Short Communications

Initial Rates of Pyruvate Transport in Mitochondria Determined by an 'Inhibitor-Stop' Technique

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(Received 4 June 1975)

An 'inhibitor-stop' technique has been developed for measuring initial rates of pyruvate transport into mitochondria. The technique uses α -cyano-3-hydroxycinnamate as the inhibitor and separates the mitochondria from the radioactive medium by Millipore filtration. Observed rates depend on availability of hydroxyl and other exchangeable anions within the mitochondrial matrix.

Recent reports suggest that pyruvate, like several other anionic metabolites, enters rat liver and heart mitochondria by a specific carrier (Papa *et al.*, 1969, 1971; Halestrap & Denton, 1974; Mowbray, 1974; Halestrap, 1975; Mowbray, 1975). It is conceivable that such a carrier might be subject to hormonal regulation, and indeed Adam & Haynes (1969) presented indirect evidence that glucagon or adrenaline injected into rats resulted in increased rates of pyruvate transport into mitochondria subsequently isolated from the livers of such rats. To test the possibility of hormonal regulation we have developed a procedure suitable for measuring initial rates of mitochondrial pyruvate transport and which allows for possible interfering factors.

Experimental

Liver mitochondria were prepared from male Wistar rats by the method of Chappell & Hansford (1972) except that Hepes* was substituted for Tris-HCl in the isolation medium. KOH was used for pH adjustment. A portion of the mitochondrial suspension (50 μ l; approx. 20 mg of protein/ml) was added to 910 μ l of a well-stirred medium containing 125 mM-KCl, 20 mM-Hepes, 2 mM-MgCl₂, 2 mM-arsenite, 5 μ g of rotenone/ml and 1 μ g of antimycin A/ml, pH 7.0 (and in most experiments 3 mM-ascorbate + 0.05 mM-tetramethylphenylenediamine), in a Perspex chamber maintained at the requisite temperature (27°C unless otherwise stated). Various substances could be added at this stage or later during a 2 min preincubation period, after which [2-¹⁴C]pyruvate was added (specific radioactivity 0.07 mCi/mmol). After various time-intervals α -cyano-3-hydroxycinnamate was added to a final concentration of 10 mM, and the mixture transferred to the funnel of a Millipore filtration apparatus containing 5 ml of the incubation medium. Rapid filtration through a

0.8 μ m- or 0.45 μ m-pore-diameter filter then followed with two further washings of 5 ml of the same medium and the filter was then dried. Liquid-scintillation counting of the filters was done with a toluene-based 'cocktail' containing 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene, 4 g and 0.12 g respectively per litre, in a Phillips PW 4510/01 liquid-scintillation counter. Computation of d.p.m. was by the channels-ratio method.

In some experiments mitochondria were preincubated in medium containing various substances before samples were transferred to the final incubation chamber. In this way the substance under test would be considerably diluted at the time when pyruvate uptake was actually occurring.

Pyruvate efflux was measured after pre-loading the mitochondria by incubation with 1.4 mM-[2-¹⁴C]-pyruvate for a period of 2 min. Then 0.8 ml of the suspension was transferred to 5 ml of well-stirred medium with various additions. After various time-intervals the medium was filtered and the radioactivity retained was determined as described above.

Glutamate dehydrogenase was determined by the method of Martin & Denton (1970) in mitochondrial suspensions in which the enzyme had been solubilized by freezing and thawing three times.

Pyruvate decarboxylation was measured as described by Adam & Haynes (1969).

Pyruvate metabolism during the uptake procedure was also studied by allowing mitochondria (samples containing 10 mg of protein) to accumulate [2-¹⁴C]pyruvate for 2 min. The mitochondria were then centrifuged down, washed twice and finally resuspended in 200 μ l of the incubation medium. Metabolites were released by freezing and thawing three times. Ethanol was added to precipitate proteins and after centrifugation supernatants were chromatographed in the system described by Lugg & Overell (1947).

O₂ uptake by mitochondria was determined by a Clark electrode in air-saturated medium containing 100 mM-KCl, 1 mM-EGTA, 2 mM-MgCl₂, 10 mM-Hepes and 2 mM-KH₂PO₄, pH 7.4.

* Abbreviations: Hepes, 2-(N-2-hydroxyethyl)piperazin-N'-yl)ethanesulphonic acid; EGTA, ethanedioxybis-(ethylamine)tetra-acetic acid.

Mitochondrial swelling was measured as described by Bradford & McGivan (1973).

Protein was determined by the method of Lowry *et al.* (1951) recommended for insoluble proteins, with bovine serum albumin as standard.

Pyruvate was measured enzymically by the method of Adam (1965).

All radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. α -Cyano-3-hydroxycinnamate was obtained from Ralph N. Emanuel, Wembley, Middx., U.K.

Results and discussion

Mitochondrial yield, respiratory efficiency and respiratory inhibition. The yield of mitochondria was 10mg of protein/g wet weight of liver. In O_2 -uptake experiments, the respiratory-control ratio with pyruvate as substrate was 4. Rotenone and antimycin A, at the concentrations used for uptake experiments, were found to abolish completely respiration due to pyruvate as judged by O_2 consumption, but ascorbate + tetramethylphenylenediamine could still support state-3 and state-4 respiration under these conditions.

Mitochondria were incubated in the medium used for uptake studies and an attempt was made to measure disappearance of enzymically assayed pyruvate from the whole system. No disappearance was detected during 2min. In other experiments production of $^{14}CO_2$ from $[1-^{14}C]$ pyruvate was measured. In the medium used by Adam & Haynes (1969) $^{14}CO_2$ output was 19.6 ± 1.8 (3) nmol/5min per mg of protein in the presence of 1.4mM- $[1-^{14}C]$ pyruvate at $30^\circ C$. In our uptake medium containing portions of the same population of mitochondria and 1.4mM- $[1-^{14}C]$ pyruvate there was no detectable increase in $^{14}CO_2$ output above that expected from 'background' in the absence of mitochondria.

Chromatography of extracts from mitochondria allowed to accumulate $[2-^{14}C]$ pyruvate revealed that none of the pyruvate in the incubation medium was transformed into malate, citrate or alanine, and less than 5% of the radioactivity in the extract was detected outside the area of the chromatogram occupied by pyruvate. It was concluded that under conditions of our pyruvate-uptake and -efflux experiments there was no detectable decarboxylation, carboxylation or transamination of pyruvate, and therefore that the radioactivity accumulated in the filtered mitochondria represented pyruvate only.

Efficiency of filtration. The protein content and glutamate dehydrogenase activity were measured in the filtrate passing through the Millipore filters after filtering typical mitochondrial samples. For the $0.45\mu m$ -pore-diameter filters $44.4 \pm 4.5\%$ (4) of the total protein and $83.3 \pm 2.6\%$ (4) of the glutamate dehydrogenase activity was retained on the filters;

for the $0.45\mu m$ -pore-diameter filters the corresponding values were $69.4 \pm 2.3\%$ (4) and over 99% (4). Uptake rates expressed as nmol of pyruvate/min per mg of mitochondrial protein were corrected for any loss of mitochondrial protein through the filters. There was no difference in such corrected rates for similar mitochondrial samples filtered through the two types of membrane filter. The radioactivity counts retained on both membranes due to incubation media only (no mitochondria present) were less than 20% of the counts due to filtered mitochondria after the shortest periods of accumulation.

Efficiency of α -cyano-3-hydroxycinnamate as an inhibitor of pyruvate transport. α -Cyano-3-hydroxycinnamate (10mM) inhibited by 80% the O_2 uptake by mitochondria incubated with 12.5mM-pyruvate. α -Cyano-3-hydroxycinnamate (5mM) inhibited by 50% the O_2 uptake under the same conditions but did not affect O_2 uptake in mitochondria incubated with 12.5mM-succinate. α -Cyano-3-hydroxycinnamate (12mM) inhibited by 50% the rate of fall of light-absorbance to mitochondrial swelling in 100mM-ammonium pyruvate. $[2-^{14}C]$ Pyruvate accumulated in mitochondria was retained after washing the mitochondria on the filters, and the presence of α -cyano-3-hydroxycinnamate did not encourage efflux of this accumulated pyruvate.

Table 1. Effect of time of addition of α -cyano-3-hydroxycinnamate and of filtration on mitochondrial content of $[2-^{14}C]$ pyruvate

Samples of a mitochondrial preparation were incubated in the medium used for pyruvate-uptake studies (see under 'Experimental') and α -cyano-3-hydroxycinnamate was added (10mM final concentration). After various intervals $[2-^{14}C]$ pyruvate was added to 1.4mM final concentration, and after further intervals the mitochondrial suspension was filtered in the usual manner and the radioactivity retained on the membrane filters was counted.

Time of addition of α -cyano-3-hydroxycinnamate before (–) or after (+) addition of $[2-^{14}C]$ pyruvate to mitochondrial suspension (s)	Time of filtration of mitochondrial suspension after addition of $[2-^{14}C]$ pyruvate (s)	$[2-^{14}C]$ Pyruvate content of mitochondria (nmol/mg)
–120	10	0.77
–60	10	0.93
–10	10	1.18
–5	10	1.00
0	10	1.02
+10	10	5.15
+20	20	11.65
+20	60	10.75
+20	120	9.75
+20	300	12.65

Fig. 1. Time-course of pyruvate uptake (a), effect of temperature (b) and concentration (c)

Pyruvate uptake was measured as described under 'Experimental'. Each part of the Figure shows results obtained with a single mitochondrial preparation. In all cases the incubation medium contained 3 mM-ascorbate + 0.05 mM-tetramethylphenylenediamine and was buffered to pH 7.0. The $[2-^{14}\text{C}]$ pyruvate concentration was 1.4 mM in (a) and (b) and as stated in (c). The incubation temperature was 27°C for experiments (a) and (c); for (b) temperatures were as indicated. (b) ■, 27°C; ●, 20°C; ▲, 4°C.

However, the inhibitor did prevent exchange of accumulated $[2-^{14}\text{C}]$ pyruvate with extramitochondrial non-radioactive pyruvate. α -Cyano-3-hydroxycinnamate at 10 mM was as effective in preventing uptake of $[2-^{14}\text{C}]$ pyruvate by mitochondria when added simultaneously with the labelled pyruvate as when the inhibitor was added 2 min before (Table 1). Further, after addition of the inhibitor the exact time of washing had no significant effect on the radioactivity counts retained in the mitochondria. We concluded from these results that α -cyano-3-hydroxycinnamate was satisfactory for use in an 'inhibitor-stop' technique.

Time-course, temperature- and concentration-dependence of mitochondrial pyruvate uptake. The time-course and effect of temperature on pyruvate uptake are shown in Fig. 1. Linearity was maintained for at least 15 s at 27°C with 1.4 mM- $[2-^{14}\text{C}]$ pyruvate in the incubation medium. In 19 such control experiments the pyruvate accumulated between 0 and 15 s was not significantly different from 3 times that accumulated between 0 and 5 s [ratio of the two accumulations 3.04 ± 0.11 (19)]. The rate of pyruvate uptake was 27.52 ± 1.55 nmol/min per mg of protein.

Replicate determinations of uptake rates in the same mitochondrial preparations sampled within 2 h of each other differed by less than 5%. Maximal accumulation reached at 2 min was 19.73 ± 0.68 nmol/mg of protein for five different mitochondrial preparations.

Fig. 1(c) shows the dependence of initial uptake rates on pyruvate concentration for a particular mitochondrial preparation.

Effects of other anions on pyruvate uptake. It has been concluded from previous work (Papa *et al.*, 1969, 1971; Papa & Paradies, 1974) that pyruvate enters mitochondria in exchange for hydroxyl anions. Our work also supports this theory in that increasing external pH above 7.0 caused an efflux of pyruvate previously accumulated within mitochondria, and also no other counterion was required to observe mitochondrial swelling due to the entry of ammonium pyruvate. To make meaningful comparisons of pyruvate-transport rates in different mitochondrial populations it is therefore advisable to stabilize the pH gradient across the mitochondrial inner membrane by allowing mitochondrial respiration. In freshly prepared mitochondria addition of 3 mM-ascorbate + 50 μM -tetramethylphenylenediamine had no effect

on the initial rate of pyruvate uptake, although it did increase accumulation during 2 min by 52%. 2,4-Dinitrophenol (50 μ M), which prevents energy conservation, whether in the form of a proton gradient or high-energy intermediates, decreased both the initial rate of pyruvate uptake and the accumulation after 2 min to 5% of the control values. We therefore included ascorbate + tetramethylphenylenediamine in all further measurements with the aim of stabilizing and maintaining the energy status during a prolonged series of incubations.

Although pyruvate-hydroxyl exchange does occur it is possible that the presence of other exchangeable anions within the mitochondrial matrix may influence observed rates of transport. One such anion may be palmitoylcarnitine (Mowbray, 1975). As in previous work (Mowbray, 1975), we have found that under certain conditions dicarboxylate anions (malate, succinate) can stimulate uptake. Thus preincubation of mitochondria for 2 min with ascorbate + tetramethylphenylenediamine and with 1 mM-malate followed by dilution of the latter to a final concentration of 0.05 mM and addition of 1.4 mM-[2- 14 C]pyruvate resulted in an increase in the initial rate of pyruvate uptake of $58 \pm 4\%$ (4) compared with the rate when no malate had been added to preincubation or incubation media. On the other hand, when 1 mM-malate was present along with 1.4 mM-[2- 14 C]pyruvate the initial rate of pyruvate uptake was depressed to $26.4 \pm 6\%$ (4) of the control rate in the absence of malate. Further, extramitochondrial malate or succinate facilitated efflux of previously accumulated intramitochondrial pyruvate, as also reported by Mowbray

(1975). We conclude from these experiments that, in assessing differences in initial rates of pyruvate transport between mitochondrial populations prepared from animals in different physiological states, the effects of both the mitochondrial pH gradient and the complement of other anions in the matrix must be taken into account.

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