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ACCUMULATION OF PYRUVATE BY ISOLATED RAT LIVER MITOCHONDRIA

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Summary

1. Various methods to measure the rate of accumulation of [3-^{14}C]pyruvate in the sucrose-impermeable space of isolated rat liver mitochondria are tested and compared with respect to their ability to distinguish between carrier-linked pyruvate transport and non-carrier-linked processes (adsorption and diffusion).

2. Evidence is presented that the cinnamic acid derivatives commonly used as specific inhibitors of the pyruvate carrier (i) do not completely abolish all carrier-mediated pyruvate transport; (ii) inhibit pyruvate adsorption, and (iii) at higher concentrations lead to a removal of previously accumulated pyruvate from the mitochondria. It is concluded that procedures which avoid the use of transport inhibitors allow more reliable estimates of carrier-linked pyruvate transport.

3. It is proposed to measure pyruvate adsorption as the accumulation of pyruvate in the presence of an uncoupler. Using this procedure, it could be shown that, with 1 mM pyruvate, adsorption represents only a small part of the total pyruvate accumulation, the main part being carrier-linked transport driven by the pH gradient across the mitochondrial inner membrane.

Introduction

Pyruvate uptake by isolated mitochondria is usually studied by determining the accumulation of radioactivity in the sucrose-impermeable space of the mitochondria following their incubation with radioactive pyruvate. The incuba-

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

tions are usually stopped by separation of the mitochondria from the medium by means of rapid centrifugation through silicone oil [1—3]. This separation step may be preceded by addition of α-cyanocinnamate or one of its derivatives which are reported to be specific inhibitors or pyruvate transport suitable for inhibitor-stop use [4,5]. In the past it has been tacitly assumed that data thus obtained represent carrier-mediated transport. However, Pande and Parvin [6] have recently emphasized that such data actually represent the sum of three distinct and simultaneously occurring processes: adsorption, diffusion and carrier-linked transport.

Since we were interested in mitochondrial pyruvate transport and engaged in studies on the modulation of the activity of the mitochondrial pyruvate carrier [7], the report of Pande and Parvin [6] led us to investigate in more detail the validity of various methods to measure mitochondrial pyruvate transport. Results presented in this paper indicate that the use of α-cyanocinnamate or its derivatives should be avoided in experiments to determine the rate of carrier-mediated pyruvate transport. As pyruvate adsorption does indeed occur, a different method to estimate its contribution to the total uptake of pyruvate is proposed in this report, viz. by measuring pyruvate accumulation in the presence of a suitable concentration of FCCP.

To avoid confusion, the following terminology will be used in the rest of this paper: 'uptake', the total accumulation of radioactivity by the mitochondria, i.e. the sum of adsorption, diffusion and carrier-linked transport; 'adsorption', all non-carrier-linked accumulation processes, i.e. the sum of adsorption and diffusion; 'transport', accumulation due to a specific carrier in the mitochondrial inner membrane. The term 'accumulation' will be used if the mechanism of the process is not specified.

Methods and Materials

Livers from female Wistar rats were homogenized in 250 mM sucrose, 5 mM Tris-HCl (pH 7.50) and 1 mM EGTA. Mitochondria were isolated as described by Myers and Slater [8], suspended in the same medium without EGTA and kept at 0°C. Mitochondrial protein was determined by a biuret method [9]. To measure pyruvate uptake, freshly isolated mitochondria (approx. 2 mg of protein) were suspended in 950 μl of an incubation medium essentially composed of 75 mM sucrose, 90 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$, 20 mM 3-(*N*-morpholino)propanesulfonate, 20 μM rotenone, 1.4 μM antimycin, 24 μM oligomycin, 6% (w/v) dextran and [6,6′-^{3}H]sucrose (1.5 μCi/ml). Additional components and pH are mentioned in the legends. After 2 min at 27°C, [3-^{14}C]pyruvate (0.15 Ci/mol) was added to a final concentration of 1 mM. Final incubation volume, 1.0 ml. At t = 2 min 15 s pyruvate uptake was stopped either by adding 100 μl of inhibitor solution (α-cyanocinnamate or α-cyano-3-hydroxycinnamate), followed at t = 2 min 35 s by centrifugation of the mitochondria through silicone oil, or by direct centrifugation of the mitochondria through silicone oil. Pyruvate adsorption was measured in parallel incubations with either inhibitor or FCCP present in the incubation medium.

In our hands, a 15 s incubation was the shortest possible period when pyruvate uptake was to be quenched by direct centrifugation. For the sake of com-

parison, pyruvate uptake was allowed the same period when the 'inhibitor-stop' technique was used. According to Titheradge and Coore [5] linearity of pyruvate uptake is maintained for at least 15 s at 27°C with 1.4 mM pyruvate in the incubation medium.

The centrifugation procedure was carried out by transferring a 0.75 ml aliquot of the incubation mixture to a 1.5 ml Eppendorf microtube containing a bottom layer of 200 μl 14% (w/v) $HClO_4$ and 18 mM unlabelled pyruvate (to prevent adsorption of ^{14}C label to denatured mitochondrial protein; cf. Ref. 2) and about 200 μl of silicone oil (specific density 1.051) above the bottom layer. The tube was spun for 120 s at 10 000 × *g* using a modified Eppendorf 3200 centrifuge with swing-out head. Radioactivity of top and bottom layer was measured in a Packard TriCarb 3255 spectrometer using a xylene-based scintillation liquid [10]; quenching was monitored with the external standard. In the experiments of Table I we also used centrifuge tubes with a bottom layer prepared according to Pande and Parvin [6]. The contents of these tubes were treated exactly as reported by the authors up to the stage of liquid scintillation counting.

[3-^{14}C]Pyruvate and [1-^{14}C]-pyruvate were purchased from New England Nuclear and [6,6'-^{3}H]sucrose was from the Radioachemical Centre, Amersham. The radioactive pyruvate was divided into 2-μCi samples, lyophilized and stored in sealed tubes at −20°C. A substantial loss (up to 50%) of pyruvate occurred within 3 months. Cellulose thin-layer chromatography with diethyl ether/90% (w/v) $HCOOH/H_2O$ (7 : 2 : 1, v/v) as developing agent revealed that this loss was due to the formation of a second radioactive compound. This unidentified compound was accumulated by rat liver mitochondria in an α-cyanocinnamate-sensitive fashion. Therefore, radioactive pyruvate was always purified by thin-layer chromatography (as described above) immediately before use.

Silicone oil (specific density 1.051) was prepared from 17 parts Dow Corning 200 (2.0 cs) and 183 parts Dow Corning 550 fluid. Nigericin was kindly donated by Dr. R.L. Hamill (Lilly Res. Lab., Indianapolis). Antimycin, ascorbic acid, dextran (M_r 40 000), oligomycin, rotenone and TMPD were obtained from Sigma; α-cyanocinnamate and α-cyano-3-hydroxycinnamate from Aldrich; pyruvic acid from Baker; FCCP, lactate dehydrogenase, nonactin, sodium pyruvate and valinomycin from Boehringer. Other chemicals were of the purest grade available. Antimycin, FCCP, nigericin, nonactin, oligomycin, rotenone and valinomycin were added to the incubation medium as ethanolic solutions, the ethanol concentration of this medium being kept constant at 1.5% (v/v).

The experiments were carried out several times with mitochondria from different rats and all incubations were in triplicate. The values shown (average ±S.D.) have been corrected for radioactivity calculated to be present in the sucrose-permeable space.

Results

In the new approach advocated by Pande and Parvin [6], the rate of pyruvate transport is calculated from the difference of pyruvate uptake and pyruvate adsorption. The latter process is measured as pyruvate accumulation in the presence of 1 mM α-cyanocinnamate to inhibit the pyruvate carrier. The former

process is measured in the absence of α-cyanocinnamate and quenched by the inhibitor-stop technique using the same inhibitor. In our experiments on the modulation of the activity of the mitochondrial pyruvate carrier [7], we had used 10 mM α-cyano-3-hydroxycinnamate as a stop-inhibitor. Thus it seemed worthwhile to compare in detail the usefulness of both inhibitors as tools in the determination of pyruvate uptake and adsorption.

In the experiments shown in Table I the following variations of the experimental conditions were tested: utilization of α-cyanocinnamate (1 mM) or α-cyano-3-hydroxycinnamate (10 mM) as inhibitors, incubation media with or without a respiratory substrate (ascorbate + TMPD), at pH 7.50 or pH 6.80, and the use of different bottom layers in the centrifuge tubes (see Methods and Materials). It is clear from Table I that much higher values for pyruvate adsorption are observed with α-cyanocinnamate as an inhibitor than with α-cyano-3-hydroxycinnamate. The calculated transport rates are, therefore, much higher when the latter inhibitor is used. Secondly, in the absence of substrate, a decrease of the external pH from 7.50 to 6.80 promotes pyruvate uptake. With α-cyanocinnamate this coincides with an increased adsorption value, whereas with α-cyano-3-hydrocycinnamate this appears to be due to an increased transport. It should be noted that the mitochondria are always isolated at pH 7.50

TABLE I

MITOCHONDRIAL ACCUMULATION OF [3-^{14}C]PYRUVATE UNDER DIFFERENT EXPERIMENTAL CONDITIONS

At t = 0 mitochondria were suspended in the standard incubation medium (pH 7.50 or 6.80) with or without 5 mM ascorbate + 50 μM TMPD (asc/TMPD) and inhibitors (1 mM α-cyanocinnamate (α-CC) or 10 mM α-cyano-3-hydroxycinnamate (α-CHC)). At t = 2 min labelled pyruvate was added. Pyruvate uptake: 100 μl inhibitor solution was added at t = 2 min 15 s. Pyruvate adsorption: inhibitor present at t = 0; 100 μl H_2O added at t = 2 min 15 s. All centrifugations were performed at t = 2 min 35 s. Centrifuge tubes had bottom layers containing either unlabelled pyruvate and $HClO_4$ (N) or a pyruvic acid/formic acid mixture (P) prepared according to Ref. 6. In the former case only pyruvate uptake was measured.

Incubation medium		Inhibitor	Bottom layer	Pyruvate accumulated (nmol/mg protein)		Calculated pyruvate transport (U—A) (nmol/mg protein)
pH	asc/TMPD			Uptake (U)	Adsorption (A)	
7.50	—	α-CC	N	4.51 ± 0.02		
			P	4.97 ± 0.09	2.68 ± 0.07	2.29 ± 0.11
	—	α-CHC	N	4.32 ± 0.07		
			P	4.58 ± 0.06	0.79 ± 0.05	3.79 ± 0.08
	+	α-CC	N	7.54 ± 0.06		
			P	7.73 ± 0.10	5.18 ± 0.04	2.55 ± 0.11
	+	α-CHC	N	5.12 ± 0.07		
			P	5.13 ± 0.16	0.96 ± 0.04	4.17 ± 0.16
6.80	—	α-CC	N	6.33 ± 0.04		
			P	6.53 ± 0.06	4.54 ± 0.07	1.99 ± 0.09
	—	α-CHC	N	5.86 ± 0.06		
			P	5.75 ± 0.15	0.94 ± 0.06	4.81 ± 0.16
	+	α-CC	N	7.51 ± 0.04		
			P	7.57 ± 0.06	5.76 ± 0.06	1.81 ± 0.08
	+	α-CHC	N	5.35 ± 0.03		
			P	5.57 ± 0.06	1.08 ± 0.04	4.49 ± 0.07

and that a lower pH of the incubation medium will impose an additional pH gradient across the mitochondrial inner membrane. Thirdly, addition of substrate stimulates pyruvate uptake (except with α-cyano-3-hydroxycinnamate at pH 6.80). Again, with α-cyanocinnamate this coincides with increased adsorption values, whereas with α-cyano-3-hydroxycinnamate the transport value is increased. In the fourth place, Table I shows that bottom layers of different composition result in very similar uptake data. We decided to keep our usual bottom layer for further experiments, partly because of the less tedious procedures involved and partly because of the better reproducibility.

In order to explain the lower adsorption values observed with α-cyano-3-hydroxycinnamate as compared with those obtained in experiments with α-cyanocinnamate, two possibilities must be considered: (i) 1 mM α-cyanocinnamate does not completely inhibit pyruvate transport; and (ii) 10 mM α-cyano-3-hydroxycinnamate partly inhibits pyruvate adsorption. Evidence that the former explanation may be correct was obtained in experiments with ionophores (Table II). In order to reduce the transmembrane pH gradient, these experiments were performed at pH 7.50 in the absence of a respiratory substrate. From Table I it can be seen that these are the conditions of minimal pyruvate uptake.

The induction of K^+ uptake by nonactin [11] or by valinomycin [12], expected to abolish the membrane potential without disturbing the pH gradient across the inner membrane, stimulates the uptake of pyruvate by the mitochondria (Table II). On the other hand, a collapse of the transmembrane pH gradient by the protonophore FCCP strongly reduces pyruvate uptake and fully prevents pyruvate transport. Similarly, pyruvate transport is inhibited by induction of K^+-H^+ exchange with nigericin [13] or with FCCP + valinomycin (not shown).

TABLE II

ACCUMULATION OF [3-^{14}C]PYRUVATE IN THE PRESENCE OF IONOPHORES

Mitochondria were suspended in the standard incubation medium (pH 7.50) with further additions as indicated: 0.40 μM nonactin; 0.14 μM valinomycin; 0.70 μM FCCP; 0.18 μM nigericin. For further experimental details, see Table I.

Inhibitor	Incubation medium	Pyruvate accumulated (nmol/mg protein)		Calculated pyruvate transport (U—A) (nmol/mg protein)
		Uptake (U)	Adsorption (A)	
1 mM α-cyanocinnamate	No further additions	4.88 ± 0.07	2.40 ± 0.04	2.48 ± 0.08
	+ Nonactin	6.97 ± 0.12	2.02 ± 0.09	4.95 ± 0.15
	+ Valinomycin	5.55 ± 0.02	2.16 ± 0.03	3.39 ± 0.04
	+ FCCP	0.74 ± 0.03	1.04 ± 0.04	<0
	+ Nigericin	0.68 ± 0.02	1.02 ± 0.03	<0
10 mM α-cyano-3-hydroxycinnamate	No further additions	4.78 ± 0.05	0.73 ± 0.08	4.05 ± 0.09
	+ Nonactin	5.53 ± 0.07	0.99 ± 0.06	4.54 ± 0.09
	+ Valinomycin	5.16 ± 0.02	1.01 ± 0.03	4.15 ± 0.04
	+ FCCP	0.66 ± 0.03	0.96 ± 0.05	<0
	+ Nigericin	0.74 ± 0.04	1.03 ± 0.02	<0

One would expect only minor effects of ionophores on pyruvate adsorption. This expectation is realized if α-cyano-3-hydroxycinnamate is applied as inhibitor, but not with α-cyanocinnamate. With the latter inhibitor the same low adsorption values are observed as with α-cyano-3hydroxycinnamate only under conditions in which pyruvate transport is fully inhibited. In conditions of active pyruvate transport, α-cyanocinnamate shows increased adsorption values. We consider these results as suggestive evidence that 1 mM α-cyanocinnamate does not completely inhibit pyruvate transport and that, therefore, in experiments with this inhibitor 'adsorption' values include some residual transport.

The second possible explanation mentioned above (inhibition of pyruvate adsorption by the cinnamate derivatives) was explored by studying pyruvate accumulation as a function of the inhibitor concentration (Fig. 1). If the inhibitory action of the cinnamates is restricted to the transport of pyruvate, one would expect that at higher inhibitor concentrations a constant level of inhibitor-insensitive pyruvate accumulation would be observed, representing pyruvate adsorption. In contrast, Fig. 1 shows a continuous decrease of pyruvate accumulation with increasing inhibitor concentrations. At inhibitor concentrations above 20 mM, pyruvate accumulation is almost completely inhibited. This means that both cinnamate derivatives inhibit pyruvate adsorption as well as pyruvate transport. Moreover, it is clear from Fig. 1 that it is impossible to select an inhibitor concentration at which transport is fully inhibited while adsorption is unaffected.

Fig. 1 also shows that there is yet another drawback associated with the use of the cinnamate derivatives. Some incubations for the measurement of (uninhibited) pyruvate uptake (indicated by the triangles in Fig. 1) were

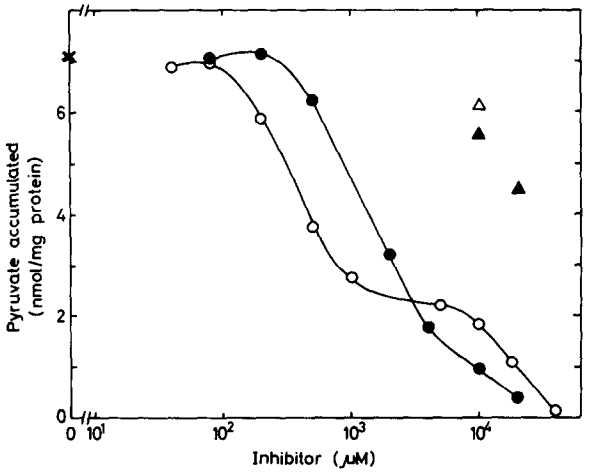

Fig. 1. Influence of α-cyanocinnamate and α-cyano-3-hydroxycinnamate on the accumulation of pyruvate by isolated mitochondria. Prior to the addition of the mitochondria at $t = 0$, the standard incubation medium (pH 6.80) was supplemented with α-cyanocinnamate (○———○) or α-cyano-3-hydroxycinnamate (●———●) at the concentrations indicated. At $t = 2$ min [3-^{14}C]pyruvate was added. Pyruvate uptake in the absence of inhibitors (X) was 7.07 ± 0.03 nmol/mg protein. All these incubations were stopped by centrifugation at $t = 2$ min 15 s. Triangles represent pyruvate uptake using α-cyanocinnamate (△) or α-cyano-3-hydroxycinnamate (▲) as stop-inhibitors at $t = 2$ min 15 s followed by centrifugation at 2 min 35 s.

stopped with the inhibitor-stop technique. All other incubations were stopped by direct centrifugation. It is clear that at high inhibitor concentrations the inhibitor-stop technique results in lower values for pyruvate uptake than direct centrifugation (indicated by the cross in Fig. 1). This would mean that pyruvate is removed from the mitochondria in the period between the addition of the stop-inhibitor (t = 2 min 15 s) and the subsequent centrifugation (t = 2 min 35 s), indicating that high concentrations of the inhibitor lead either to an extrusion of previously accumulated pyruvate from the matrix space or to a removal of adsorbed pyruvate from the mitochondria.

Taking together all the vagaries associated with the cinnamates, it is clear that the use of these inhibitors in determining the rate of pyruvate transport should be avoided. Direct centrifugation of the mitochondria through silicone oil is, therefore, the method of choice to stop the incubations. By this method unambiguous data on pyruvate uptake can be obtained. The question then remains how to correct these data reliably for the adsorption of pyruvate. Since FCCP was found to inhibit pyruvate transport completely (Table II), its effect on the accumulation of labelled pyruvate by mitochondria was investigated more closely. Fig. 2 shows that, in contrast to the effects of α-cyanocinnamate and α-cyano-3-hydroxycinnamate (Fig. 1), at increasing concentrations of the uncoupling agent, the FCCP-insensitive accumulation of pyruvate is indeed reaching a constant level. In our opinion, this level offers a fair estimate of the non-carrier-linked accumulation of pyruvate, i.e. of pyruvate adsorption as defined in the introduction.

TABLE III

COMPARISON OF VARIOUS METHODS TO DETERMINE MITOCHONDRIAL PYRUVATE TRANSPORT

At t = 0 mitochondria were suspended in the standard incubation medium (pH 7.50 or 6.80). At t = 2 min [3-^{14}C]pyruvate was added. Pyruvate uptake was quenched at t = 2 min 15 s either by direct centrifugation or by addition of a stop-inhibitor. In the latter case the mitochondria were removed from the medium by centrifugation at t = 2 min 35 s. Pyruvate adsorption was measured in parallel incubations in which transport was inhibited either by 0.7 μM FCCP or by a cinnamate derivative. α-CC, α-cyanocinnamate; α-CHC, α-cyano-3-hydroxycinnamate.

pH	Uptake quenched by	Adsorption measured in the presence of	Pyruvate accumulated (nmol/mg protein)		Calculated rates of pyruvate accumulation (nmol/min per mg protein)	
			Uptake (U)	Adsorption (A)	Uptake (U)	Transport (U—A)
7.50	centrifugation	FCCP	5.99 ± 0.01	1.00 ± 0.03 *	23.96 ± 0.04	19.96 ± 0.12
	stop-inhibitor	1 mM α-CC	4.99 ± 0.04	2.37 ± 0.04 **	19.96 ± 0.16	10.48 ± 0.24
	stop-inhibitor	2 mM α-CHC	6.09 ± 0.12	3.29 ± 0.03 **	24.36 ± 0.48	11.20 ± 0.48
	stop-inhibitor	10 mM α-CHC	4.76 ± 0.08	1.07 ± 0.06 **	19.04 ± 0.32	14.76 ± 0.40
6.80	centrifugation	FCCP	7.07 ± 0.03	1.32 ± 0.06 *	28.28 ± 0.12	23.00 ± 0.28
	stop-inhibitor	11 mM α-CC	7.01 ± 0.05	4.85 ± 0.03 **	28.04 ± 0.20	8.64 ± 0.24
				2.77 ± 0.04 *		
	stop-inhibitor	2 mM α-CHC	7.62 ± 0.11	3.89 ± 0.03 **	30.48 ± 0.44	14.92 ± 0.44
				3.22 ± 0.06 *		
	stop-inhibitor	10 mM α-CHC	5.58 ± 0.07	1.00 ± 0.09 **	22.32 ± 0.28	18.32 ± 0.44
				0.98 ± 0.02 *		

* Adsorption incubations terminated by centrifugation at t = 2 min 15 s.

** Adsorption incubations terminated by centrifugation at t = 2 min 35 s.

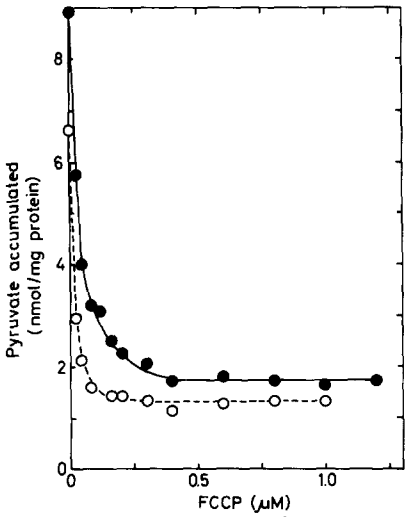

Fig. 2. Accumulation of [3-^{14}C]pyruvate as a function of FCCP concentration in the standard incubation medium. Pyruvate accumulation was stopped by centrifugation. ○- - - - -○, pH 7.50; ●———●, pH 6.80.

Table III summarizes data on pyruvate transport rates obtained by various methods. We prefer to stop incubations by direct centrifugation and to correct for adsorption by measurement of pyruvate accumulation in the presence of a suitable concentration of FCCP (cf. Fig. 2). Using these procedures, pyruvate transport rates of 19.96 and 23.00 nmol/min per mg protein can be calculated at pH 7.50 and 6.80, respectively (lines 1 and 5). Similarly, it is demonstrated that under these conditions (with 1 mM pyruvate) adsorption accounts for less than 20% of the total uptake of pyruvate. Values for transport rates obtained with either α-cyanocinnamate or α-cyano-3-hydroxycinnamate are substantially lower. Comparison of adsorption values in lines 5, 7 and 9 demonstrates again that during the 15 s incubation period a residual transport occurs in the presence of inhibitor. Comparison of lines 6 and 7 and of 8 and 9 shows that accumulation of pyruvate in the presence of inhibitor continues until the mitochondria are separated from the medium. The latter process will also introduce errors in uptake values determined by the inhibitor-stop technique. The very divergent transport rates calculated from experiments in which the cinnamates are used at concentrations currently reported in the literature affirm our conclusion that these inhibitors are not suitable for this purpose.

Discussion

Pande and Parvin [6] were the first to draw attention to the marked adsorption of pyruvate on isolated mitochondria and to the errors that this phenomenon may introduce in studies on the pyruvate-transporting system of mitochondria. According to these authors, an account can be made for the adsorption of pyruvate by determining the accumulation of pyruvate in the presence and absence of 1 mM α-cyanocinnamate, a specific inhibitor of pyruvate transport. By assuming that the inhibitor-sensitive accumulation is indicative of trans-

port whereas the inhibitor-insensitive accumulation represents adsorption, these authors concluded that the ratio of adsorption to transport is as high as 17:1 at 1 mM pyruvate.

In this paper we demonstrate that the correction proposed by Pande and Parvin [6] will lead to rather arbitrary results since the inhibitory action of 1 mM α-cyanocinnamate is neither complete nor restricted to the transport process. An incomplete inhibition of the pyruvate carrier will result in an overestimation of the adsorption correction and, consequently, in the calculation of too low rates of the carrier-linked transport process. The error is even promoted by the procedure of Pande and Parvin [6] to initiate pyruvate uptake by addition of the mitochondria rather than by adding the labelled pyruvate. As shown by Halestrap [14], it takes time for the inhibitors to block the pyruvate carrier. In fact, the use of this initiation procedure may well explain why Pande and Parvin [6] failed to observe α-cyanocinnamate-sensitive transport of [1-^{14}C]pyruvate. In our procedure, the mitochondria are 'preincubated' for 2 min in the presence of the inhibitors before pyruvate is added. This preincubation period also serves to allow a complete binding of the various metabolic inhibitors to their mitochondrial sites of action. We feel that an extensive metabolism of pyruvate will obscure pyruvate uptake rather than that it is a prerequisite for it, as suggested by Pande and Parvin [6]. Using an incubation medium (pH 6.80) without respiratory substrate, we found that the 2 min preincubation resulted in a subsequent production of [$^{14}CO_2$] from [1-^{14}C]-pyruvate as low as 0.88 ± 0.04 (n = 4) nmol/min per mg protein at 27°C. Under these conditions the disappearance of pyruvate from the incubation (assayed enzymically) amounted to 1.91 ± 0.48 (n = 4) nmol/min per mg protein (cf. Ref. 5). Comparison of these figures with those in Table III shows that in our experiments pyruvate metabolism will not cause substantial errors.

It may be worthwhile to mention here that much higher adsorption values were observed with crude [3-^{14}C]pyruvate than with the purified compound.

The same drawbacks which invalidate the use of α-cyanocinnamate for the adsorption correction, also make it unsuitable for inhibitor-stop use. Fortunately, rapid centrifugation offers a reliable alternative. The different results obtained with both methods can be seen from Table III. With our modified Eppendorf centrifuge, the mitochondria reach the acid bottom layer within 2—3 s, which is of the same order as the mixing time for pyruvate and for the stop-inhibitors. A more serious problem connected with the centrifugation procedure is that it does not allow shorter incubation periods than 15 s. Although Titheradge and Coore [5] found that at 27°C the rate of pyruvate uptake is constant for at least 15 s, to obtain true initial rates of pyruvate transport one might be forced to perform the assays at even less physiological temperatures below 27°C.

As an alternative method for the assay of pyruvate adsorption, we propose to measure pyruvate accumulation in the presence of a suitable concentration of FCCP, taking into account that the relative concentration of uncoupler to protein should be considered. Results similar to those with FCCP can be obtained with nigericin. We realize that with this method no correction will be made for energy-dependent adsorption of pyruvate, but we consider it highly unlikely that such a process occurs. It remains unexplained why Pande and

Parvin [6] failed to observe any effect of FCCP on the α-cyanocinnamate-sensitive accumulation of pyruvate.

If rapid centrifugation through silicone oil is used to stop the accumulation of labelled pyruvate by the mitochondria, and if the adsorption of pyruvate on the mitochondria is assayed by measuring pyruvate accumulation in the presence of FCCP, it can be demonstrated that, with 1 mM pyruvate, adsorption accounts for only 17 or 19% of the total uptake of pyruvate at pH 7.50 or 6.80, respectively.

The use of ionophores to measure pyruvate adsorption assumes that the mechanism of pyruvate transport is an energy-independent direct exchange of pyruvate anions for hydroxyl ions (or a symport of pyruvate anions and protons), as described by Papa and coworkers [1,3] and by Halestrap [4,14]. Since no experimental data are available which are incompatible with such a mechanism for the action of the mitochondrial pyruvate carrier, we consider this assumption justified. In fact, we feel that many results reported in this paper, such as the effects of pH, substrate, ionophores and uncoupler, support the existence of a specific pyruvate-hydroxyl exchange carrier catalysing a transport of pyruvate into the mitochondrial matrix space driven by the pH gradient across the mitochondrial membrane.

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