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The Mitochondrial Pyruvate Carrier

KINETICS AND SPECIFICITY FOR SUBSTRATES AND INHIBITORS

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1. Studies on the kinetics of pyruvate transport into mitochondria by an 'inhibitor-stop' technique were hampered by the decarboxylation of pyruvate by mitochondria even in the presence of rotenone. Decarboxylation was minimal at 6°C. At this temperature the K_m for pyruvate was 0.15mM and $V_{max.}$ was 0.54nmol/min per mg of protein; α-cyano-4-hydroxycinnamate was found to be a non-competitive inhibitor, K_i 6.3μM, and phenylpyruvate a competitive inhibitor, K_i 1.8mM. 2. At 100μM concentration, α-cyano-4-hydroxycinnamate rapidly and almost totally inhibited O_2 uptake by rat heart mitochondria oxidizing pyruvate. Inhibition could be detected at concentrations of inhibitor as low as 1μM although inhibition took time to develop at this concentration. Inhibition could be reversed by diluting out the inhibitor. 3. Various analogues of α-cyano-4-hydroxycinnamate were tested on rat liver and heart mitochondria. The important structural features appeared to be the α-cyanopropenoate group and the hydrophobic aromatic side chain. α-Cyanocinnamate, α-cyano-5-phenyl-2,4-pentadienoate and compound UK 5099 [α-cyano-β-(1-phenylindol-3-yl)acrylate] were all more powerful inhibitors than α-cyano-4-hydroxycinnamate showing 50% inhibition of pyruvate-dependent O_2 consumption by rat heart mitochondria at concentrations of 200, 200 and 50nM respectively. 4. The specificity of the carrier for its substrate was studied by both influx and efflux experiments. Oxamate, 2-oxobutyrate, phenylpyruvate, 2-oxo-4-methylpentanoate, chloroacetate, dichloroacetate, difluoroacetate, 2-chloropropionate, 3-chloropropionate and 2,2-dichloropropionate all exchanged with pyruvate, whereas acetate, lactate and trichloroacetate did not. 5. Pyruvate entry into the mitochondria was shown to be accompanied by the transport of a proton (or by exchange with an OH^- ion). This proton flux was inhibited by α-cyano-4-hydroxycinnamate and allowed measurements of pyruvate transport at higher temperatures to be made. The activation energy of mitochondrial pyruvate transport was found to be 113kJ (27kcal)/mol and by extrapolation the rate of transport of pyruvate at 37°C to be 42nmol/min per mg of protein. The possibility that pyruvate transport into mitochondria may be rate limiting and involved in the regulation of gluconeogenesis is discussed. 6. The transport of various monocarboxylic acids into mitochondria was studied by monitoring proton influx. The transport of dichloroacetate, difluoroacetate and oxamate appeared to be largely dependent on the pyruvate carrier and could be inhibited by pyruvate-transport inhibitors. However, many other halogenated and 2-oxo acids which could exchange with pyruvate on the carrier entered freely even in the presence of inhibitor.

The existence of a specific carrier for pyruvate in mitochondria has been in dispute (Papa *et al.*, 1971; Zahlten *et al.*, 1972; Paradies & Papa, 1973; Bakker & Van Dam, 1974). However, the recent discovery that α-cyano-4-hydroxycinnamate powerfully and specifically inhibits pyruvate entry into mitochondria (Halestrap & Denton, 1974) not only demonstrates the existence of such a carrier but should allow the kinetic properties of the carrier to be studied by the 'inhibitor-stop' technique. This technique was originally devised by Pfaff & Klingenberg (1968) to study the kinetics of adenine nucleotide translocation and has since been used to study the transport of succinate (Quagliarello *et al.*, 1969), citrate (Robinson *et al.*, 1971; Palmieri *et al.*, 1972*a*), 2-oxoglutarate (Palmieri *et al.*, 1972*b*) and glutamate (Bradford & McGivan, 1973). In the present paper, I report some kinetic studies on the transport of pyruvate into rat liver mitochondria. Various inhibitors of the transport process are investigated to gain further insight into the specificity of the carrier. Evidence is presented that pyruvate transport is associated with a stoicheiometric proton flux. This proton flux is used to study the rate of pyruvate transport under conditions where the 'inhibitor-stop' technique was found to be inappropriate, such as at higher temperatures. Proton flux is also used to identify

other carboxylic acids whose entry into the mitochondria largely depends on the pyruvate transporter. Dichloroacetate, difluoroacetate and oxamate are such acids.

Experimental

Materials

Chemicals. Except where stated below, enzymes, substrates and coenzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. and all other chemicals from BDH Chemicals Ltd., Poole, Dorset, U.K. α-Cyano-4-hydroxycinnamic acid, α-cyano-3-hydroxycinnamic acid, α-cyano-4-methyl-2-pentenoic acid, α-fluorocinnamic acid, α-thio-2-furanpyruvic acid, 2,3-dibromopropionic acid, difluoroacetic acid and rotenone were obtained from Ralph N. Emmanuel, Wembley, Middx. HA0 1PY, U.K. Antimycin A, glyoxylic acid and the sodium salts of phenylpyruvate and 2-oxo-4-methylpentanoate were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. 2-Chloropropionic acid and 3-chloropropionic acid were from Kodak Ltd., Kirby, Liverpool, U.K., and 2,2′-dichloropropionate was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Compound UK 5099 [α-cyano-β-(1-phenylindol-3-yl) acrylate] was a kind gift from Pfizer Ltd., Sandwich, Kent. All radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [3-^{14}C]Pyruvate was treated as follows. The solid was dissolved in water, divided into 3μCi samples, freeze-dried and stored in sealed tubes at −20°C. After 3 months' storage chromatographic analysis [18h descending chromatography in 1M-ammonium formate (pH5.0)–propanol–water (3:13:5, by vol.)] showed that more than 95% of the ^{14}C was present as pyruvate (R. H. Cooper & D. A. Walsh, unpublished work).

Mitochondria. Rat liver mitochondria were prepared in sucrose medium [sucrose (0.25M), Tris–HCl (5mM) and ethanedioxybis(ethylamine)tetra-acetate (EGTA) (2mM), pH7.6] as described by Chappell & Hansford (1972). Rat heart mitochondria were prepared similarly except that the initial homogenization was performed in Polytron PT10 OD homogenizer for 15s at one-quarter the maximum speed. Mitochondrial protein contents were determined by a modified biuret method (Gornall *et al.*, 1949).

Methods

Kinetic studies by 'inhibitor-stop' method. The determination of the kinetics of pyruvate transport followed a procedure similar to that used by Bradford & McGivan (1973) for studying glutamate transport. Rat liver mitochondria (approx. 8mg of mitochondrial protein) were suspended in 1ml of medium composed of 0.125M-KCl, 0.02M-Tris–HCl, 10μM-rotenone, 10μM-antimycin A and [6,6′-^{3}H]sucrose (1μCi/ml), pH6.8 at 6°C. At zero time [3-^{14}C]pyruvate (0.1μCi/ml) was added at the desired concentration. At the appropriate time transport was stopped by the addition of 0.5mM-α-cyano-4-hydroxycinnamate, the mitochondria were sedimented by centrifugation for 1min at 15000$g_{av.}$ in an Eppendorf 3200 centrifuge and the mitrochondrial [3-^{14}C]-pyruvate was determined as described by Halestrap & Denton (1974). In experiments where pyruvate efflux was being investigated the procedure was identical except that the mitochondrial pellet obtained after centrifugation was resuspended by vortexing in fresh medium (0.125M-KCl–0.02M-Tris–HCl – 10μM-rotenone – 10μM-antimycin A, pH6.8) with other additions as indicated. After incubation at 6°C for the required time the mitochondria were re-sedimented by centrifugation and the mitochondrial pyruvate content was determined.

Measurement of proton fluxes. Mitochondrial proton fluxes were studied by using a Radiometer model 26pH meter fitted with chart recorder (0.1pH unit/full scale deflexion). Mitochondria (approx. 50mg of mitochondrial protein) were suspended in 3ml of medium (0.14M-KCl–0.5mM-Tris–HCl–10μM-rotenone, pH6.8) in a water-jacketed vessel at the required temperature. The pH was readjusted to 6.8 and then substrate (also at pH6.8) added to 2mM and the change in pH recorded. Proton fluxes were calibrated by addition of a standard amount of HCl to the incubation medium.

Results and Discussion

'Inhibitor-stop' studies on pyruvate transport

Before using α-cyano-4-hydroxycinnamate for 'inhibitor-stop' studies of pyruvate transport it was necessary to establish whether its inhibition was both total and sufficiently rapid. Results of experiments designed to investigate this are shown in Table 1. In these and all subsequent experiments pyruvate accumulation by mitochondria was produced by using a pH gradient as described by Bradford & McGivan (1973). Mitochondria were prepared at pH7.6 and incubated at pH6.8. The accumulation of pyruvate under these conditions was similar to that obtained when succinate oxidation was used to drive pyruvate accumulation (Halestrap & Denton, 1974; Halestrap *et al.*, 1974), and avoided the presence of other carboxylic acid anions which might interfere with the kinetics of pyruvate transport.

It is also assumed that ^{14}C label taken up by the mitochondria represents pyruvate. This assumption

Table 1. *Characterization of the use of α-cyano-4-hydroxycinnamate for 'inhibitor-stop' kinetic studies*

Rat liver mitochondria (approx. 8mg of protein) were suspended in 1ml of medium {0.125M-KCl–0.02M-Tris–HCl–10μM-rotenone–10μM-antimycin A and [6,6′-^{3}H]sucrose (1μCi/ml), pH6.8} at 6°C. Pyruvate transport was started by the addition of [3-^{14}C]pyruvate (0.25mM; 0.1μCi/ml) and stopped by the addition of α-cyano-4-hydroxycinnamate (0.5mM) before sedimentation of the mitochondria by centrifugation and analysis of the pellet for pyruvate as described under 'Methods'. Further details are given in the Table and in the Experimental section. Results are given as the means±S.E.M. of four separate observations.

Experimental conditions	Mitochondrial substrate content (nmol/mg of protein) 6°C	30°C
Pyruvate and α-cyano-4-hydroxycinnamate added together 5min before centrifugation	<0.01	<0.01
Pryuvate added 1min after α-cyano-4-hydroxycinnamate and 5min before centrifugation	<0.01	<0.01
Pyruvate added 5min before centrifugation, α-cyano-4-hydroxycinnamate added 15s before centrifugation	1.95±0.01	0.476±0.196
Pyruvate added 10min before centrifugation, α-cyano-4-hydroxycinnamate added 5min before centrifugation	1.48±0.07	0.076±0.064

is supported by the following evidence. First, in previous experiments both the influx and efflux of ^{14}C label into and out of the mitochondria were matched by corresponding fluxes of enzymically assayable pyruvate (Halestrap & Denton, 1974; Halestrap *et al.*, 1974). Both movement of ^{14}C label and of enzymically assayable pyruvate were specifically blocked by α-cyano-4-hydroxycinnamate. Secondly, the rate of pyruvate entry into mitochondria at 6°C measured by proton flux and thus independent of ^{14}C label (Fig. 4) agrees with that measured radioactively (Figs. 1 and 2).

At both 6° and 30°C, 0.5mM-α-cyano-4-hydroxycinnamate totally prevented pyruvate entry into mitochondria whether the inhibitor was added before or at the same time as the pyruvate. The inhibition was both complete and very rapid at this concentration of inhibitor. If the mitochondria were left for 5min in the presence of pyruvate, pyruvate accumulated, but no further accumulation occurred after the inhibitor was added. Rather it appeared that pyruvate was actually lost from the mitochondria during this time. At 30°C the loss was almost total within 5min. This suggested that some form of metabolism of the pyruvate was occurring which produced a metabolite that could leave the mitochondria even in the presence of α-cyano-4-hydroxycinnamate. Under identical conditions to those used in the experiments of Table 1 the rates of pyruvate carboxylation and decarboxylation by intact rat liver mitochondria were determined. Pyruvate carboxylation was assayed by adding $NaH^{14}CO_3$ (0.1mM; 5μCi/ml) to the incubation medium and measuring the incorporation of ^{14}C into acid-stable material as described by Walter & Stucki (1970). No detectable rate of carboxylation was found at either 6° or 30°C under these conditions, which might be expected in the absence of appreciable HCO_3^-, phosphate or ATP. Decarboxylation was studied by adding [1-^{14}C]pyruvate (0.5μCi/μmol) to the incubation medium and measuring the release of $^{14}CO_2$ as described by Cooper *et al.* (1974). Under identical conditions to those used for determining mitochondrial pyruvate uptake the rate of decarboxylation (expressed as nmol of CO_2 produced/min per mg of mitochondrial protein and the mean of two observations) was 0.04 at 6°C and 2.42 at 30°C. These rates of decarboxylation could account for the loss of pyruvate seen after the addition of α-cyano-4-hydroxycinnamate in the experiments of Table 1. The decarboxylation of pyruvate occurred despite the presence of rotenone and antimycin and probably represents the rate of an NAD^+-independent side reaction of pyruvate dehydrogenase which may produce acetoin. This reaction can proceed at about 1% of the rate of the overall pyruvate dehydrogenase reaction (Cooper *et al.*, 1974). No way has been found of preventing this decarboxylation and I have therefore been restricted in the use of the 'inhibitor-stop' technique to kinetic studies at 6°C. At this temperature experiments can be carried out sufficiently rapidly to prevent any serious interference by decarboxylation.

The rate of uptake of pyruvate into mitochondria at 6°C, pH6.8 and 0.15mM-pyruvate is shown in Fig. 1(*a*). The uptake showed apparent first-order kinetics (Fig. 1*b*) as has also been described for other mitochondrial transport mechanisms (Pfaff *et al.*, 1969; Quagliariello *et al.*, 1969; Robinson *et al.*, 1971; Bradford & McGivan, 1973), and this allows the initial rate of pyruvate entry into the mitochondria to be calculated. By performing such experiments at various concentrations of pyruvate, the concentration-dependence of uptake was shown to obey Michaelis–Menten kinetics as indicated by the Lineweaver–Burk plot of Fig. 2(*a*). The K_m value for pyruvate was determined as 0.15±0.02mM and $V_{max.}$ as 0.54±0.03nmol/min per mg of mitochondrial

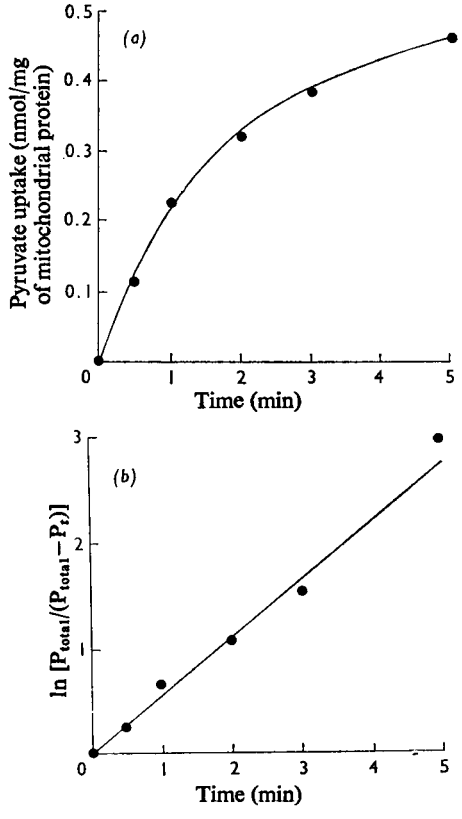


Fig. 1. *Time-course of pyruvate uptake by liver mitochondria*

Pyruvate uptake (*a*) was determined as described in the legend to Table 1. The pH was 6.8, the temperature 6°C and pyruvate was added at 0.15mM. Total uptake of pyruvate at equilibrium (P_{total}) was estimated, by extrapolation of data in (*a*), to be 0.49nmol/mg of mitochondrial protein. P_t represents the pyruvate uptake at time *t*. From (*b*) the first-order rate constant was calculated to be 0.59min^{-1} and hence the initial rate of pyruvate uptake was determined from the first-order rate equation $v = k$ (P_{total}) to be 0.29nmol/min per mg of mitochondrial protein.

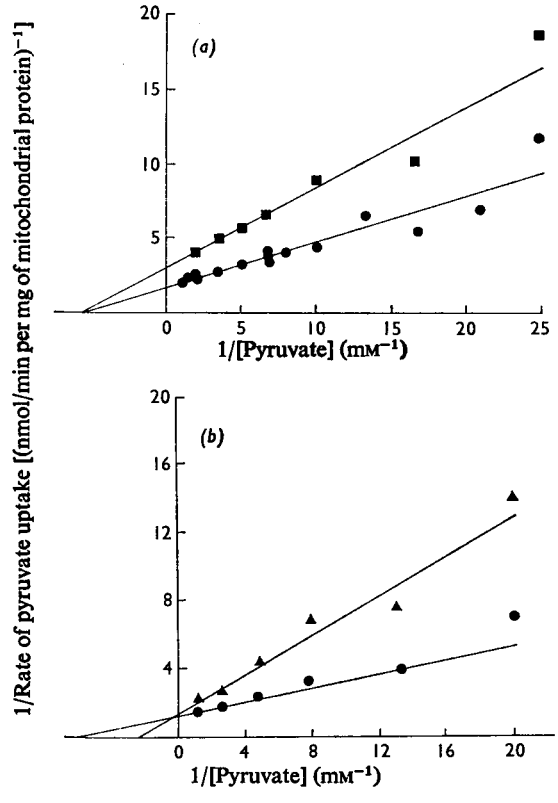


Fig. 2. *Concentration-dependence of pyruvate uptake by liver mitochondria*

Pyruvate accumulation was measured at pH6.8 and 6°C as described in the legend to Table 1 at the concentrations of pyruvate indicated. Initial rates of uptake were calculated as described in the legend to Fig. 1 and are plotted as Lineweaver–Burk plots. (*a*) ●, Control; ■, mitochondria preincubated for 3min with α-cyano-4-hydroxycinnamate (5μM) before addition of pyruvate. (*b*) ●, Control; ▲, with 1.5mM-phenylpyruvate added with the pyruvate.

protein. These results were obtained from 15 separate observations over a range of pyruvate concentrations from 0.04 to 1.0mM, the errors being calculated by least-squares fit. Inhibition of pyruvate transport by α-cyano-4-hydroxycinnamate was non-competitive with a K_i of 6.3μM (Fig. 2*a*) whereas that by phenylpyruvate was competitive with a K_i of 1.8mM (Fig. 2*b*). Previous work (Halestrap *et al.*, 1974) had already indicated that phenylpyruvate inhibited mitochondrial pyruvate transport and it was suggested that this might be important in the pathology of phenylketonuria.

Inhibition of pyruvate-dependent O_2 uptake by rat heart mitochondria

To investigate the nature of the inhibition by α-cyano-4-hydroxycinnamate more fully and to identify other inhibitors of pyruvate transport, pyruvate oxidation by intact rat heart mitochondria was studied by using an oxygen electrode and low concentrations of inhibitor. Fig. 3 shows some typical oxygen-electrode traces for rat heart mitochondria oxidizing pyruvate. The mitochondria were incubated with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone rather than coupled with ADP to prevent any changes in pyruvate dehydrogenase

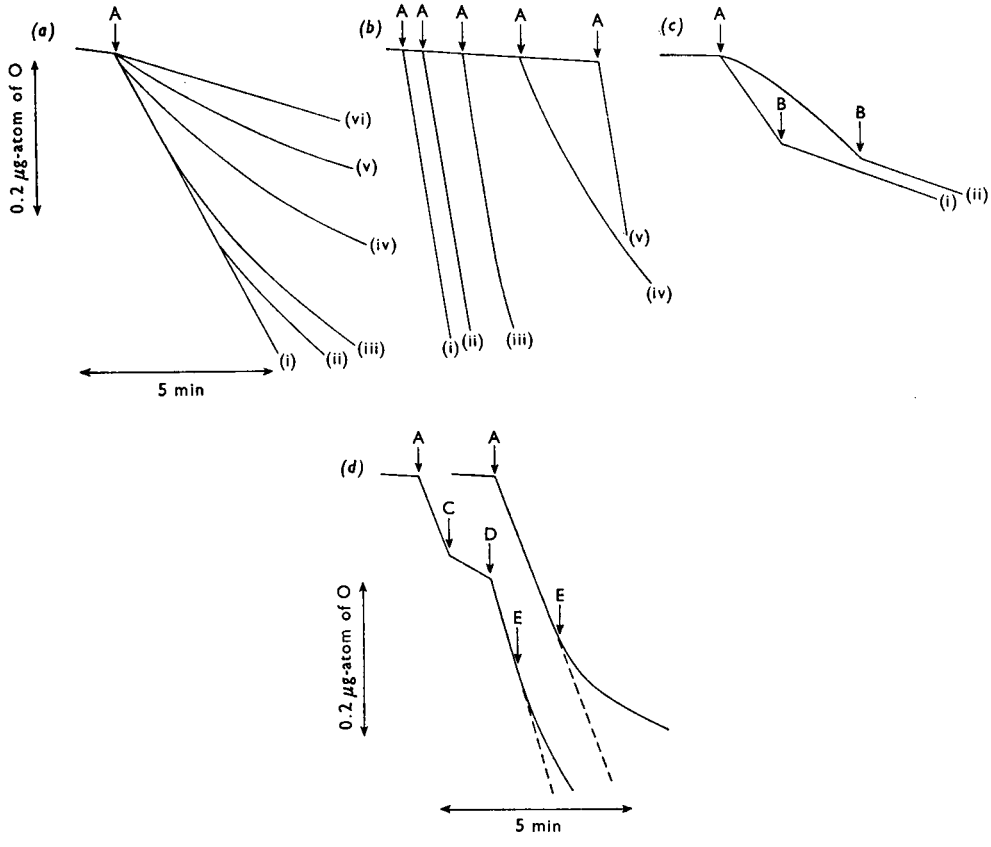


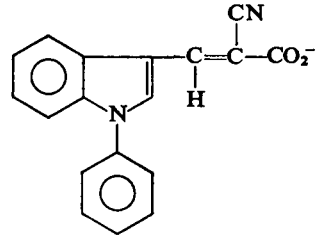
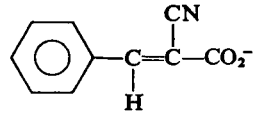
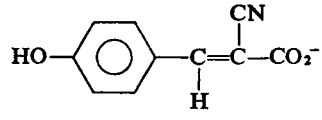
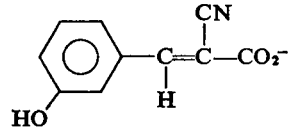
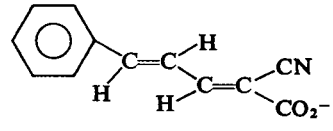
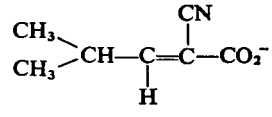
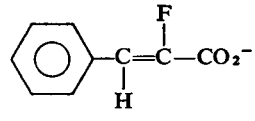
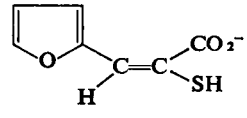
Fig. 3. *α-Cyano-4-hydroxycinnamate inhibition of pyruvate-dependent O_2 uptake by rat heart mitochondria*

Rat heart mitochondria (about 0.25mg of protein) were incubated in 1ml of medium (125mM-KCl–25μM-malate–2mM-potassium phosphate–20mM-Tris–HCl, pH7.4) at 30°C in a water jacketed vessel. Uncoupler (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was present at 0.1μM, and after a steady state of O_2 uptake was obtained pyruvate (2mM) was added (A). (*a*) α-Cyano-4-hydroxycinnamate was added at the same time as pyruvate at concentrations of 0μM (i), 1.0μM (ii), 1.5μM (iii), 2.0μM (iv), 3.0μM (v) and 200μM (vi). (*b*) α-Cyano-4-hydroxycinnamate (1.0μM) was added 0.5min (ii), 1.5min (iii) or 3.0min (iv) before the addition of pyruvate. In curves (i) and (v) no inhibitor was present and pyruvate was added at 1 and 5min respectively after the addition of mitochondria to the incubation medium. (*c*) Mitochondria (40mg of protein/ml) were incubated in sucrose medium (0.25M sucrose–20mM-Tris–HCl–2mM-EGTA, pH7.6) for 30min at 0°C in the presence (ii) or absence (i) of 20μM-α-cyano-4-hydroxycinnamate before measuring the pyruvate-dependent O_2 uptake as above. At B, α-cyano-4-hydroxycinnamate (0.2mM) was added. (*d*) Additions were made as follows: A, pyruvate (2mM); C, albumin (2mg/ml); D, ADP (2mM); E, α-cyano-4-hydroxycinnamate (20μM).

activity occurring through changes in mitochondrial ATP concentration (Martin *et al.*, 1972). No inhibition of O_2 uptake was seen at 0.5μM-α-cyano-4-hydroxycinnamate, but if the concentration was increased to 1.0μM some inhibition became apparent during the incubation. Inhibition was greater and became evident more rapidly at higher concentrations. If the inhibitor was present for several minutes before pyruvate was added, inhibition was apparent sooner after the addition of the pyruvate than if both inhibitor and pyruvate were added simultaneously. These results might indicate that inhibition was caused by the slow formation of a covalent bond between α-cyano-4-hydroxycinnamate and the carrier, a process whose rate would depend on the concentration of inhibitor. If inhibition involved covalent-bond formation the amount of inhibition at equilibrium might be expected to depend on the concentration of mitochondria since it would be likely that the inhibitor would titrate out the carrier. I was unable to find any evidence for this. Further, α-cyanocinnamate can almost totally

Table 2. *Sensitivity of pyruvate transport in rat heart mitochondria to inhibition by various analogues of α-cyano-4-hydroxycinnamate*

Pyruvate oxidation was studied in the presence of uncoupler as described in the legend to Fig. 3. When a steady state of O_2 uptake was obtained after the addition of 0.2mM-pyruvate, inhibitor was added until 50% inhibition was obtained. Each experiment was repeated at least twice.

Inhibitor	Structure	Concentration giving 50% inhibition (μM)
Compound UK 5099		0.050
α-Cyanocinnamate		0.20
α-Cyano-4-hydroxycinnamate		1.5
α-Cyano-3-hydroxycinnamate		1.5
α-Cyano-5-phenyl-2,4-pentadienoate		0.20
α-Cyano-4-methyl-2-pentanoate		>1000
α-Fluorocinnamate		200
α-Thio-2-furanpyruvate		500

inhibit pyruvate transport at concentrations about one order of magnitude lower than α-cyano-4-hydroxycinnamate (Table 2). This would seem to discount the possibility that α-cyano-4-hydroxycinnamate can titrate out the pyruvate carrier. In Fig. 3(*c*) the reversibility of pyruvate-transport

inhibition is shown, which further weakens the likelihood that α-cyano-4-hydroxycinnamate inhibition is by covalent linkage. By incubating the mitochondria with inhibitor and then diluting the mitochondria into the oxygen-electrode incubation medium the rate of O_2 uptake gradually increased from an almost totally inhibited rate to one approaching the rate of control mitochondria preincubated in the absence of inhibitor. Thus inhibition by α-cyano-4-hydroxycinnamate would appear to be non-competitive and yet reversible, as is the case for the inhibition of the adenine nucleotide transporter by bongkrekic acid (Klingenberg, 1970). Such inhibition could be due to the inhibitor binding non-covalently, but slowly, to some site of the carrier protein other than the active centre and causing a distortion of the carrier sufficient to make it ineffective. An alternative explanation would be that the inhibitor reacts covalently with the carrier, but the product is hydrolysed slowly. Such a process actually occurs in the normal catalytic mechanism of serine proteases where a serine residue is esterified and the ester is then hydrolysed (see Williams, 1969). The particular K_i value measured for an inhibitor acting in this way would depend on the relative rates of the covalent bond formation and its hydrolysis under the particular conditions used. At present I have no means of distinguishing these two inhibitory mechanisms.

Inhibition of pyruvate transport by analogues of α-cyano-4-hydroxycinnamate

To investigate the structural features of α-cyano-4-hydroxycinnamate that make it a powerful inhibitor of pyruvate transport, various analogues were tested on rat heart mitochondria oxidizing pyruvate. The concentrations of the analogues required to give about 50% inhibition of O_2 uptake are shown in Table 2. In no case was oxoglutarate oxidation affected by the presence of the inhibitor. Two main structural features would appear to be important, the nitrile group and the aromatic side chain. Thus α-fluorocinnamate and α-thio-2-furanpyruvate were about 1000 times less powerful inhibitors than α-cyanocinnamate, whereas α-cyano-4-methyl-2-propenoate showed no inhibition at concentrations as high as 1 mM. The important aspects of the aromatic group were less-well defined. α-Cyanocinnamate was at least 20-fold more powerful an inhibitor than its 3- or 4-hydroxyl derivatives which might indicate that the hydrophobic character was important. Addition of two carbons to the side chain as in α-cyano-5-phenyl-2,4-pentadienoate seemed to have little effect on the potency of inhibition. Compound UK 5099, a close analogue of α-cyanocinnamate but with a larger aromatic group, increased the potency to give one-half maximum inhibition at about 50 nM. It would seem possible that the presence of the hydrophobic aromatic moiety locks the active α-cyanopropenoate grouping into the necessary inhibitory position on the carrier. The geometric isomers used in these studies are those readily obtainable by the normal synthetic routes (Cope *et al.*, 1941; Zabicky, 1961; Le Moal *et al.*, 1966) and the correct structures are shown in Table 2. Measurements of pyruvate uptake into rat liver mitochondria were performed to test the effect of the most active inhibitors on pyruvate transport into mitochondria more directly (Table 3). The results of these experiments confirm the relative potency of the various analogues as inhibitors of pyruvate transport, although the actual concentrations of inhibitor required to inhibit pyruvate transport in liver mitochondria at 6°C appeared to be slightly higher than those seen for heart mitochondria at 30°C. Table 3 also shows the effects of albumin (10 mg/ml) on the inhibition of pyruvate transport by α-cyano-4-hydroxycinnamate (0.1 mM). Inhibition

Table 3. *Effect of various inhibitors on pyruvate entry into rat liver mitochondria*

Pyruvate uptake was studied at 6°C as described in the legend to Table 1. Uptake was stopped 2.5 min after the addition of pyruvate by the addition of α-cyano-4-hydroxycinnamate (0.5 mM). Pyruvate was present at 0.2 mM in Expts. 1 and 3 and at 1.0 mM in Expts. 2 and 4. All results are expressed as the means ± S.E.M. of four separate observations with significance, when compared with control incubations in the absence of inhibitor, of: $*P<0.01$; $**P<0.001$.

Expt.	Additions	Pyruvate uptake (nmol/mg of protein)
1	None	0.581 ± 0.032
	5 μM-α-Cyano-3-hydroxycinnamate	0.381 ± 0.025*
	5 μM-α-Cyano-4-hydroxycinnamate	0.331 ± 0.040*
	5 μM-α-Cyanocinnamate	0.068 ± 0.011**
2	None	1.256 ± 0.092
	2 μM-α-Cyanocinnamate	0.267 ± 0.046**
	2 μM-α-Cyano-4-hydroxycinnamate	1.092 ± 0.054
	2 μM-Compound UK 5099	0.317 ± 0.021**
	2 μM-α-Cyano-5-phenyl-2,4-pentadienoate	0.188 ± 0.054**
3.	None	0.450 ± 0.30
	2 mM-Phosphoenolpyruvate	0.404 ± 0.019
	0.2 μM-Compound UK 5099	0.190 ± 0.028**
	0.1 mM-Compound UK 5099	0.044 ± 0.023**
	2 mM-Dichloroacetate	0.196 ± 0.020**
4	None	0.854 ± 0.088
	0.1 mM-α-Cyano-4-hydroxycinnamate	0.117 ± 0.050**
	Albumin (10 mg/ml)	1.088 ± 0.079
	Albumin (10 mg/ml) + 0.1 mM-α-cyano-4-hydroxycinnamate	0.513 ± 0.058*

was still appreciable, although slightly diminished. This was confirmed by the experiments shown in Fig. 3(*d*) where pyruvate oxidation by uncoupled rat heart mitochondria was studied. The addition of albumin (2mg/ml) largely inhibited O_2 uptake apparently by binding the carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, since addition of ADP renewed O_2 uptake. Almost total inhibition of O_2 uptake could now be produced if 20μM-α-cyano-4-hydroxycinnamate was added, but the inhibition appeared to take longer to develop than in the absence of albumin.

Table 3 also shows the effects of dichloroacetate and phosphoenolpyruvate on pyruvate transport into rat liver mitochondria. Dichloroacetate appeared to inhibit pyruvate uptake quite significantly when present at 2mM in the presence of 0.2mM-pyruvate whereas 2mM-phosphoenolpyruvate had no effect under these conditions. Work in this laboratory has already shown that phenylpyruvate and α-oxo-4-methylpentanoate inhibit entry of pyruvate into mitochondria (Halestrap *et al.*, 1974; Fig. 2*b*). To study this inhibition in more detail, experiments on the exchange of preloaded mitochondria were performed. In these experiments mitochondria were incubated with pyruvate in the usual manner for 5min before being sedimented by centrifugation and resuspended in fresh buffer. The mitochondria were left in this fresh buffer for 3min, during which time pyruvate efflux could occur. α-Cyano-4-hydroxycinnamate was then added to stop efflux, the mitochondria were sedimented by centrifugation and the pyruvate remaining in the mitochondria was analysed as usual. Addition of any compound to the efflux buffer that can exchange with pyruvate on the carrier should increase efflux and thus decrease the amount of pyruvate remaining in the mitochondria. The results of such exchange experiments are shown in Table 4 and support these predictions. In Expt. 1 both pyruvate and phenylpyruvate at 2mM caused a considerable increase in the efflux of [^{14}C]pyruvate from the mitochondria, whereas 2mM-acetate was without effect. This experiment demonstrates both pyruvate–pyruvate and phenylpyruvate–pyruvate exchange on the carrier as shown previously (Paradies & Papa, 1973; Halestrap *et al.*, 1974) and is consistent with the competitive nature of phenylpyruvate inhibition of transport (Fig. 2*b*). In contrast, in Expt. 3 both α-cyano-4-hydroxycinnamate and compound UK 5099 decreased the rate of pyruvate efflux from the mitochondria, as expected from the non-competitive nature of their inhibition (Fig. 2*a*). Lactate (2mM) was without effect on efflux (Expt. 2), which agrees with the lack of effect of α-cyano-4-hydroxycinnamate on the uptake of lactate by mitochondria (Halestrap & Denton, 1974). Similarly phosphoenolpyruvate (2mM) showed no stimulation of

Table 4. *Pyruvate efflux from mitochondria*

Rat liver mitochondria (approx. 8mg of protein/ml) were incubated at 6°C in buffered KCl medium, pH6.8, containing [6,6'-^{3}H]sucrose (2μCi/ml) and rotenone (10μM) for 5min in the presence of [3-^{14}C]pyruvate (0.1μCi/ml) at 0.5mM (Expts. 1 and 2), 0.2mM (Expt. 3) or 1.0mM (Expt. 4). After centrifugation the mitochondria were resuspended in fresh buffer without [^{3}H]sucrose, incubated at 6°C for 3min in the presence of the additions indicated, re-centrifuged and the mitochondrial pellet was analysed for [3-^{14}C]pyruvate. Results are expressed as the means±S.E.M. of four separate observations with significance, compared with control incubations with no additions, of: *$P<0.02$; **$P<0.01$; ***$P<0.001$.

Expt.	Additions to efflex medium	Pyruvate remaining in mitochondria (nmol/mg of protein)
1	None	0.430±0.025
	2mM-Pyruvate	0.172±0.015**
	2mM-Phenylpyruvate	0.204±0.008**
	2mM-Acetate	0.381±0.033
2	None	0.403±0.010
	2mM-Lactate	0.438±0.003
	2mM-Dichloroacetate	0.280±0.015**
3	None	0.160±0.004
	2mM-Phosphoenolpyruvate	0.145±0.002
	0.5mM-α-Cyano-4-hydroxycinnamate	0.284±0.017***
	0.1mM-Compound UK 5099	0.262±0.012***
4	Control	0.875±0.071
	5mM-2-Oxo-4-methylpentanoate	0.283±0.017***
	5mM-Acetate	0.550±0.06 *

pyruvate efflux, which is at variance with the results of Paradies & Papa (1973). However, the difference in charge between phosphoenolpyruvate and pyruvate would make it unlikely that they should enter on the same carrier. Rather it is believed that phosphoenolpyruvate enters the mitochondria on the citrate carrier (Robinson, 1971). α-Oxo-4-methylpentanoate increases the efflux of pyruvate from preloaded mitochondria (Expt. 4) but this effect is only seen at quite high (5mM) concentrations of α-oxo-4-methylpentanoate. Table 3 shows that dichloroacetate inhibited pyruvate entry into mitochondria and in Table 4 (Expt. 2) it can be seen that dichloroacetate (2mM) also enhances the efflux of pyruvate from mitochondria. This result indicates that dichloroacetate–pyruvate exchange can occur and thus that dichloroacetate can enter mitochondria on the pyruvate carrier.

Use of proton fluxes to study pyruvate transport

Since the entry of pyruvate into the mitochondria is independent of the addition of any other anions

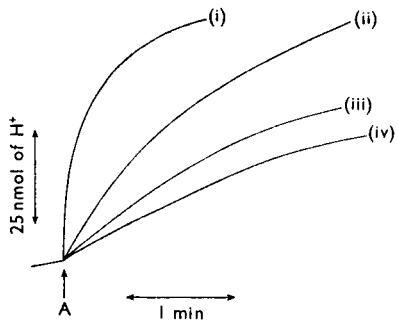


Fig. 4. *Pyruvate- and dichloroacetate-dependent proton fluxes in rat liver mitochondria*

Proton fluxes were measured as described in the Experimental section. Additions: (i) dichloroacetate (2mM) added at A; (ii) pyruvate (2mM) added at A; (iii) compound UK 5099 (50μM) present, dichloroacetate (2mM) added at A; (iv) compound UK 5099 (50μM) present, pyruvate (2.0mM) added at A.

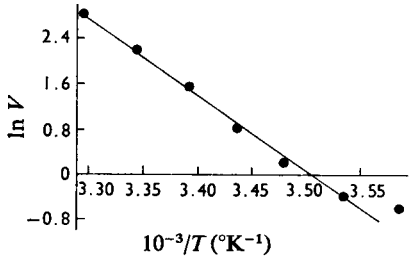


Fig. 5. *Temperature-dependence of pyruvate-associated proton flux into liver mitochondria*

Measurement of proton fluxes was performed as described in the Experimental section. Pyruvate was added at 2mM. The initial rate of pyruvate uptake (V) (expressed as ng-ions of H^+/min per mg of protein) was calculated from the first-order rate constant as described for Fig. 1.

it would appear that it must either cross the membrane with a proton or in exchange for an OH^- ion. In either cases it should be possible to measure an increase in the pH of the extramitochondrial medium as pyruvate enters the mitochondria. This has been shown for the transport of other carrier-dependent anions (McGivan & Klingenberg, 1971). Such apparent proton fluxes could be seen as pyruvate entered the mitochondria (Fig. 4) and these fluxes were greatly inhibited by α-cyano-4-hydroxycinnamate. Further, this entry of protons followed first-order kinetics and could be analysed to give the initial rate of proton flux as described in the legend to Fig. 1. At 6°C and 2mM-pyruvate, the initial rate of proton flux was determined to be 0.61nmol/min per mg of mitochondrial protein (mean of three observations agreeing within 10%). This compares very favourably with the $V_{max.}$ value of 0.54nmol/min per mg for pyruvate entry into the mitochondria at 6°C measured directly (Figs. 1 and 2) and would indicate a stoicheometry of 1 H^+ ion to 1 pyruvate molecule as expected. By studying the rate of proton flux at various temperatures between 6° and 30°C the temperature-dependence of pyruvate transport was investigated and is shown in Fig. 5 as an Arrhenius plot. The plot is linear, yielding an activation energy of 113kJ (27kcal)/mol with a possible break in the linearity between 10° and 6°C. Such a change in activation energy between these temperatures has been recorded for the adenine nucleotide translocator (Klingenberg, 1970) and might reflect a transition in the membrane structure.

Entry of other monocarboxylic acids into the mitochondria

The entry of any other carboxylic acid into the mitochondria on the pyruvate transporter should also be accompanied by the translocation of protons, and this proton flux should be inhibited by α-cyano-4-hydroxycinnamate. Identification of a carboxylic acid that is transported almost exclusively by this means should therefore be possible. The experiments recorded in Table 5 attempted to explore this possibility. As expected weak acids such as acetate and propionate entered the mitochondria extremely fast and the accompanying proton flux was not inhibited by compound UK 5099. It is thought that such acids enter liver mitochondria as the free acid and without any carrier requirements (Chappell & Haarhof, 1966; Chappell & Crofts, 1966; Bakker & Van Dam, 1974). Acids that showed some apparent dependence on the pyruvate carrier were dichloroacetate, difluoroacetate, oxamate and possibly 2,2-dichloropropionate. All these acids also enhanced the exchange of pyruvate from mitochondria, which further suggests that these acids are substrates for the transporter. Such exchange studies showed that several α-oxo acids other than pyruvate may also use the carrier, although the proton fluxes associated with their entry were not inhibited by pyruvate transport inhibitors. 2-Oxobutyrate, phenylpyruvate and 2-oxo-4-methylpentanoate fit into this category. It would appear that although these acids can use the pyruvate carrier they are also able to cross the membrane as the free acid relatively rapidly under the conditions used. The pK values of these acids (and hence the concentration of free acid present) are unlikely to be dissimilar to that of pyruvate. Why the membrane should exert a barrier to pyruvate and not to these other 2-oxo acids is not known but it is possible that the solubilities of the

Table 5. *Liver mitochondrial proton fluxes and pyruvate exchange induced by various monocarboxylic acids*

Proton fluxes were measured at pH 6.8 as described in the Experimental section and legend to Fig. 5. The temperature was 15°C and the potassium salt of the carboxylic acid was added at 2mM. Where appropriate compound UK 5099 was present at 50μM. Results are given as the mean of two experiments agreeing within 10%. Pyruvate exchange was measured at 6°C as described in the legend of Table 4. Liver mitochondria were preloaded in 0.2mM-pyruvate and efflux was allowed to continue for 3min in the presence or the absence of monocarboxylate (2mM). Pyruvate exchange is expressed in terms of efflux of pyruvate from the mitochondria caused by the presence of monocarboxylate as a percentage of the total pyruvate remaining in the mitochondria incubated in the absence of carboxylate. pK_a values, where available, were taken from Kortum *et al.* (1961) except that of α-cyano-4-hydroxycinnamate which was determined by titration. The results are given as the means ±S.E.M. for four separate observations with significance, compared with control rate, of: $*P<0.02$; $**P<0.01$; $***P<0.001$.

Monocarboxylic acid anion	pK_a	Rate of proton flux into mitochondria (ng-ions of H^+/min per mg of protein) Control	With compound UK 5099	Inhibited rate as % of control rate	Pyruvate exchange (% in 3min)
Pyruvate	2.49	1.2	0.3	25	60.0±1.3***
Acetate	4.75	>30	>30	100	11.5±2.3*
Propionate	4.90	>30	>30	100	—
Lactate	3.86	5.7	5.7	100	(−)9.1±3.2
β-Hydroxybutyrate	4.70	1.1	1.0	90	(−)6.5±4.0
Monochloroacetate	2.81	6.0	5.7	95	30.1±5.1**
Dichloroacetate	1.29	5.3	0.5	10	34.6±2.7***
Trichloroacetate	0.70	4.3	4.1	95	2.3±5.6
2-Chloropropionate	2.83	>30	>30	100	33.0±2.0***
3-Chloropropionate	3.98	>30	>30	100	34.2±2.00***
2,2-Chloropropionate	—	12.5	9.5	76	18.3±6.4
Difluoroacetate	—	8.0	4.1	51	26.5±4.3**
2-Oxobutyrate	—	>30	>30	100	44.1±3.0***
2-Oxo-4-methylpentanoate	—	19.4	17.5	89	16.0±2.7**
Phenylpyruvate	—	9.7	9.7	100	47.0±1.8***
Oxamate	—	8.7	2.4	28	41.2±2.2***
Glyoxalate	2.98	<0.05	<0.05	—	—
Glycollate	3.90	1.0	1.1	110	—
α-Cyano-4-hydroxycinnamate	3.83	9.7	8.8	91	(−)44.7±9.1*

free acids in the membrane are greatly different or that other membrane carriers exist. The same anomaly was present for various halogenated acids tested where again pK_a values seem to give little correlation to the ease of entry of these acids into the mitochondria. Thus 2-chloropropionate with pK_a 2.89 entered the mitochondria faster than lactate (pK_a 3.86) and was not dependent on the pyruvate carrier. Similarly although dichloroacetate appeared to almost totally depend on the pyruvate carrier for entry, 2,2-dichloropropionate was much less dependent and could enter quite rapidly under conditions where the pyruvate carrier was inhibited. Even more anomalous was the observation that trichloroacetate (pK_a 0.7) entered the mitochondria quite fast and independently of the pyruvate carrier whereas DL-β-hydroxybutyrate (pK_a 4.70) entered only slowly, also independently of the carrier. This latter observation was confirmed with the use of DL-β-hydroxy[U-^{14}C]butyrate and is at variance with the suggestion of Land & Clark (1974) that β-hydroxybutyrate transport in brain mitochondria is blocked by inhibition of the pyruvate transporter. It is possible that a study of the relative solubilities of the free acids in the membrane may provide a clue to these apparent inconsistencies. From exchange studies many of the halogenated acids appeared to be substrates for the pyruvate carrier even if their transport was not exclusively carrier-dependent. Thus monochloroacetate, dichloroacetate, difluoroacetate, 3-chloropropionate, 2-chloropropionate and 2,2-dichloropropionate all enhanced pyruvate exchange. This specificity bears a close relationship to the specificity of pyruvate dehydrogenase kinase to inhibition by various pyruvate analogues. Thus the isolated kinase is inhibited to much the same extent by monochloroacetate, dichloroacetate, trichloroacetate, difluoroacetate, 2-chloropropionate, 3-chloropropionate and 2,2-dichloropropionate (Whitehouse *et al.*, 1974). Of these inhibitors dichloroacetate, 2,2-dichloropropionate and difluoroacetate are effective at extremely low concentrations on the activation of pyruvate dehydrogenase in isolated heart mitochondria (Whitehouse *et al.*, 1974). It is possible that the K_m and $V_{max.}$ values of the pyruvate carrier for these halogenated carboxylic acids might provide some explanation of these differences in sensitivity. At low concentrations the rate of diffusion of the

Table 6. *Kinetic constants of anion translocation into rat liver mitochondria*

Values in parentheses are activation energies in kcal/mol.

Anion-transporting system	$V_{max.}$ at 9°C (nmol/min per mg of protein)	Extrapolated $V_{max.}$ at 37°C (nmol/min per mg of protein)	K_m for named substrate (mM)	Activation energy (kJ/mol)		Reference
ADP–ATP	70	2373	0.012	88–96	(21–23)	Pfaff *et al.* (1969)
Succinate	50	1694	0.83	91	(22)	Quagliariello *et al.* (1969)
Oxoglutarate	43	1146	0.046	84.7	(20.5)	Palmieri *et al.* (1972*a*)
Citrate	23	575	0.09	84.0	(20.1)	Palmieri *et al.* (1972*b*)
Phosphate	80	—	0.25	—	—	J. D. McGivan & M. Klingenberg (unpublished work)
Glutamate	5.0	20.5	4.0	38	(8.8)	Bradford & McGivan (1973)
Pyruvate	0.6	42	0.15	113	(27)	Present paper

various acids across the membrane, which is directly proportional to the concentration, might be very slow. However, the rate of transport of the carboxylic acid on the carrier could still be quite rapid if the K_m and $V_{max.}$ for transport were of appropriate values.

Conclusions

The kinetic properties of the rat liver pyruvate transported reported in this paper are compared with those of other mitochondrial transport systems in Table 6. The K_m (0.15mM) and activation energy [113kJ (27kcal)/mol] of the pyruvate carrier seem to be in much the same range as the values reported for other mitochondrial carriers, but the $V_{max.}$ (0.54nmol/min per mg of protein at 6°C) is very much lower. At physiological temperature (37°C) a $V_{max.}$ value of 42nmol/min per mg of protein can be calculated from the data of Fig. 5. At this temperature the maximum rate of transport is still very much lower than all the other transport systems except that for glutamate. Bradford & McGivan (1973) suggested that glutamate transport may be the rate-limiting step in glutamate metabolism, and it would seem possible that similar considerations may apply to pyruvate transport. In the perfused rat liver stimulated with glucagon or fatty acids, gluconeogenesis from lactate can reach values of 100–200μmol/h per g wet wt. (Exton & Park, 1969; Chan & Freedland, 1972), which would require pyruvate to be transported across the mitochondrial membrane at a rate of approximately 65–125nmol/min per mg of mitochondrial protein. This would appear to be greater than the maximum rate of pyruvate transport catalysed by the pyruvate carrier. When it is considered that the concentration of pyruvate may be of the order of 200–400nmol/g wet wt. of liver under these conditions (see Exton, 1972) it would appear unlikely that the carrier could operate at more than 75% of its maximum velocity anyway. Halestrap & Denton (1975) present some evidence that pyruvate transport may be limiting in pyruvate carboxylation by isolated intact rat liver mitochondria. Thus it would seem possible that pyruvate transport might be a rate-limiting step in gluconeogenesis under certain conditions and therefore a point of control. Glucagon is known to stimulate gluconeogenesis from lactate and pyruvate at some point between the conversion of pyruvate into phosphoenolpyruvate but as yet the exact location of this activation is unknown (Exton & Park, 1968, 1969; Exton, 1970, 1972; Ui *et al.*, 1973). Pyruvate transport would seem one possible candidate for such regulation and Adam & Haynes (1969) have already suggested this as a result of experiments showing an increase in pyruvate carboxylation by mitochondria from livers of animals treated with glucagon or adrenalin. However, I have been unable to demonstrate any increase in the rate of pyruvate transport by mitochondria prepared from livers of rats treated with glucagon as described by Adam & Haynes (1969) or after 48h starvation.

The inhibition of pyruvate transport by α-cyano-4-hydroxycinnamate and its analogues would appear to be exceptionally potent in comparison with inhibitors of other carboxylic carriers. Thus α-cyanocinnamate and compound UK 5099 are highly effective non-competitive inhibitors with K_i values of less than 1μM. In comparison, butylmalonate, phenylsuccinate and 2-ethyl citrate (competitive inhibitors of malate, oxoglutarate and citrate transport respectively) are used in 'inhibitor-stop' studies at concentrations of 10–20mM (Robinson, 1971; Palmieri *et al.*, 1972*a*,*b*). The potency of the inhibition of pyruvate transport by α-cyanocinnamate derivatives shows some similarity to the inhibition of adenine nucleotide transport by bongkrekic acid (Klingenberg, 1970); both are non-competitive and reversible. However, the mechanism by which α-cyanocinnamate and its analogues cause their inhibition is not known. For the majority of studies on pyruvate transport the inhibitor of choice would appear to be α-cyanocinnamate. It is considerably more potent than the

4-hydroxyl derivative and does not absorb greatly at 340nm.

The specificity of the pyruvate carrier would seem to be rather broad in that, although 2-oxo acids appear to be the preferred substrates, various halogenated carboxylic acids are also transported. In each case the transport of the acid is accompanied by a proton (or by exchange with an OH^- ion). Pyruvate itself can exist in keto, enol and diol forms of which the enol form is present only to a very small extent (less than 2%) in comparison to the keto and diol forms (Becker, 1964; Pocker *et al.*, 1969). Halestrap & Denton (1974) suggested that α-cyano-4-hydroxycinnamate might be an analogue of the enol form of pyruvate and that this might be the reason for its ability to inhibit pyruvate transport at such low concentrations. However, in the light of the broad specificity of the carrier to various halogenated carboxylic acids, this proposal would now seem unlikely.

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