Malate Exchange Between the Cytosol and Mitochondria

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(Received 16 October 1972)

1. By comparing the relative isotopic yields in glucose and CO₂ from precursors of mitochondrial and cytosolic malate, it is evident that the rate of isotopic exchange between these compartments is rapid. 2. A variety of potential inhibitors of malate exchange were tested, but no specific and effective inhibitor of the isotopic exchange has been found. 3. Compounds such as *n*-butylmalonate and *p*-iodobenzylmalonate, which have been used as inhibitors of the malate–phosphate transport system in isolated mitochondria, do not appear to be sufficiently specific to be useful in studies with intact cells.

Malate has been established as an important carrier of reducing equivalents from the mitochondria to the cytosol in gluconeogenic tissues (Lardy *et al.*, 1965; Krebs *et al.*, 1967). Studies with isolated mitochondria have established the existence of several malate-transport systems in the mitochondrial membrane (Chappell, 1968), and inhibitors of these carrier systems have been found. The possibility that certain of these transport systems may operate as rate-limiting steps in the intact cell has been proposed (Williamson *et al.*, 1970), mainly on the basis of evidence involving the same inhibitors used with isolated mitochondria. In the present paper we examine the rate of exchange of isotopically labelled malate between the cytosol and the mitochondria in the intact kidney-cortex cell, and the effects of various compounds on this exchange rate.

Methods

Kidney cortex segments from Wistar rats were prepared by the method of Guder & Wieland (1970). Segments (125 mg wet weight) were incubated in 2 ml of phosphate–salts buffer (Krebs *et al.*, 1963) for 2 h at 37°C with 100% O₂ as the initial gas phase, together with the labelled and unlabelled substrates. Acetate was added in all experiments to stimulate gluconeogenesis (Krebs *et al.*, 1965). The 25 ml incubation flasks were stoppered with serum caps from which a plastic cup was suspended. To stop the incubation, 0.5 ml of 0.5 M-H₂SO₄ was injected into the medium and 0.3 ml of phenethylamine–water (1:1, v/v) was injected into the hanging cup. The flasks were shaken for 2.5 h to collect ¹⁴CO₂. The medium was put through a 1 cm × 5 cm Zeo-Karb 225 (H⁺ form; 50–100 mesh) column on top of a Dowex 1 (acetate form; 100–200 mesh) column; these were then washed with water until 20 ml was collected. One sample of this was counted for the yield of isotopically

labelled glucose and another was used for glucose analysis by the procedure of Slein (1965).

DL-[3-¹⁴C]Aspartate was obtained from Dhom (North Hollywood, Calif., U.S.A.). L-[U-¹⁴C]Malate, [2,3-¹⁴C]- and [1,4-¹⁴C]-succinate were obtained from Amersham/Searle (Arlington Hts., Ill., U.S.A.). [5-¹⁴C]- and [4-¹⁴C]-Citrate were synthesized enzymically from [1-¹⁴C]- and [2-¹⁴C]-acetate by using acetate kinase, phosphotransacetylase and citrate synthase. L-[3-¹⁴C]Malate was made from DL-[3-¹⁴C]-aspartate by using glutamate oxalacetate transaminase and malate dehydrogenase. *p*-Iodobenzylmalonate was obtained from K & K Laboratories (Hollywood, Calif., U.S.A.). *n*-Butylmalonate came from Aldrich Chemical Co. (San Leandro, Calif., U.S.A.). Other malate analogues came from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Results and Discussion

Using a simple model we have provided evidence that the rate of exchange of isotopically labelled malate between the cytosol and mitochondria is rapid in the intact kidney cortex (Rognstad, 1970). These experiments were done by using high concentrations of malate as a substrate, and it was possible that exchange rates were stimulated under these conditions. To estimate exchange rates with a more normal gluconeogenic substrate present, we have used tracer amounts (approx. 0.1 μmol) of ¹⁴C-labelled malate, which will enter the cell in the cytosolic space, and of ¹⁴C-labelled compounds that are precursors of mitochondrial malate, such as succinate, citrate and glutamate. From the various tracer compounds we have determined the isotopic yield in glucose, which is the primary end-product of cytosolic malate metabolism, and the isotopic yield in CO₂, which is the primary end-product of mitochondrial malate metabolism (with some ¹⁴CO₂ also

being produced in cytosolic reactions). If malate exchange between the cytosol and mitochondria were slow, marked differences should arise in the isotopic yields obtained from [^{14}C]malate and [^{14}C]succinate. ^{14}C from malate would largely go to glucose, while ^{14}C from succinate would label primarily CO_2 . If exchange rates were fast, the ratio of the isotopic yield in glucose to that in CO_2 would be similar from both compounds.

Table 1 shows that this ratio of isotopic yields is indeed about the same from ^{14}C -labelled malate and from ^{14}C -labelled substrates that are metabolized to malate in the mitochondria. This indicated that the malate exchange rate, even with normal physiological substrates such as lactate and fructose, is very fast. Table 1 also shows that the isotopic exchange rate does not appear to be affected by the presence of

1 mM-*p*-iodobenzylmalonate. This compound is an inhibitor of dicarboxylate anion transport in isolated mitochondria (Robinson & Williams, 1969).

Exchange reactions tend to make difficult the determination of pathways of net carbon flow by isotopic techniques, and a specific inhibitor of the malate exchange would be of value. We have tested the effect of various malate analogues on the exchange rate. However, as seen in Table 1, none of the compounds yet tested show significant effects on the isotopic exchange rate, while all show definite inhibitory effects on gluconeogenesis from lactate. The inhibition of gluconeogenesis is shown by the decreased isotopic yield of tracer in glucose, and is also found by analysis (results not shown). The increased yield of tracer in CO_2 probably does not reflect an increased rate of oxidation. In fact, inhibition of the

Table 1. *Isotopic yields in glucose and CO_2 from mitochondrial and cytosolic sources of malate*

Details of the incubation are given in the Methods section. All incubations contained 10 mM-acetate in addition to the substrates shown in the Table. About 1 μCi and about 0.1 μmol of labelled substrates was used in each flask.

Expt. no.	Unlabelled substrate and inhibitors	Labelled substrate	^{14}C yields (% of added ^{14}C)		Ratio of yields $\left(\frac{[\text{glucose}]}{[\text{CO}_2]}\right)$
			[Glucose]	[CO_2]	
K-170	L-Lactate (20 mM)	L-[U- ^{14}C]Malate	38.2	42.7	0.90
		[U- ^{14}C]Succinate	39.1	41.8	0.93
		[4,5- ^{14}C]Citrate	31.8	34.7	0.92
K-185	L-Lactate (10 mM)	L-[U- ^{14}C]Malate	41.3	43.3	0.95
		[U- ^{14}C]Succinate	38.2	39.9	0.96
		[4,5- ^{14}C]Citrate	34.0	35.6	0.95
	D-Fructose (10 mM)	L-[U- ^{14}C]Malate	23.2	62.3	0.73
		[U- ^{14}C]Succinate	22.0	60.3	0.73
		[4,5- ^{14}C]Citrate	15.7	51.9	0.38
K-210	L-Lactate (5 mM)+L-malate (5 mM)	L-[3- ^{14}C]Malate	49.0	22.8	2.15
		[2,3- ^{14}C]Succinate	47.8	21.9	2.18
K-197	L-Lactate (10 mM)	L-[U- ^{14}C]Malate	28.4	39.4	0.72
		[U- ^{14}C]Succinate	28.3	40.4	0.70
		[4,5- ^{14}C]Citrate	24.7	33.2	0.74
	L-Lactate (10 mM)+ <i>p</i> -iodobenzyl- malonate (1 mM)	L-[U- ^{14}C]Malate	22.0	45.3	0.49
		[U- ^{14}C]Succinate	22.6	45.8	0.49
		[4,5- ^{14}C]Citrate	15.9	32.2	0.49
K-206	L-Lactate (10 mM)	L-[U- ^{14}C]Malate	30.7	47.1	0.65
		[U- ^{14}C]Succinate	30.9	47.6	0.65
	L-Lactate (10 mM)+chloromalate (10 mM)	L-[U- ^{14}C]Malate	17.6	47.0	0.37
	L-Lactate (10 mM)+ β -methyl- malate (10 mM)	[U- ^{14}C]Succinate	20.2	52.3	0.39
		L-[U- ^{14}C]Malate	11.3	63.8	0.18
	L-Lactate (10 mM)+citramalate (10 mM)	[U- ^{14}C]Succinate	12.1	67.4	0.18
		L-[U- ^{14}C]Malate	13.7	65.8	0.21
	L-Lactate (10 mM)+mesotartrate (10 mM)	[U- ^{14}C]Succinate	11.2	68.7	0.16
		L-[U- ^{14}C]Malate	4.7	65.2	0.07
		[U- ^{14}C]Succinate	4.4	59.4	0.07

Table 2. *Relative isotopic yields in glucose and CO₂ as a function of time*

Incubations in parallel flasks contained 10mM-L-lactate plus 10mM-acetate as the unlabelled substrates. The kidney cortex segments (125mg wet wt.) were preincubated for 10min with the unlabelled substrates before injection of the tracer substrates into the medium. The incubations were terminated at the times (after addition of tracer) shown in the table.

Labelled substrate	Time (min)	¹⁴ C yields (% of added ¹⁴ C)		Ratio of yields $\left(\frac{[\text{Glucose}]}{[\text{CO}_2]}\right)$
		[Glucose]	[CO ₂]	
L-[U- ¹⁴ C]Malate	5	14.1	15.4	0.92
[U- ¹⁴ C]Succinate	5	16.7	18.0	0.93
L-[U- ¹⁴ C]Malate	10	21.7	24.5	0.89
[U- ¹⁴ C]Succinate	10	25.7	29.2	0.88
L-[U- ¹⁴ C]Malate	15	27.0	31.1	0.87
[U- ¹⁴ C]Succinate	15	31.5	37.3	0.85
L-[U- ¹⁴ C]Malate	30	30.5	36.7	0.83
[U- ¹⁴ C]Succinate	30	37.3	44.3	0.84
L-[U- ¹⁴ C]Malate	90	35.4	43.0	0.82
[U- ¹⁴ C]Succinate	90	37.8	46.1	0.82

tricarboxylic acid cycle by these compounds is the probable cause of the inhibition of gluconeogenesis. The increase of isotopic yield in CO₂ probably reflects (1) a relatively larger block in the competing synthetic pathway and (2) an increase in the mitochondrial oxaloacetate specific radioactivity because of decreased conversion of unlabelled pyruvate into oxaloacetate. At low concentrations, fluorocitrate, a known inhibitor of the tricarboxylic acid cycle, shows similar effects on isotopic CO₂ yields when lactate is the unlabelled substrate (R. Rognstad, unpublished work).

The data of Table 1 were taken from experiments of 2h duration. Utilization of the tracer-level substrates was found to be nearly complete in 30min (Table 2). In this experiment the ratio of isotopic yield in glucose to that in CO₂ from both L-[U-¹⁴C]-malate and [U-¹⁴C]succinate was measured in incubations of various time periods. Although the ratio decreased slightly as the incubations became longer, the ratios from both labelled substrates remained similar. It should perhaps be stressed that the approach used is basically different from the methods generally used in measuring rates of exchange of metabolites into and out of isolated mitochondria, which are dependent upon rapid and precise measurement of intra- and extra-mitochondrial isotope distribution in short-term experiments. Our approach is based on the continuous competition of pathways for the labelled metabolite involved and assumes a reasonably steady state. Thus, the duration of the experiment in principle is not particularly important. Absolute steady state is actually not required either, but it would then be important that the metabolism of the different isotopes added does not occur at

different times, e.g. that one is not metabolized perhaps entirely in an initial transient phase and the other slowly throughout the incubations. We have found that the rates of gluconeogenesis with kidney cortex segments are nearly linear over a 2h period, provided that the substrate is not exhausted.

As mentioned, *n*-butylmalonate and *p*-iodobenzylmalonate inhibit the malate-phosphate transport system in isolated mitochondria. *n*-Butylmalonate was used in studies with intact cells and its effects were interpreted on the basis of a specific inhibition at this transport step (Williamson *et al.*, 1970). Table 3 shows the effect of these compounds on gluconeogenesis in the kidney cortex from different substrates. They inhibit gluconeogenesis from pyruvate, which would be consistent with a block in malate transport, since malate outflow from the mitochondria is the accepted pathway. Gluconeogenesis from lactate is also inhibited. Since aspartate outflow is involved here (Rognstad & Katz, 1970), it is difficult to see why a specific inhibitor of malate transport should affect gluconeogenesis. Gluconeogenesis from L-malate is inhibited to a similar degree by these compounds. Here, where the gluconeogenic pathway occurs entirely in the cytosol, it is even more difficult to see why a specific inhibitor of malate transport between the mitochondria and cytosol should affect gluconeogenesis. It would be easier to explain these results on the basis of an inhibitory effect at another site, probably in the tricarboxylic acid cycle. Indeed, Robinson & Williams (1969) have shown that *n*-butylmalonate may have other inhibitory sites of action in isolated mitochondria. We urge that interpretations based on the use of these

Table 3. *Effect of n-butylmalonate and p-iodobenzylmalonate on gluconeogenesis in the kidney cortex*

Substrates were present at 10mM concentration together with 10mM-acetate.

Glucogenic substrate	<i>n</i> -Butylmalonate concn. (mM)	<i>p</i> -Iodobenzylmalonate concn. (mM)	Glucose formation (μ mol/125mg per 2h)	Percentage inhibition
L-Lactate	—	—	3.52	
L-Lactate	—	1.0	1.36	61
Pyruvate	—	—	2.94	
Pyruvate	—	1.0	1.83	39
L-Malate	—	—	3.47	
L-Malate	—	1.0	1.60	54
L-Lactate	—	—	3.31	
L-Lactate	10	—	1.98	40
Pyruvate	—	—	4.22	
Pyruvate	10	—	2.44	42
L-Malate	—	—	3.64	
L-Malate	10	—	1.85	49

compounds in the intact cell be considered highly tentative until further studies of the specificity of their inhibitory actions are made.

The rapid rate of exchange of isotopically labelled malate, as pointed out previously (Rognstad, 1970), will tend also to cause isotopic mixing of the mitochondrial and cytosolic NADH pools (in regard to tritium on C-4 of the nicotinamide). This follows because the mitochondrial and cytosolic malate dehydrogenase isoenzymes are very active and this will tend to bring the malate in each compartment close to equilibrium with the NADH in the space. Since the primary product of mitochondrial NAD³H metabolism is ³HHO, the malate exchange pathway will cause considerable ³HHO production even from compounds which form NAD³H in the cytosol, such as [2-³H]-glycerol. Production of ³HHO from these substrates thus by no means reflects simple net transport of cytosolic NAD³H into the mitochondria and oxidation there, as has been assumed (Carnicero *et al.*, 1972). In addition to this detritiation by an exchange pathway, marked detritiation also can occur in the cytosol by exchange pathways, e.g. the aldolase, triosephosphate isomerase system (Rose & Warms, 1969; Katz & Rognstad, 1966).

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