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Pyruvate transport by thermogenic-tissue mitochondria

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1. Mitochondria isolated from the thermogenic spadices of *Arum maculatum* and *Sauromatum guttatum* plants oxidized external NADH, succinate, citrate, malate, 2-oxoglutarate and pyruvate without the need to add exogenous cofactors. 2. Oxidation of substrates was virtually all via the alternative oxidase, the cytochrome pathway constituting only 10–20% of the total activity, depending on the stage of spadix development. 3. During later stages of spadix development, pyruvate oxidation was enhanced by the addition of aspartate. This was caused by acetyl-CoA condensing with oxaloacetate, produced from pyruvate/aspartate transamination, and so decreasing feedback inhibition of pyruvate dehydrogenase. 4. Pyruvate oxidation was inhibited by the long-chain acid maleimides AM5–11, but not by those with shorter polymethylene side groups, AM1–4. 5. The α-cyanocinnamate derivatives UK5099 [α-cyano-β-(1-phenylindol-3-yl)acrylate] and CHCA [α-cyano-4-hydroxycinnamate] inhibited pyruvate-dependent O_2 consumption and the carrier-mediated uptake of pyruvate across the mitochondrial inner membrane. Characteristics of non-competetive inhibition were observed for CHCA, whereas for UK5099 the results were more complex, suggesting a very low rate of dissociation of the inhibitor–carrier complex. 6. A comparison of the values of $V_{max.}$ and K_m for oxidation and transport suggested that it was the latter which controls the overall rate of pyruvate oxidation by mitochondria from both tissues.

INTRODUCTION

In the thermogenic spadices of *Arum maculatum* and *Sauromatum guttatum* plants, the breakdown of storage compounds, mostly in the form of starch (Meeuse, 1984), leads to the production of large quantities of cytosolic pyruvate. Further metabolism of this keto acid in the tricarboxylic acid cycle, by pyruvate dehydrogenase (located in the matrix), requires the movement of pyruvate across the mitochondrial inner membrane. The rate of influx or the rate of oxidation may therefore exert a considerable control over the flux through the tricarboxylic acid cycle and the subsequent alternative oxidase-dependent heat generation. This is necessary for the volatilization of odoriferous insect-attracting compounds, mainly amines, which are required for the pollination of these species (Meeuse, 1984).

The characteristics of pyruvate translocation in mammalian mitochondria, including its sensitivity to the compounds α-cyano-4-hydroxycinnamate (CHCA) and α-cyano-β-(1-phenylindol-3-yl)acrylate (UK5099), have been well documented (Halestrap & Denton, 1974; Halestrap, 1975, 1978; Paradies & Papa, 1975, 1977; Halestrap *et al.*, 1980; Shearman & Halestrap, 1984). There are few data available, however, on pyruvate transport into plant mitochondria. It does appear to involve a specific carrier which mediates the electroneutral uptake of pyruvate, driven by ΔpH (Proudlove & Moore, 1982), is inhibited non-competitively by CHCA (Day & Hanson, 1977) and also by UK5099 (Proudlove & Moore, 1984; Brailsford *et al.*, 1986). The rapid rate of pyruvate-dependent O_2 consumption by thermogenic-tissue mitochondria, which does not appear to require the addition of 'sparker' metabolites or exogenous cofactors (Proudlove & Moore, 1984), allows a more accurate estimate of these rates of oxidation to be made over a wide range of inhibitor concentrations, particularly when the cinnamate concentration is high and the pyruvate concentration is low.

In the present paper, therefore, we have studied the characteristics of pyruvate-dependent O_2 uptake by and the accumulation of this keto acid into the matrix of mitochondria isolated from thermogenic spadices. In order to assess whether transport of pyruvate across the inner membrane may limit its subsequent oxidation by the mitochondrial electron-transport chain, and hence overall tricarboxylic acid-cycle turnover, we have investigated the effects of the α-cyanocinnamate derivatives, CHCA and UK5099, on both of these processes.

EXPERIMENTAL

Materials

Corms of *Sauromatum guttatum* were purchased from Bees Ltd., Chester, U.K., stored at 4 °C until required and germinated by transfer to a glasshouse at 20–25 °C. Spadices were harvested after approx. 6 weeks, when they showed maximum heat production and amine volatilization (Meeuse, 1984). *Arum maculatum* inflorescences, in the stage of development as near as possible to that designated γ (James & Beevers, 1950), were collected from local woodlands and used the same day. Silicone oils (AR200 and AR20) and UK5099 were gifts from Wacker-Chemie, Munich, W. Germany, and Pfizer (U.K.), Sandwich, Kent, U.K., respectively, and the *N*-polymethylenecarboxymaleimides were synthesized by Dr. D. G. Griffiths at the University of Canterbury (Griffiths *et al.*, 1981). CHCA was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Radioisotopes,

Abbreviations used: CHCA, α-cyano-4-hydroxycinnamate; UK5099, α-cyano-β-(1-phenylindol-3-yl)acrylate; Δp, protonmotive force.
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[1-^{14}C]pyruvate, [^{14}C]sorbitol and $^{3}H_2O$, were purchased from Amersham International, Amersham, Bucks, U.K., and all other chemicals from Sigma Chemical Co., Poole, Dorset, U.K., or BDH Chemicals, Chadwell Heath, Essex, U.K. Immediately after the [1-^{14}C]pyruvate was opened, it was dissolved in water and divided into 5 μCi samples, which were rapidly vacuum-desiccated and stored at −20 °C until used. This was normally within 3–4 months.

Isolation of mitochondria

Spadices of *A. maculatum* were cleaned by gently abrading them with cotton wool soaked in iced water and those of *S. guttatum* by rinsing with cold tap water (the surface coloration of the latter could not be removed without damaging the tissue). It was found that mitochondrial activity was unaffected if surface anthocyanins were left intact, and in later preparations spadices were not washed. Approx. 50–100 g of tissue was cut (5 mm transverse sections) into 200 ml of 0.3 M-mannitol/1 mM-EDTA/0.2% (w/v) bovine serum albumin/4 mM-cysteine/20 mM-Mops, pH 7.6. After tissue disruption, with a Moulinex 66 homogenizer (Moulinex, Alencon, France) for approx. 20 s, the mitochondria were isolated by differential centrifugation as previously described (Moore & Proudlove, 1983; Proudlove & Moore, 1984). The final pellets were resuspended in a small volume of isolation medium, minus cysteine, to a protein concentration of 55–65 mg/ml.

Oxygen-electrode experiments

Substrate-dependent O_2 consumption was measured polarographically at 25 °C with a Hansatech O_2 electrode (Hansatech, King's Lynn, Norfolk, U.K.). Reactions were performed in 1 ml of medium A (0.3 M-mannitol/10 mM-KCl/5 mM-$MgCl_2$/10 mM-Mops/10 mM-KH_2PO_4, pH 7.2) and contained 1–3 mg of mitochondrial protein. Other additions were made as indicated in the text. The electrode was calibrated by assuming the concentration of O_2 in air-saturated distilled water to be 254 μM, at 25 °C (Estabrook, 1967).

Pyruvate-transport experiments

The movement of pyruvate across the mitochondrial inner membrane was investigated by monitoring the accumulation of [1-^{14}C]pyruvate in the matrix by using the silicone-oil centrifugal-filtration technique (Moore & Proudlove, 1983; Proudlove & Moore, 1982, 1984). All incubations were carried out at 20–22 °C in 0.3 M-mannitol/10 mM-KCl/5 mM-$MgCl_2$/10 mM-Mops, pH 7.2 (medium U).

Time-dependent pyruvate uptake

Incubations were performed in 400 μl polypropylene tubes (Hughes and Hughes, Romford, Essex, U.K.). Into these were pipetted 20 μl of 10% (v/v) $HClO_4$, 70 μl of silicone oil [AR200/AR20, 10:1 (v/v)] and 50 μl of medium U, containing 0.2 nmol of antimycin A/mg of mitochondrial protein, 100 μM-n-propyl gallate (both added to prevent pyruvate oxidation), 1 mM-ATP (added to generate a Δp to drive uptake), 20 nmol of [1-^{14}C]-pyruvate (0.5 μCi of 25 mCi/mmol) and 80 nmol of unlabelled pyruvate. Reactions were initiated by the addition of mitochondria (50 μl at 8 mg of mitochondrial protein/ml) and terminated by the addition of 50 μl of 30 μM-UK5099 (ice-cold). Mitochondria were pelleted through the silicone oil by centrifuging (5–7 s after the addition of UK5099 inhibitor-stop) for 50 s in a Beckman Microfuge B. Each time point was repeated three times per experiment.

Concentration-dependent pyruvate uptake

Approx. 0.6–1.5 mg of mitochondrial protein (in 100 μl portions) was added to a 100 μl volume of medium U, containing 0.2 nmol of antimycin A/mg of protein, 100 μM-n-propyl gallate, 1 mM-ATP and double the required final concentration of CHCA or UK5099. Reactions were initiated after 2 min by the addition of 50 μl of pyruvate solution, containing 0.5 μCi of [1-^{14}C]-pyruvate (25 mCi/mmol) and sufficient unlabelled pyruvate to give the required concentration, and terminated after a further 15 s by the addition of 100 μl of 50 μM-UK5099 (ice-cold). Triplicate 100 μl samples were pipetted into 0.4 ml tubes containing $HClO_4$ and silicone oil, and mitochondria were pelleted as described for time-dependent uptake.

Sample treatment

Tubes were frozen at −17 °C, cut through the oil layer, and supernatant and pellet fractions were mixed with 2 ml of Optiphase 'Safe' scintillant (LKB, South Croydon, Surrey, U.K.). Radioactivity in all samples was determined with a Beckman LS1800 liquid-scintillation spectrometer, the H# technique (Beckman LS Systems Operating Manual) being used to correct for quench. Control values for the amount of protein pelleted through the oil layer (normally 40–60%) and non-specific adhesion of label (determined by incubations where the inhibitor-stop UK5099 was added before pyruvate) were taken into account when calculating the uptake of pyruvate into the matrix. This volume was measured by subtracting the volume permeable to [^{14}C]-sorbitol from that permeable to $^{3}H_2O$, average values being 1.5–2.3 μl/mg of protein.

Metabolism of [1-^{14}C]pyruvate

Mitochondria were incubated essentially as described for time-dependent uptake. The $HClO_4$, silicone oil and unlabelled pyruvate were omitted, the mitochondrial protein was increased to 1 mg and the [1-^{14}C]pyruvate to 2.5 μCi (1 mM). Incubations were stopped at set times by the addition of 50 μl of 30 μM-UK5099, rapidly followed by 200 μl of ethanol. Samples from each time point were analysed by t.l.c. (Proudlove & Thurman, 1981) and, up to 1 min incubation, showed only one radioactive spot, corresponding to pyruvate.

Protein determinations

All proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard. Samples were taken from the initial resuspension, the diluted mitochondrial suspension used for uptake experiments and from supernatant and pellets after silicone-oil centrifugal filtration. Recovery controls were run, minus radioactivity, as the first and last incubations per experiment. Samples (50 μl) of supernatants and whole pellets, obtained by cutting tubes at the oil/acid interface, were mixed in water, and total protein was determined in each.

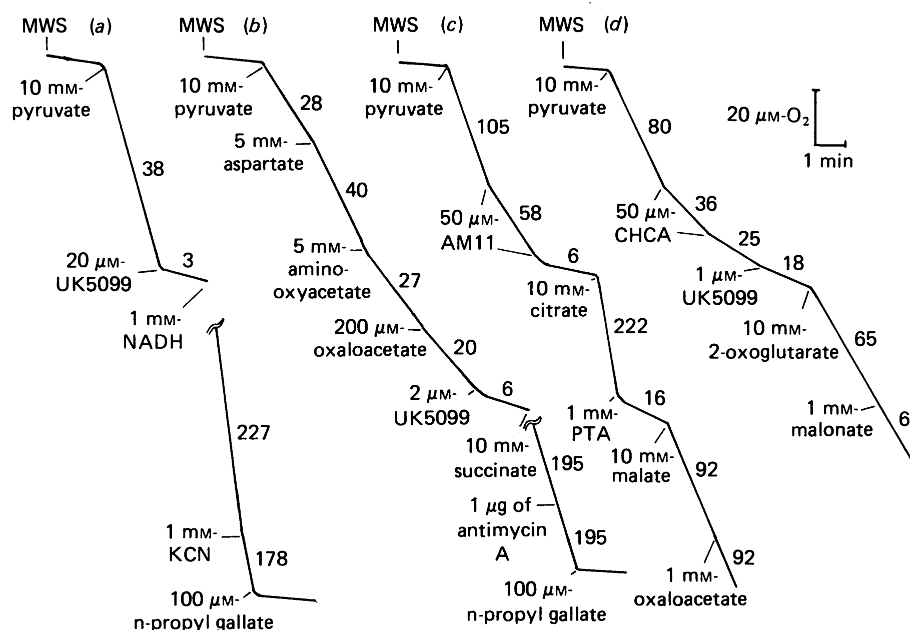

Fig. 1. Substrate-dependent O_2 consumption by thermogenic-tissue mitochondria and the effect of several inhibitors

After their isolation by differential centrifugation, mitochondria (MWS) from the spadices of *Sauromatum guttatum* (*a*, 1.16 mg of protein) and *Arum maculatum* (*b*, 0.97 mg of protein; *c* and *d*, 0.53 mg of protein) were incubated in 1 ml of medium A (0.3 M-mannitol/10 mM-KCl/5 mM-$MgCl_2$/10 mM-Mops/10 mM-KH_2PO_4, pH 7.2), and O_2 consumption was measured polarographically at 25 °C with a Hansatech O_2 electrode. Additions were made as indicated, and numbers by traces refer to the rates of O_2 consumption, in nmol of O_2/min per mg of mitochondrial protein. Breaks in traces *a* and *b* represent increases in chart speed. Abbreviation: PTA, phthalonic acid.

RESULTS AND DISCUSSION

Mitochondria isolated from thermogenic spadices oxidize most tricarboxylic acid-cycle intermediates, including pyruvate (Proudlove & Moore, 1984). The rates of substrate oxidation were generally higher than those found for washed mitochondria from other tissues, and there was no requirement for 'sparker' metabolites or exogenous cofactors, necessary for dehydrogenase activity in some mitochondria (Douce & Neuburger, 1987; Fig. 1). In mitochondria isolated from *S. guttatum*, pyruvate-dependent O_2 consumption was potently inhibited by 20 μM-UK5099, but was readily re-established by the addition of exogenous NADH (Fig. 1*a*). The rate of NADH oxidation was essentially cyanide-insensitive, but was inhibited by n-propyl gallate.

With certain preparations of *A. maculatum* mitochondria, notably from the end of the season when many spadices were at the δ–ϵ stage of development (James & Beevers, 1950), pyruvate was oxidized more slowly (Fig. 1*b*) than by mitochondria from spadices in the γ stage (Figs. 1*c* and 1*d*). This rate could, however, be enhanced by the addition of 5 mM-aspartate. Reversal of this stimulation by 5 mM-amino-oxyacetate, a general transaminase inhibitor (Givan, 1980), suggests that it was the supply of oxaloacetate that was limiting pyruvate oxidation. Aspartate transamination with pyruvate produces oxaloacetate and alanine, and the former condenses with acetyl-CoA, a product of pyruvate dehydrogenation, to synthesize citrate. Presumably the removal of matrix acetyl-CoA decreases feedback inhibition of this dehydrogenase (Moller & Palmer, 1984) and results in a mixture of pyruvate- and citrate-dependent O_2 consumption, citrate also being transported and readily oxidized by these mitochondria (Fig. 1*c*). Direct addition of oxaloacetate (200 μM) had an inhibitory, rather than stimulatory, effect on pyruvate oxidation (Fig. 1*b*). This result may be explained by oxaloacetate being transported across the inner membrane (Proudlove & Moore, 1984; Ebbighausen *et al.*, 1985), lowering the endogenous concentration of NADH, as a result of the $K_{eq.}$ for malate dehydrogenase (Moller & Palmer, 1984), and so limiting the flux through the respiratory chain. This imbalance in oxaloacetate supply may be an aspect of tissue senescence as the spadices 'burn out' in the δ–ζ stage. Even under these conditions, however, the oxidation rate is sensitive to UK5099. In this situation, the respiratory rate was re-established by the addition of succinate.

Several of the *N*-polymethylenecarboxymaleimides [trivial names 'acid maleimides' (AM) followed by a number denoting the number of methylene groups in the side chain (Griffiths *et al.*, 1981)] were found to decrease pyruvate-dependent O_2 consumption in *A. maculatum* mitochondria (Griffiths *et al.*, 1983). These compounds allow the maleimide residue to penetrate into the membrane, the depth of penetration being determined by the length of the polymethylene chain, and uncontrolled entry into hydrophobic regions being prevented by the ionized carboxy group (Griffiths *et al.*, 1983). The greatest inhibition was seen with AM11 (Fig. 1*c*), although shorter-chain maleimides also inhibited, the degree of inhibition being proportional to chain length, with no inhibitory effects observed below AM5 (results not shown). These data suggest that, as has been reported for mammalian mitochondria, where *N*-ethyl- and *N*-

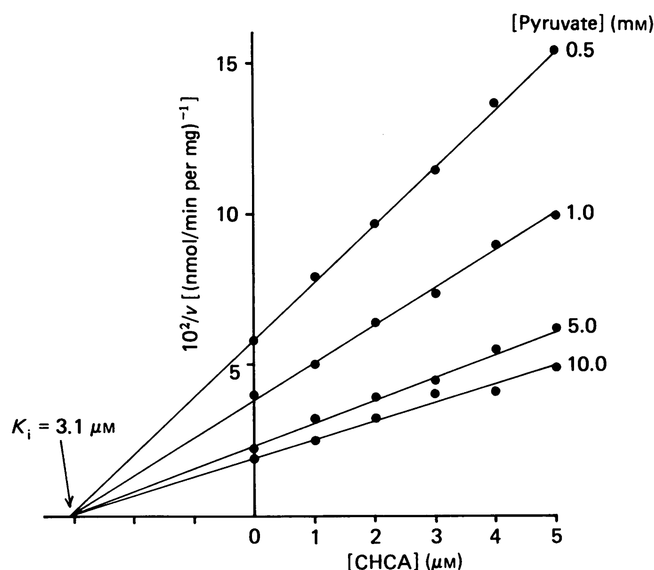

Fig. 2. Dixon plot for the effect of CHCA on pyruvate oxidation by *Arum maculatum* mitochondria

Mitochondria were isolated from *Arum maculatum* spadices, and 0.5–2 mg of protein was incubated for 2 min, in medium A plus the appropriate concentration of CHCA, as described in Fig. 1. O_2 consumption was initiated by the addition of the relevant concentration of pyruvate, and rates were measured over the first 3 min. Each point is the mean of at least one determination from 15 separate preparations of mitochondria. Standard deviations from the mean for each point, which were generally within 5–10 % of the means, were not included, to avoid over-crowding of the data in the Figure. Regression coefficients for each line were all greater than 0.98.

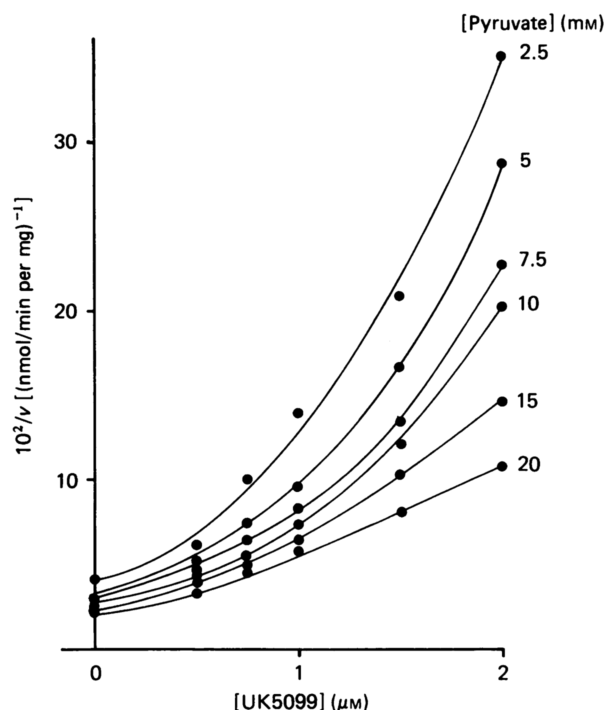

Fig. 3. Dixon plot for the effect of UK5099 on pyruvate oxidation by *Sauromatum guttatum* mitochondria

Mitochondria were isolated from the spadices of *Sauromatum guttatum*, and 1–3 mg of protein was incubated for 2 min, in medium A plus the appropriate concentration of UK5099, as described in Fig. 1. O_2 consumption was initiated by the addition of the relevant concentration of pyruvate and rates were measured over the first 3 min. Each point is the mean of at least one determination from eight separate preparations of mitochondria. Standard deviations from the mean for each point, which were generally within 5–10 % of the means, were not included, to avoid over-crowding of the data in the Figure.

phenyl-maleimide inhibit pyruvate accumulation (Papa & Paradies, 1974; Thomas & Halestrap, 1981), there is a thiol group associated with the pyruvate transporter of plant mitochondria. Inhibition by the short-chain *N*-ethylmaleimide, but not by AM1–4, occurs because the former freely partitions into the lipid domain of the membrane, whereas the latter are prevented from doing so by their polar carboxy group (Griffiths *et al.*, 1983). It is interesting that a direct estimation of the effect of AM11 on pyruvate transport by *S. guttatum* mitochondria showed a measurable inhibition of pyruvate uptake, but it was not as marked as seen for oxidation studies. Preincubating mitochondria for 2 min with 1 mM-AM11 decreased the uptake of 5 mM-pyruvate by only 61 % (result not shown), whereas 100 μM-AM11 decreased the oxidation of 10 mM-pyruvate by 95 % within a similar time period (Fig. 1*c*). This suggests that the maleimide was reacting with protein thiol groups associated with both pyruvate transport and pyruvate dehydrogenation, and prompts the speculation that, as found for the glycine decarboxylase complex in pea leaf mitochondria (Moore *et al.*, 1984) and as reported for castor-bean endosperm mitochondria (Reid *et al.*, 1975), the pyruvate dehydrogenase complex may be located, at least partially, in the mitochondrial inner membrane of these tissues.

Fig. 1(*d*) illustrates that the cinnamate derivative CHCA, at micromolar concentrations, also inhibits pyruvate oxidation by *A. maculatum* mitochondria. The inhibition is, however, less pronounced than that seen for equimolar concentrations of UK5099. The specificity of cinnamate inhibition of pyruvate oxidation was emphasized by the restoration of O_2 consumption after the addition of other substrates oxidized via Complex I (citrate, 2-oxoglutarate and malate) as well as those for Complex II (succinate) and the external NADH dehydrogenase (Fig. 1). These results indicate that neither compound was exerting its effect by a non-specific inhibition of the respiratory chain, but that inhibition was specific to pyruvate oxidation. Results in Fig. 1 also show that, whereas cyanide and antimycin A have little or no effect on substrate oxidation, mitochondria from both tissues are highly sensitive to n-propyl gallate, demonstrating that O_2 uptake was catalysed by the alternative terminal oxidase.

As reported previously for thermogenic tissue mitochondria (Proudlove & Moore, 1984), citrate-dependent O_2 consumption was inhibited by phthalonic acid. This had no effect on malate transport and oxidation, but it did prevent the entry of oxaloacetate (Fig. 1*c*). Oxaloacetate would have inhibited malate oxidation by a reversal of malate dehydrogenase. The finding that the oxidation of 2-oxoglutarate was insensitive to malonate

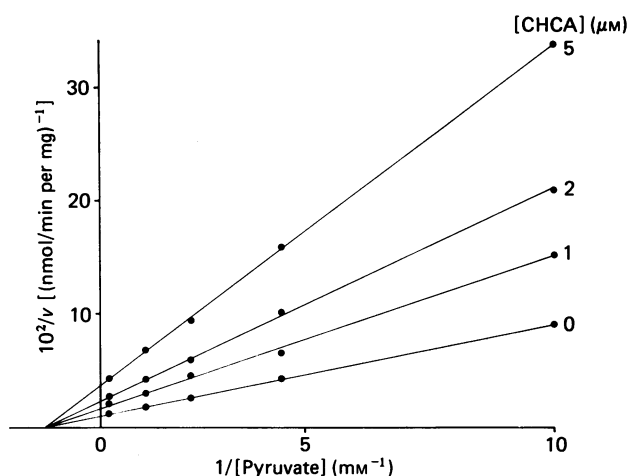

Fig. 4. Lineweaver–Burk plot for the effect of CHCA on the uptake of pyruvate into the matrix of *Arum maculatum* mitochondria

Incubations were carried out at 20–22 °C in 200 μl of medium containing 0.3 M-mannitol, 10 mM-KCl, 5 mM-$MgCl_2$, 10 mM-Mops (pH 7.2), 0.2 nmol of antimycin A/mg of protein, 100 μM-n-propyl gallate, 1 mM-ATP, the specified concentration of CHCA and 0.6–1.5 mg of mitochondrial protein. After a 2 min preincubation, reactions were initiated by the addition of 50 μl of pyruvate solution, containing 0.5 μCi of [1-^{14}C]pyruvate (25 mCi/mmol) and unlabelled pyruvate to give the required concentration, terminated after a further 15 s by the addition of 100 μl of 50 μM-UK5099 (at 4 °C), and mitochondria were removed from the bathing medium by pelleting them through silicone oil. Radioactivity in pellet and supernatant, and hence uptake, were determined as outlined in the Experimental section. Each point is the mean of at least one determination from six separate preparations of mitochondria. Regression coefficients for each line were all greater than 0.98.

(Fig. 1*d*) indicates that it was this compound which was being oxidized and not succinate.

A Dixon plot for the effect of CHCA on pyruvate oxidation by *Arum maculatum* mitochondria showed non-competitive kinetics (Fig. 2), with an estimated K_i of 3.1 ± 0.2 μM. This was similar to the response and K_i reported for the inhibition of pyruvate oxidation by CHCA in rat liver (6.3 μM; Halestrap, 1975) and rat heart (2 μM; Shearman & Halestrap, 1984) mitochondria and that calculated from the data for etiolated maize mitochondria (approx. 3 μM; Day & Hanson, 1977). When the rate of pyruvate oxidation was measured in the presence of various concentrations of UK5099, no simple pattern of inhibition was found, a Dixon plot of the results being clearly non-linear (Fig. 3). This is comparable with data published by Shearman & Halestrap (1984) for ADP-stimulated pyruvate-dependent O_2 uptake by rat heart mitochondria over an equivalent range of UK5099 concentrations. These authors varied pyruvate oxidation by changing the amount of mitochondrial protein at constant pyruvate concentrations. Controlling the rate of pyruvate-dependent O_2 consumption, by varying the substrate concentration at a constant protein concentration, and obtaining the same non-linear response suggests that the mode of inhibition is similar in both types of mitochondria. The decrease in pyruvate-dependent O_2 consumption by α-cyanocinnamates may reflect an inhibition of pyruvate movement across the inner membrane, an inhibition of pyruvate oxidation by pyruvate dehydrogenase, or a mixture of both. In studies to date, for mammalian (Halestrap, 1975) and plant (Day & Hanson, 1977; Proudlove & Moore, 1982; Walker & Oliver, 1983; Brailsford *et al.*, 1986) mitochondria, these compounds appear to inhibit specifically a pyruvate carrier situated in the inner membrane.

Pyruvate uptake by *A. maculatum* mitochondria followed first-order kinetics, over the first 30 s, when measured by inhibitor-stop as described in the Experimental section (results not shown). In the presence of antimycin A and n-propyl gallate there was also no apparent metabolism of [1-^{14}C]pyruvate in this time period, suggesting that it was the initial rate of transport that was being observed. We calculated the initial rate of reaction, from $v = k(P_{max.})$ [see Brailsford *et al.* (1986) for details], to be 4.6 nmol/min per mg of mitochondrial protein.

That pyruvate influx was carrier-mediated was denoted by substrate saturation of initial rates of uptake (see data for uninhibited rates in Figs. 4 and 5) and specific inhibition by cinnamate derivatives. The kinetics of inhibition by CHCA and UK5099 were similar to those

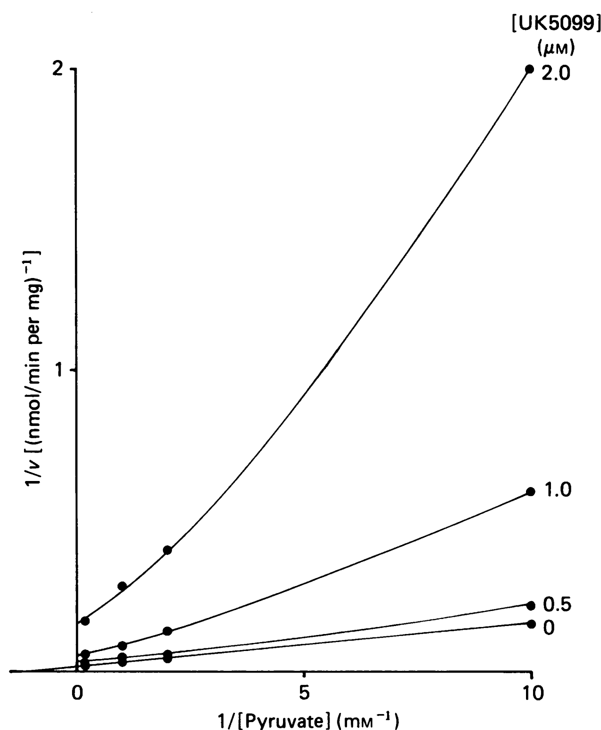

Fig. 5. Lineweaver–Burk plot for the effect of UK5099 on the uptake of pyruvate into the matrix of *Arum maculatum* mitochondria

The initial rates of pyruvate accumulation were determined essentially as described in the legend for Fig. 4. Mitochondria were preincubated for 2 min with various concentrations of UK5099, and reactions were terminated by the addition of 50 μM-UK5099 (at 4 °C). Each point is the mean of at least one determination from four separate preparations of mitochondria.

Table 1. Kinetic constants of pyruvate oxidation and transport by thermogenic-tissue mitochondria

The rates of pyruvate oxidation by mitochondria from both tissues were measured, in medium A alone, as described in Figs. 2 and 3. Transport of pyruvate into the mitochondrial matrix was measured as outlined in Fig. 4, except that the cinnamate derivatives were omitted from the 2 min preincubation and *S. guttatum* mitochondria were also used to study concentration-dependent pyruvate uptake ($n = 3$). The Michaelis–Menten constants, $V_{max.}$ and K_m, were calculated from Eadie–Hofstee plots of these data, and results are means ± S.D.

	$V_{max.}$ (nmol uptake/min per mg of mitochondrial protein)		K_m (mM-pyruvate)	
Tissue	O_2	Pyruvate	Oxidation	Uptake
A. maculatum	52.6 ± 2.8	83.4 ± 6.3	1.4 ± 0.1	0.8 ± 0.1
S. guttatum	48.2 ± 4.1	82.1 ± 7.3	2.1 ± 0.5	1.2 ± 0.6

observed for oxidation. A Lineweaver–Burk plot for the effect of different concentrations of CHCA on pyruvate uptake (Fig. 4) indicated that this compound inhibited non-competitively, as found for oxidation (Fig. 2), with an estimated K_i of 2.2 ± 0.2 μM (Dixon plot; not shown). When UK5099 was substituted for CHCA, non-linear kinetics, as found during oxidation studies (Fig. 3), were again observed (Fig. 5). Such non-linear responses for the inhibition of pyruvate oxidation and transport are in contrast with the report by Brailsford *et al.* (1986), for UK5099 inhibition of State-3 pyruvate-dependent O_2 consumption in castor-bean endosperm mitochondria. These authors interpreted their results to indicate that this α-cyanocinnamate derivative was a non-competitive inhibitor, similar to CHCA but with a much lower K_i. Their use of lower concentrations of UK5099 may explain this discrepancy. The lowest concentration used in the present study (0.5 μM) was 10 times higher than the greatest concentration used by Brailsford *et al.* (1986). Under these conditions, the points in a Lineweaver–Burk plot do tend to linearity. It is only under conditions where the amount of UK5099 in the incubation is relatively high and that of pyruvate is relatively low that maximum deviation from linearity is observed. Careful scrutiny of data in Brailsford *et al.* (1986), indeed, may persuade one to conclude that the points do not describe straight lines, but show the trends reported in the present study. Further experiments will be necessary to clarify this point.

An explanation of the non-linear inhibition kinetics of UK5099 on pyruvate oxidation by rat heart mitochondria (Shearman & Halestrap, 1984) was that the inhibitor was non-competitive and had a K_i much lower than the concentration of binding sites or carrier molecules. This suggestion was supported by computer reiteration [presumably arithmetic errors in their eqn. (3) were corrected], whereby calculated values could be closely associated with those observed. In some ways, a simpler view would be that UK5099 dissociated only very slowly from the carrier–inhibitor complex. This would therefore constitute a rate-limiting step in the binding, transport and oxidation of pyruvate. Under conditions of high [substrate]/low [inhibitor], when most of the UK5099 was bound, there are still sufficient free substrate-binding sites available to react with pyruvate and kinetics tend to Michaelis–Menten. As the ratio was changed, to low [substrate]/high [inhibitor], the experimental points deviated from linearity, UK5099 having a far greater inhibitory effect than would have been predicted from Michaelis–Menten kinetics. Under these conditions more of the carrier molecules existed as the carrier–inhibitor complex, and pyruvate binding and transport were a function of the dissociation of this complex and the successful formation of the carrier–substrate complex. This argument may also be applied to conditions where the substrate concentration remains constant and the number of carrier molecules is varied (Shearman & Halestrap, 1984).

The question still remains as to the possible regulatory role of transport in the oxidation of pyruvate by thermogenic-tissue mitochondria. Results presented in Table 1, which gives the values for $V_{max.}$ and K_m, may be interpreted in two ways. According to Day & Hanson (1977), the complete oxidation of 1 mol of pyruvate to CO_2 and water consumes 2.5 mol of O_2. The estimated $V_{max.}$ for pyruvate uptake by both *A. maculatum* and *S. guttatum* mitochondria was approx. 83 nmol/min per mg of protein, and this would permit a maximum rate of O_2 consumption of 207.5 nmol/min per mg of protein. As shown in Table 1, the values of $V_{max.}$ calculated for O_2 uptake by *A. maculatum* and *S. guttatum* mitochondria were approx. 53 nmol/min per mg of protein, only one-quarter of the rate predicted. [Although higher rates of pyruvate oxidation were recorded (Fig. 1), the mean from at least 15 separate determinations (Fig. 2) was that reported in Table 1.] Alternatively, if 1 mol of pyruvate produced 1 mol of NADH, which in turn reduced 0.5 mol of O_2, then a transport rate of approx. 105 nmol of pyruvate uptake/min per mg of protein would be needed to support the measured rates of O_2 consumption. This value is similar to the estimated $V_{max.}$ for transport, and the discrepancy between the two may be explained by differences in the number of replicates used to determine each, and by the errors involved in estimating kinetic parameters from straight-line derivations of the Michaelis–Menten equation. From these calculations, we would therefore conclude that the rate of pyruvate movement across the mitochondrial inner membrane had a regulatory role in the overall velocity of pyruvate oxidation by these organelles.

The correlation between the values of $V_{max.}$ for oxidation and transport found in this investigation were much closer than has been previously reported for plant mitochondria (Day & Hanson, 1977; Brailsford *et al.*,

1986). For etiolated maize mitochondria the rate of pyruvate uptake was 21 nmol/min per mg of protein at 2 mM-pyruvate, whereas State-3 pyruvate oxidation at this concentration was approx. 173 nmol of O_2 consumed/min per mg of protein [assuming a 10-fold error for $1/v$ in Fig. 3 of Day & Hanson (1977)]. Castor-bean endosperm mitochondria showed a State-3 pyruvate-dependent O_2-consumption rate of 33 nmol/min per mg of protein, yet the $V_{max.}$ for pyruvate uptake was only 0.95 nmol/min per mg of protein (Brailsford *et al.*, 1986). This was discovered to be due to oxidation of pyruvate and export of radiolabel in the form of citrate and malate. Metabolism of matrix pyruvate by these mitochondria may have been expected, as uptake was measured in the presence of ADP and in the absence of any respiratory inhibitor. Energizing mitochondria with ascorbate/*NNN'N'*-tetramethyl-*p*-phenylenediamine or ATP and inhibiting respiration with antimycin A and n-propyl gallate, as was done in the present work, should enable a more accurate estimate of the kinetics of pyruvate transport into these mitochondria.

The rapid oxidation of pyruvate, by mitochondria isolated from thermogenic spadices, therefore involved its transport across the inner membrane on a carrier which was specifically inhibited by certain long-chain acid maleimides and the α-cyanocinnamate derivatives CHCA and UK5099. The acid maleimides appeared also to inhibit pyruvate dehydrogenase, whereas the kinetics of cinnamate inhibition reflected those of the transporter. Whereas CHCA inhibited non-competitively, in accordance with Michaelis–Menten kinetics, the pattern of UK5099 inhibition was more complex, suggesting a low dissociation constant for the carrier–inhibitor complex. A comparison between the maximum rates of pyruvate transport and pyruvate-dependent O_2 uptake indicated that the former is the main rate-determining component of pyruvate oxidation.

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