from pyruvate. The inaccessibility to some substrates of the cyanide-insensitive alternative terminal oxidase has also been reported in cassava, sweet potato and spinach leaf mitochondria (Tomlinson & Moreland, 1975; Douce *et al.*, 1977; Huq & Palmer, 1978). Furthermore, the action of dibutylchloromethyltin chloride on the oxidation of substrates in mung-bean mitochondria (Moore *et al.*, 1980) suggests that the oxidation of malate and succinate occurs through a pool of quinone which is functionally distinct from that relating to the oxidation of external NADH. In common with this finding, our data are difficult to rationalize on the basis of a homogeneous pool of quinone which mediates the flow of electrons between all the dehydrogenases and the two oxidase systems. These results therefore require one to hypothesize some sort of functional compartmentation of the ubiquinone pool.

Inhibition of pyruvate oxidation in castor-bean mitochondria by the α -cyanocinnamates

The inhibition of pyruvate oxidation by a series of α -cyanocinnamate analogues was studied. The titres of these compounds required to induce 50% inhibition of oxygen uptake (I_{50}) are shown in Table 2. The values reported are similar to those found previously in rat liver mitochondria (Halestrap, 1975). The compounds listed in Table 2 do not affect the oxidation of succinate, added NADH or malate, and hence they have no effect on the activity of the electron-transport chain or the associated dehydrogenases.

UK5099 is a very potent inhibitor of pyruvate metabolism in castor-bean mitochondria, 50% inhibition being achieved at a titre of 0.9 nmol of inhibitor/mg of mitochondrial protein. However, it was found that, as the pyruvate concentration was increased above 5 mM, the oxidation of pyruvate became increasingly insensitive to UK5099. At 40 mM-pyruvate, its oxidation could no longer be inhibited by UK5099, even at titres of 30 nmol/mg of mitochondrial protein. This has been reported previously in rat heart mitochondria (Pande & Parvin, 1978), and indicates that at higher concentrations

Fig. 2. Non-competitive inhibition of pyruvate oxidation by compound UK5099

Mitochondria (1 mg of protein) were incubated in an oxygen electrode for 3 min, with reaction buffer (0.25 M-mannitol, 15 mM-KCl, 5 mM-MgCl₂, 5 mM-K₂HPO₄, pH 7.2) containing CoA (50 μ M), thiamin pyrophosphate (100 μ M), malate (1.5 mM), ADP (0.7 mM) and the desired concentration of compound UK5099. Pyruvate (5 mM) was then added and the rate of pyruvate-dependent O₂ consumption determined as described in Table 2. The data are presented as a Lineweaver-Burk plot at various concentrations of UK5099; ●, 0 (control); ■, 17 nM; ▲, 50 nM.

the entry of pyruvate involves a α -cyanocinnamate-insensitive carrier-independent mechanism.

We have investigated the mechanisms and evaluated the appropriate kinetic constants for the inhibition of pyruvate oxidation by α -cyanocinnamic acid and its derivatives. Mitochondria were preincubated for 3 min with increasing concentrations of the α -cyanocinnamates, and the pyruvate-dependent O₂ consumption at various concentrations of pyruvate was determined with an oxygen electrode. At least four separate experiments over a range of pyruvate concentrations from 0.05 to 0.4 mM were performed, the errors being calculated by least-squares fit. Data typical of those obtained with all of the α -cyanocinnamates are presented as a Lineweaver-Burk plot (Fig. 2) of results with UK5099. The dissociation constant of the enzyme-inhibitor complex (K_i) for each of the α -cyanocinnamates studied are shown in Table 2. We conclude that α -cyanocinnamate and its analogues are potent non-competitive inhibitors of pyruvate oxidation. This is in agreement with the mechanism reported for the inhibition by the α -cyanocinnamates of the oxidation of pyruvate by corn mitochondria (Day & Hanson, 1977b). The inhibitory effects of the α -cyanocinnamates could not be reversed by the addition of monochloroacetic acid, as reported for rat liver mitochondria by Halestrap (1978).

From the nature of the kinetics observed, it is clear that the complex between the α -cyanocinnamates and their site of action is freely reversible within the time scale of these experiments. However, in a further series of experiments, castor-bean mitochondria were incubated with an inhibitory titre of UK5099 (0.9 nmol/mg of protein) for 30 min at 0 °C. These UK5099-treated mitochondria were then diluted 100-fold into an

oxygen-electrode vessel. This dilution led to a concentration that would not normally inhibit pyruvate oxidation. However, there was no reversal of the inhibition by UK5099 of pyruvate oxidation. Control experiments showed that preincubation of the mitochondria with UK5099 did not inhibit the oxidation of NADH or succinate. It appears that the 30 min incubation period of UK5099 with the mitochondria has allowed the interaction between the inhibitor and its active site to be stabilized by other, slower, reactions. The formation of an irreversible complex between the α -cyanocinnamates and their site of action should facilitate the identification of proteins involved with pyruvate transport in castor-bean mitochondria.

These results differ from those reported for the inhibition by the α -cyanocinnamates of pyruvate transport into rat liver mitochondria, where (1) both competitive (Paradies & Papa, 1975, 1977) and non-competitive (Halestrap, 1975) mechanisms have been observed, (2) the inhibition of pyruvate oxidation could be reversed by the addition of monochloroacetic acid (Halestrap, 1978), and (3) the inhibition of pyruvate oxidation observed after 30 min incubation of rat liver mitochondria with α -cyano-4-hydroxycinnamate at 0 °C as described above could be reversed by a similar dilution procedure (Halestrap, 1975). Thus the model envisaged by Halestrap (1978), where the α -cyanocinnamates interact reversibly with a thiol group on the inner surface of the mitochondrial inner membrane, does not appear to be apposite for the interaction of α -cyanocinnamates with the inner membrane of castor-bean mitochondria. Inspection of the experimental protocol used by Halestrap (1975) to demonstrate the reversibility of the inhibition by the α -cyanocinnamates of pyruvate oxidation in rat liver mitochondria shows that (a) when the α -cyanocinnamate is added directly to the oxygen electrode the titre was 6 nmol/mg of mitochondrial protein, a titre capable of giving 50% inhibition of pyruvate oxidation, whereas (b) when the α -cyanocinnamate was preincubated with the mitochondria at 0 °C for 30 min the titre was 0.5 nmol/mg of mitochondrial protein. Thus it is unlikely that any irreversible inhibition of pyruvate oxidation would have occurred under these conditions.

Accumulation of pyruvate by castor-bean mitochondria

We have studied the effects of the α -cyanocinnamates on pyruvate uptake directly by using the silicone-oil-centrifugation assay described in the Experimental section. The preincubation of mitochondria with *N*-ethylmaleimide (1 mM) before addition of [2-¹⁴C]pyruvate was used as a means of determining the radiolabelled pyruvate that accumulates in the extra-matrix space. It was found that the carry-over observed increased linearly with increasing concentrations of added [2-¹⁴C]-pyruvate, and was unaffected by increases in the period of mitochondrial incubation with the radiolabelled pyruvate from 30 s to 4 min.

The time course of pyruvate uptake into the mitochondria at 30 °C is shown in Fig. 3(a). Extrapolation of these data showed the maximum uptake of pyruvate ($P_{\max}$) to be 1.03 nmol/mg of mitochondrial protein. The addition of 1 μ M-antimycin A to the incubation medium did not significantly increase the amounts of pyruvate accumulated by the mitochondria. The uptake showed apparent first-order kinetics (Fig. 3b), as has

Fig. 3. Time course of pyruvate accumulation by castor-bean mitochondria

Mitochondria (2–3 mg of protein) were suspended in 2.1 ml of reaction buffer at 30 °C in a stirred vessel with CoA (50 μ M), thiamin pyrophosphate (100 μ M), malate (1.5 mM) and ADP (0.7 mM). Pyruvate transport was started by the addition of [2-¹⁴C]pyruvate (0.275 mM, 0.25 μ Ci/ml), and stopped after the appropriate incubation time by the addition of 10 μ M-UK5099. A 0.7 ml sample was taken and the mitochondria were separated from the suspending medium by silicone-oil centrifugation. The radioactive content of the mitochondrial pellet was determined as described in the Experimental section. By extrapolation of the data in Fig. 3(a), the maximum uptake of pyruvate ($P_{\max}$) was determined as 1.03 nmol/mg of mitochondrial protein. By using this value it is possible to derive the first-order rate constant (k) from Fig. 3(b), where P_t represents the pyruvate uptake at time t . The initial rate of pyruvate uptake can be determined from the first-order rate equation, $v = k(P_{\max})$.

also been described for other mitochondrial transport mechanisms (Pfaff *et al.*, 1969; Quagliariello *et al.*, 1969; Bradford & McGivan, 1973). The rate constant (k) was 0.65 min⁻¹. The initial rate of reaction was determined from the first-order rate equation, $v = k(P_{\max})$, as 0.62 nmol/min per mg of mitochondrial protein.

This accumulation of pyruvate by castor-bean mitochondria could be inhibited by compound UK5099. The concentration of UK5099 required to achieve 50% inhibition of pyruvate accumulation was 1 nmol/mg of

Fig. 4. Dependence of the rate of pyruvate uptake on the pyruvate concentration

Pyruvate accumulation was measured at 30 °C at a range of pyruvate concentrations of 0.025 to 0.400 mM. The assays were performed and the initial rates of uptake calculated as described in the legend to Fig. 3.

mitochondrial protein. This is in close agreement with that required to inhibit pyruvate oxidation by a similar amount (Table 2).

To determine the kinetics of pyruvate uptake, mitochondria were incubated with pyruvate at concentrations in the range 0.025–0.400 mM, and the initial rates of uptake determined as above. The accumulation was shown to observe Michaelis–Menten kinetics as indicated by the Lineweaver–Burk plot (Fig. 4). The K_m value for pyruvate was 0.10 mM and the V_{max} 0.95 nmol/min per mg of mitochondrial protein.

It is clear, however, that the observed rates of pyruvate accumulation are insufficient to account for the pyruvate oxidation rates found in the oxygen-electrode studies (see Table 1). Furthermore, the extents of pyruvate accumulation are unaffected by the addition of 1 μ M-antimycin A to the incubation medium. The loss of radiolabel from antimycin A-inhibited mitochondria which are accumulating [2- 14 C]pyruvate has been reported previously in rat liver mitochondria (Halestrap, 1975). It was attributed to the decarboxylation of the pyruvate by an NAD^+ -independent side reaction of pyruvate dehydrogenase. We could find no evidence for such a reaction in castor-bean mitochondria.

We have further studied the apparent loss of radiolabel from castor-bean mitochondria by preincubating the mitochondria for 3 min in an oxygen-electrode cell, in the presence or absence of either 10 μ M-UK5099 or 1 μ M-antimycin A as described in the legend to Fig. 3. The assay was started by the addition of [2- 14 C]pyruvate (0.1 mM). After the appropriate incubation period, samples were taken and the mitochondria sedimented by centrifugation. Samples of the supernatants were then analysed by two-dimensional t.l.c. on cellulose plates as described in the Experimental section. This procedure allows the separation of tricarboxylic acid-cycle intermediates. Standard samples of carboxylic acids were found

Table 3. Metabolism of [2- 14 C]pyruvate by castor-bean mitochondria

Mitochondria (2 mg of protein) were incubated for 3 min in reaction buffer (4.0 ml) as described in the legend to Fig. 3. Control incubations also contained either 10 μ M-UK5099 or 1 μ M-antimycin A. To initiate the assay [2- 14 C]pyruvate (0.25 μ Ci/ml) was added to a final concentration of 0.1 mM. After the appropriate time, 0.6 ml samples were removed and the mitochondria sedimented by centrifugation at 12000 g in a microfuge. Samples (6 μ l) of the supernatants were then analysed by two-dimensional t.l.c. on cellulose plates, and the radioactivity contents of the appropriate spots determined as described in the Experimental section.

Incubation time (s)	Additions	14 C-labelled compound in supernatant (nmol)		
		Pyruvate	Citrate	Malate
30	—	240	63	45
60	—	208	97	65
180	—	134	112	66
300	—	41	207	103
30	10 μ M-UK5099	341	13	7
300	10 μ M-UK5099	337	15	8
30	1 μ M-Antimycin A	328	7	4
300	1 μ M-Antimycin A	322	10	7

to have R_F values in each dimension comparable with those reported previously (Myers & Huang, 1969), and were resolved sufficiently to allow their elution without cross contamination.

The data in Table 3 show that in the absence of UK5099 there is, in the suspension medium, a gradual loss of radiolabel associated with pyruvate, and a concomitant increase in radiolabel associated with extra-mitochondrial malate and citrate. The ratio of the amount of radiolabel in citrate to that in malate varies with the period of incubation, and rises from 1.4:1 after 30 s incubation to 2.0:1 after 300 s incubation. In the incubations where 10 μ M-UK5099 or 1 μ M-antimycin A had been added before the addition of pyruvate, most of the radiolabel remained associated with pyruvate.

In all the experiments almost 90% of the radiolabel loaded on the thin-layer plates could be accounted for in pyruvate, malate and citrate. There were no significant amounts of radiolabel in other tricarboxylic acid-cycle intermediates or in the form of CO_2 . There was also a good correlation between the observed O_2 consumption and the amounts of malate and citrate present in the supernatant.

Although antimycin A does not have a significant effect on the amounts of pyruvate accumulated by the mitochondria, there is an almost total inhibition of the metabolism of pyruvate to malate and citrate in the presence of antimycin A (Table 3). Thus, although pyruvate can be accumulated in the presence of antimycin A, the continued uptake of pyruvate by castor-bean mitochondria appears to be linked to the metabolism of the pyruvate, as has been reported in rat heart mitochondria (Pande & Parvin, 1978). However, in the absence of antimycin A the carbon from C-2 and C-3 of pyruvate does not accumulate appreciably, as it is removed from the mitochondrial matrix as malate and

citrate. The observation that no appreciable radiolabel can be found in CO_2 also suggests that no cycling of the $[^{14}\text{C}]$ malate occurs. It seems likely therefore that internal $[^{14}\text{C}]$ malate will exchange for external P_i on the malate- P_i antiporter (Palmieri *et al.*, 1971), whereas internal $[^{14}\text{C}]$ citrate could exchange for the unlabelled malate in the incubation mixture on the malate-citrate antiporter (McGivan & Klingenberg, 1971; Papa *et al.*, 1971). The observation that little of the $[^{14}\text{C}]$ malate produced from the $[2\text{-}^{14}\text{C}]$ pyruvate is recycled via the tricarboxylic acid cycle, and that malate is required to 'spark' the oxidation of the $[2\text{-}^{14}\text{C}]$ pyruvate, suggests that two separate pools of malate may be present in the mitochondria.

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REFERENCES

- Bonner, W. D. (1967) *Methods Enzymol.* **10**, 126–133
- Bradford, N. M. & McGivan, J. D. (1973) *Biochem. J.* **134**, 1023–1029
- Chappell, J. & Beevers, H. (1983) *Plant Physiol.* **72**, 434–440
- Coleman, J. O. D. & Palmer, J. M. (1972) *Eur. J. Biochem.* **26**, 499–509
- Day, D. A. & Wiskich, J. T. (1974) *Plant Physiol.* **53**, 104–109
- Day, D. A. & Hanson, J. B. (1977*a*) *Plant Physiol.* **59**, 139–144
- Day, D. A. & Hanson, J. B. (1977*b*) *Plant Physiol.* **59**, 630–635
- Douce, R., Mannella, C. A. & Bonner, W. D. (1973) *Biochim. Biophys. Acta* **292**, 105–116
- Douce, R., Moore, A. L. & Neuberger, M. (1977) *Plant Physiol.* **60**, 625–628
- Halestrap, A. P. (1975) *Biochem. J.* **148**, 85–96
- Halestrap, A. P. (1978) *Biochem. J.* **172**, 377–387
- Halestrap, A. P. & Denton, R. M. (1974) *Biochem. J.* **138**, 313–316
- Halestrap, A. P., Scott, R. D. & Thomas, A. P. (1980) *Int. J. Biochem.* **11**, 97–105
- Huq, S. & Palmer, J. M. (1978) in *Plant Mitochondria* (Ducet, G. & Lance, C., eds.), pp. 225–232, Elsevier/North-Holland Biomedical Press, Amsterdam
- Jackson, C., Dench, J. E., Hall, D. O. & Moore, A. L. (1979) *Plant Physiol.* **64**, 150–153
- McGivan, J. D. & Klingenberg, M. (1971) *Eur. J. Biochem.* **20**, 392–399
- Moore, A. L., Linnett, P. E. & Beechey, R. B. (1980) *J. Bioenerg. Biomembr.* **12**, 309–323
- Myers, W. F. & Huang, K. Y. (1969) *Methods Enzymol.* **13**, 431–434
- Neuburger, M., Day, D. A. & Douce, R. (1984) *Arch. Biochem. Biophys.* **229**, 253–258
- Palmieri, G., Prezioso, G., Quagliariello, E. & Klingenberg, M. (1971) *Eur. J. Biochem.* **232**, 66–71
- Pande, S. V. & Parvin, R. (1978) *J. Biol. Chem.* **253**, 1565–1573
- Papa, S. & Paradies, G. (1974) *Eur. J. Biochem.* **49**, 265–274
- Papa, S., Lofrumento, N. E., Kanduc, D., Paradies, G. & Quagliariello, M. (1971) *Eur. J. Biochem.* **22**, 134–143
- Paradies, G. & Papa, S. (1975) *FEBS Lett.* **52**, 149–152
- Paradies, G. & Papa, S. (1977) *Biochim. Biophys. Acta* **462**, 333–346
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Pfaff, E., Heldt, H. W. & Klingenberg, M. (1969) *Eur. J. Biochem.* **10**, 484–493
- Quagliariello, E., Palmieri, F., Prezioso, G. & Klingenberg, M. (1969) *FEBS Lett.* **4**, 251–254
- Schonbaum, G. R., Bonner, W. D., Storey, B. T. & Bahr, J. T. (1971) *Plant Physiol.* **44**, 115–128
- Thomas, A. P. & Halestrap, A. P. (1981) *Biochem. J.* **196**, 471–479
- Tomlinson, P. F. & Moreland, D. E. (1975) *Plant Physiol.* **55**, 365–369
- Vaartjes, W. J., Geelen, M. J. H. & Van Den Bergh, S. G. (1979) *Biochim. Biophys. Acta* **548**, 38–47

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