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TRANSFER OF REDUCING EQUIVALENTS ACROSS THE MITOCHONDRIAL MEMBRANE

I. HYDROGEN TRANSFER MECHANISMS INVOLVED IN THE REDUCTION OF PYRUVATE TO LACTATE IN ISOLATED LIVER CELLS

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SUMMARY

1. The reduction of pyruvate to lactate has been studied in isolated liver cells in order to elucidate the mechanims involved in the transfer of reducing equivalents from mitochondria to cytosol.

2. Manipulation of the cytosolic oxaloacetate concentration did not support the malate–oxaloacetate cycle as being responsible for the transfer of reducing equivalents out of the mitochondria: a. With pyruvate plus oleate present 2 mM Amytal caused a 10-fold decrease in the oxaloacetate concentration, but had only a small inhibitory effect on lactate production. Oleate was essential in order to prevent disintegration of the cells in the presence of Amytal. b. Quinolinate, an inhibitor of phosphoenolpyruvate carboxylase (GTP : oxaloacetate carboxylyase, transphosphorylating, EC 4.1.1.32), caused a several-fold increase in the oxaloacetate concentration but inhibited lactate production from pyruvate; this was accompanied by an increased reduction of mitochondrial pyridine nucleotides.

3. *p*-Chlorophenyl pyruvate, an inhibitor of pyruvate carboxylase (pyruvate: carbondioxide ligase, ADP, EC 6.4.1.1), also inhibited lactate production from pyruvate.

4. It is postulated that with pyruvate as substrate, recycling of carbon via pyruvate carboxylase, phosphoenolpyruvate carboxylase and pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) is an important, energy-requiring, mechanism for the transfer of the proportion of NADH not directly associated with gluconeogenesis.

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INTRODUCTION

Since the original observation of Lehninger [1] that the mitochondrial membrane is impermeable to NADH, a number of pathways have been proposed for the transfer of reducing equivalents across the mitochondrial membrane (see refs 2 and 3 for reviews). In mammalian tissues the experimental evidence indicates that malate plays an important role as carrier of reducing equivalents across the mitochondrial membrane in both directions [4–11]. Thus it has been concluded that in rat liver and rat heart the transfer of NADH from cytosol to mitochondria occurs predominantly via the malate–aspartate cycle, first proposed by Borst [2]. This conclusion is based on the observation that transaminase inhibitors like cycloserine and aminooxyacetate inhibit processes like ethanol [9] and lactate oxidation [6, 7].

When both reducing equivalents and the C_4 carbon skeleton must be exported from the mitochondria for extramitochondrial synthetic purposes, as in the case of gluconeogenesis from pyruvate, it is probable that a net efflux of malate from the mitochondria occurs [6, 8, 11]. When only a net transfer of NADH from mitochondria to cytosol is required without a concomitant net transfer of carbon, a pathway can be visualized in which malate leaves the mitochondria and returns as oxaloacetate, thus completing a malate–oxaloacetate cycle [12].

A restriction of the malate–oxaloacetate cycle is imposed by the limited permeability of the mitochondrial membrane towards external oxaloacetate [13, 14], although at high oxaloacetate concentrations high transport rates can be obtained [15]. This complication is avoided if oxaloacetate is transported into the mitochondria as aspartate. However, the movement of aspartate in energized mitochondria is essentially unidirectional and directed out of the mitochondria [16, 17], thus making the malate–aspartate cycle functional only in the direction of inward transfer of reducing equivalents. Indeed, no inhibition of lactate production from pyruvate is found in isolated liver cells upon addition of difluorooxaloacetate, a transaminase inhibitor [10]. Furthermore, lactate production from pyruvate in perfused hearts is not affected by addition of aminooxyacetate [18]. With lactate as substrate, however, aminooxyacetate inhibits lactate uptake by more than 70 % [18]. Berry and Kun [10] observed that fluoromalate, an inhibitor of both malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.77) and of the dicarboxylate translocator in mitochondria, inhibits the reduction of pyruvate to lactate in isolated liver cells. They concluded that the malate–oxaloacetate cycle is partly responsible for the net transfer of NADH from mitochondria to cytosol.

Gimpel et al. [15] reported a K_m of 80–130 μM and a V of 80–270 nmoles/min per mg protein at 30 °C for oxaloacetate translocation in isolated rat liver mitochondria. Since in rat liver in vivo the concentration of oxaloacetate is 10 μM or lower [19]. transport rates of maximally 1.8 μmoles/min per g wet weight can be calculated (assuming that 1 g wet weight is equivalent to 60 mg mitochondrial protein [20]). If isolated liver cells are incubated with pyruvate, the rate of lactate production is 1–2 μmoles/min per g wet weight at 37 °C (this paper; see also ref. 10). Thus, if the transfer of reducing equivalents from mitochondria to cytosol is mediated by the malate–oxaloacetate cycle, the oxaloacetate concentration in the cytosol must be at least 10 μM. Therefore, on kinetic grounds, it is to be expected that variations in the cytosolic oxaloacetate concentration would affect the rate of hydrogen transport

out of the mitochondria. In the experiments to be described the concentration of oxaloacetate in the cytosol was decreased by adding Amytal and increased by adding quinolinate, an inhibitor of phosphoenolpyruvate carboxylase (GTP : oxaloacetate carboxylyase, transphosphorylating, EC 4.1.1.32) [21]. The results indicate that a simple malate–oxaloacetate cycle occurs only to a limited extent, if at all. Instead, a pathway for NADH transfer from mitochondria to cytosol is proposed which involves recycling of pyruvate between pyruvate carboxylase, phosphoenolpyruvate carboxylase and pyruvate kinase; this pathway is therefore energy dependent.

METHODS AND MATERIALS

Isolation of liver cells

Liver cells were prepared from livers of 20–24-h-fasted male rats (Sprague–Dawley) weighing 180–220 g, according to the procedure of Berry and Friend [22], as modified by Johnson et al. [23]. The perfusion technique used was the same as described by Williamson et al. [24]. The cells were used immediately after isolation.

Incubation conditions

The cells were suspended in Krebs–Ringer bicarbonate buffer (pH 7.4) fortified with 4 % (w/v) defatted serum albumin. Usually an amount of cells equivalent to 20–50 mg wet weight per ml of incubation medium was used. All results are expressed per g wet weight, using the factor 3.77 for conversion of the trichloroacetic acid-insoluble dry weight to wet weight [10]. The incubation volume was 2.5–4 ml in 25-ml plastic Erlenmeyer flasks which were gassed with 95 % O_2 plus 5 % CO_2, stoppered, and shaken vigorously at 37 °C. Aliquots of the cell suspension were removed at 15 or 20 min intervals and added to $HClO_4$ (final concentration, 3.5 %). After removing the denatured protein by centrifugation in the cold, the pH of the supernatant was adjusted to about 6.5 with 6 M KOH plus 0.5 M PIPES (piperazine-*N*-*N*′-bis[2-ethanesulphonic acid]) and recentrifuged to remove the precipitated $KClO_4$. In some cases the reaction was terminated by centrifugation of the cells into 14 % $HClO_4$ (w/v) through a layer of silicone oil (specific gravity 1.05) essentially as described for the separation of mitochondria from a medium [25]. For proper separation of the cells, it was found necessary to add dextran (mol. wt 117 000) in a final concentration of 60 mg/ml prior to centrifugation of the cells.

Assays

Metabolites were determined according to standard enzymic procedures [26,27]. Oxaloacetate was measured within 2 h after neutralization of the samples. Extracellular lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) was routinely measured in the different incubations at the end of the experiment after centrifuging the cells at $50 \times g$ for 2 min. To determine total lactate dehydrogenase, either sonicated or homogenized cells were used. Extracellular lactate dehydrogenase was also measured in the stock suspension of the cells kept at 0 °C.

Chemicals and enzymes

Collagenase was obtained from Worthington Biochemical Corp., and hyaluronidase from Sigma Chemical Co. Defatted serum albumin (Pentex, Fraction V)

from Miles Laboratories, Inc., was dialysed against three changes of Krebs–Ringer bicarbonate buffer (from which $CaCl_2$ was omitted) prior to use in the Ca^{2+}-free preperfusion of the liver with collagenase (EC 3.4.4.19) and hyaluronidase (EC 3.3.1.35), and also in the various incubations. In the latter case, extra Ca^{2+} was added to correct for the absence of Ca^{2+} in the albumin solution which was added as a 20 % (w/v) solution. Quinolinate was obtained from Aldrich Chemical Co. *p*-Chlorophenylpyruvate was obtained from Smith, Kline and French Laboratories. *n*-Butylmalonate was prepared from the diethyl ester as described by Vogel [28]. Sodium amobarbital (Amytal) was obtained from Eli Lilly and Co. All enzymes and cofactors were purchased from Sigma Chemical Co. or Boehringer Mannheim Corp.

RESULTS

The effect of Amytal on lactate production from pyruvate

In Table I the effect of increasing concentrations of Amytal on the metabolism of pyruvate is shown. Oleate was present. At 2 mM Amytal, the β-hydroxybutyrate/acetoacetate ratio increased from 0.49–0.71, and glucose production and the level of ATP were greatly depressed, while that of ADP did not change significantly. Lactate production, however, was only slightly inhibited by this concentration of Amytal, despite the fact that the oxaloacetate concentration decreased more than 10-fold (see further). It was only at concentrations of Amytal of 4 mM or higher that lactate formation decreased significantly. However, at these high concentrations of the inhibitor, considerable leakage of lactate dehydrogenase took place, indicating that the cells were damaged.

Similar results were obtained in the absence of oleate (Table II), except that the inhibitory effects of Amytal became evident at lower Amytal concentration. Also the leakage of lactate dehydrogenase from the cells took place at a lower Amytal concentration. As in the presence of oleate, addition of Amytal led to an increased

TABLE I

THE EFFECT OF AMYTAL ON THE METABOLISM OF PYRUVATE IN THE PRESENCE OF OLEATE

Rat-liver cells were incubated as indicated in Materials and Methods in the presence of 10 mM pyruvate, 0.5 mM oleate and Amytal at the concentrations indicated. Reaction time, 30 min. β-OH, β-hydroxybutyrate; Ac. ac., acetoacetate; OAA, oxaloacetate; LDH, lactate dehydrogenase. The leakage of lactate dehydrogenase from the stock suspension of cells after 60 min storage at 0 °C was 9 %.

Amytal (mM)	ΔGlucose (μmoles/g wet wt)	ΔLactate (μmoles/g wet wt)	ATP (μmoles/g wet wt)	ADP (μmoles/g wet wt)	Malate (μmoles/g wet wt)	OAA (μmoles/g wet wt)	β-OH/ Ac.ac.	% leakage of LDH
0	12.9	32.0	2.12	0.77	1.00	0.011	0.49	9
1	8.9	28.1	1.81	1.00	1.13	0.007	0.53	11
2	4.7	25.1	1.31	0.89	0.63	< 0.001	0.71	11
4	1.8	15.7	0.59	1.10	0.51	< 0.001	2.50	21
6	1.6	11.9	0.18	0.80	0.52	< 0.001	5.62	38

TABLE II

THE EFFECT OF AMYTAL ON THE METABOLISM OF PYRUVATE

Rat-liver cells were incubated as indicated in Methods and Materials in the presence of 10 mM pyruvate and Amytal at the concentrations indicated. Reaction time, 30 min. Abbreviations: Ac.ac., acetoacetate; β-OH, β-hydroxybutyrate.

Amytal (mM)	ΔGlucose (μmoles/g wet wt)	ΔLactate (μmoles/g wet wt)	ATP (μmoles/g wet wt)	ADP (μmoles/g wet wt)	β-OH/ Ac.ac.	% leakage of LDH
0	15.9	33.4	2.21	0.72	0.35	20
1	5.3	30.8	1.56	1.19	0.55	20
2	1.0	16.1	0.45	1.63	1.31	41
4	1.1	12.0	0.18	0.65	3.95	69
6	0.6	9.0	<0.01	0.48	7.22	77

β-hydroxybutyrate/acetoacetate ratio, indicating a more reduced state of the mitochondria, as expected. As the Amytal concentration was raised to 2 mM the level of ATP decreased while that of ADP increased. At still higher levels of Amytal not only ATP but also ADP decreased. In the same experiment it was found that with 6 mM Amytal present total NAD decreased from a control value of 774 to 408 nmoles/g wet weight and total NADP from 422 to 267 nmoles/g wet weight. Separate experiments (not shown) indicated that the extracellular fluid contained sufficient NADase activity to destroy the NAD and NADP that might have leaked from the cells, thus accounting for the decrease in total NAD(P) at high Amytal concentration.

In addition to the increased leakage of lactate dehydrogenase and loss of pyridine nucleotides, microscopic inspection of the cells revealed severe disruption of the plasma membrane at high Amytal concentrations. Rupture of the plasma membrane may also be partially responsible for the high inhibition of lactate production from pyruvate under energy-poor conditions in the experiments of Berry [29] (see also Discussion).

In the experiment carried out in the presence of oleate (Table I) malate and oxaloacetate levels were measured. The levels of these metabolites, particularly oxaloacetate, decreased as the Amytal concentration was increased to 2 mM or higher. Although the oxaloacetate concentration decreased from 11 to < 1 nmole/g wet weight in the presence of 2 mM Amytal, lactate production decreased by only 25 %.

Using the kinetic data of Gimpel et al. [15], it can be calculated that in the presence of 2 mM Amytal, when the concentration of oxaloacetate is 1 μM or less (see Table I), the malate–oxaloacetate cycle can account for 25 % or less of the lactate formed. Moreover, it should be pointed out that the K_m value for oxaloacetate translocation in mitochondria as reported by Gimple et al. [15] was measured in the absence of competing anions. The results of the experiments reported in this section, then, lead us to conclude that mechanisms other than the malate–oxaloacetate cycle must be responsible for the transfer of reducing equivalents from the mitochondria to the cytosol under these conditions.

The effect of quinolinate on pyruvate metabolism

In another series of experiments the concentration of oxaloacetate in the cyto-

TABLE III

THE EFFECT OF QUINOLINATE ON PYRUVATE METABOLISM

Rat-liver cells were incubated as indicated in Methods and Materials with 10 mM pyruvate and quinolinate at the concentrations indicated. The incubations were terminated by centrifugation–filtration of the cells through silicone oil (see Methods). Reaction time, 30 min, ATP, ADP, malate and oxaloacetate were measured in the cell extract. Glucose, lactate and the ketone bodies were measured in the extracellular fluid. Abbreviations: these are the same as in Tables I and II. The leakage of lactate dehydrogenase from the stock suspension of cells after storage at 0 °C for 60 min was 16 %.

Quinolinate (mM)	ΔGlucose (μmoles/g wet wt)	ΔLactate (μmoles/g wet wt)	Malate (μmoles/g wet wt)	OAA (μmoles/g wet wt)	ADP (μmoles/g wet wt)	ATP/ ADP	Malate/ OAA	β-OH/ Ac.ac.	% leakage of LDH
0	22.7 (21.2±0.9)*	52.2 (47.0±3.4)*	0.30	< 0.002	0.71	5.17	> 300	0.22	21
1.5	20.3	45.1	0.49	0.003	0.70	5.91	163	0.22	12
3	19.9	42.9	0.60	0.013	0.73	5.10	46	0.37	16
5	18.1	36.2	0.73	0.026	0.66	4.86	28	0.32	18
7.5	15.5 (15.5±0.9)**	30.9 (33.4±4.5)**	0.85	0.030	0.66	5.20	28	0.36	21

* Numbers in parenthesis are the means ± S.E. of 11 separate experiments.

** Numbers in parenthesis are the means ± S.E. of 4 separate experiments.

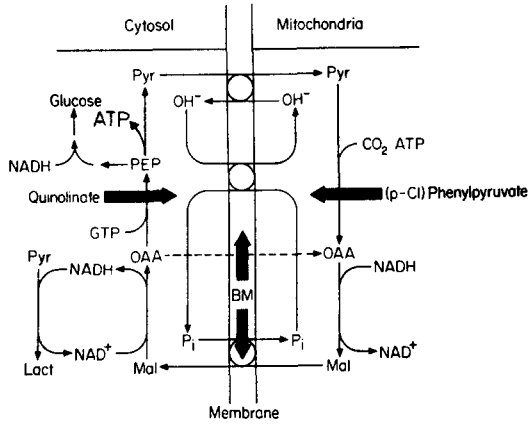

Fig. 1. Mechanism for the transport of reducing equivalents from mitochondria to cytosol. Sites of inhibition by quinolinate, *p*-chlorophenylpyruvate and butylmalonate are depicted by the solid arrows. Evidence for an exchange of pyruvate with hydroxyl has been obtained by Papa et al. [32] (see also Brouwer et al. [33]; contrast ref. 34). OAA, oxaloacetate.

sol was increased by addition of quinolinate (Table III). In order to determine if the increased oxaloacetate was truly intracellular, the incubations were terminated by centrifuging the cells through silicone oil into an acid layer (see Methods section).

As expected, increasing concentrations of quinolinate caused a graded inhibition of glucose production, although the inhibition was never complete (cf. ref. 30), even after prolonged incubation (not shown). The decrease in glucose production was accompanied by a large increase in malate and oxaloacetate levels (Table III; cf. ref. 21). Despite the increase in intracellular oxaloacetate levels from 2 to about 30 nmoles/g wet weight, a decrease in lactate production was also observed. From the fact that the β-hydroxybutyrate/acetoacetate ratio increased and the total malate/oxaloacetate ratio decreased upon addition of quinolinate, it can be concluded that transport of reducing equivalents out of the mitochondria is inhibited by quinolinate. The levels of ATP and ADP did not change significantly with quinolinate (Table III), indicating the absence of any non-specific effect of the inhibitor on energy metabolism. Also, the amount of lactate dehydrogenase found in the extracellular fluid at the end of the incubations was not altered by quinolinate*.

These results strongly suggest that cycling of carbon via pyruvate carboxylase, phosphoenolpyruvate carboxylase and pyruvate kinase is responsible for transport of hydrogen from mitochondria to cytosol (see Fig. 1). Although this process has often been indicated as 'futile' or useless cycling, it is clear from Fig. 1 that the net effect is an energy-dependent transfer of reducing equivalents from mitochondria to cytosol (see also ref. 35).

* Klahr and Schoolwerth [31] have suggested that quinolinate inhibits not only phosphoenolpyruvate carboxylase but also glutamate dehydrogenase in kidney. However, we have evidence that quinolinate does not affect glutamate dehydrogenase in intact liver cells (Meijer, A. J., Ohkawa, K. and Williamson, J. R., in preparation).

The effect of p-chlorophenylpyruvate on pyruvate metabolism

In order to test further if the cycle shown in Fig. 1, operates, the effect of *p*-chlorophenylpyruvate*, an inhibitor of pyruvate carboxylase, was tested. Since data concerning the specificity of *p*-chlorophenylpyruvate in intact cells were not available, the effect of the inhibitor was studied in various systems.

Table IV shows that *p*-chlorophenylpyruvate inhibited gluconeogenesis in the presence of pyruvate or alanine. In these systems the conversion of pyruvate to oxaloacetate is an essential step in the chain of reactions leading to formation of glucose. Indeed, parallel to the inhibition of gluconeogenesis, malate levels decreased, which is consistent with inhibition at the level of pyruvate carboxylase. With glutamine as the substrate, where pyruvate carboxylase is not involved in gluconeogenesis, *p*-chlorophenylpyruvate did not inhibit glucose formation. It can therefore be concluded that of the enzymes involved in gluconeogenesis only pyruvate carboxylase is affected by *p*-chloropenylpyruvate.

TABLE IV

THE EFFECT OF *p*-CHLOROPHENYLPYRUVATE ON GLUCONEOGENESIS AND ON THE TRANSFER OF REDUCING EQUIVALENTS FROM MITOCHONDRIA TO CYTOSOL

Rat-liver cells were incubated as indicated in Materials and Methods with 1 mM oleate and with 10 mM pyruvate, 10 mM alanine or 10 mM glutamine where present. In addition 3 mM ornithine was added with each of the latter two substrates. *p*-Chlorophenylpyruvate was added at the concentrations indicated .Reaction time, 30 min. Abbreviations: Pyr, pyruvate; Ala, alanine; Gln, glutamine; Cløpyr, *p*-chlorophenylpyruvate.

Additions	ΔGlucose (μmoles/g wet wt)	ΔLactate (μmoles/g wet wt)	ATP (μmoles/g wet wt)	Malate (μmoles/g wet wt)
Pyr	31.2	85.0	2.41	1.58
Pyr + 3 mM Cløpyr	23.7	69.3	2.20	0.97
Pyr + 5 mM Cløpyr	17.0	58.7	2.10	0.61
Ala	20.1	2.4	2.25	0.10
Ala + 3 mM Cløpyr	13.0	5.9	2.37	0.08
Ala + 5 mM Cløpyr	8.6	5.2	2.24	0.04
Gln	13.2	–	2.21	0.30
Gln + 3 mM Cløpyr	16.1	–	2.06	0.69
Gln + 5 mM Cløpyr	15.5	–	1.93	0.72
None	1.2	–	2.09	0.01

p-Chlorophenylpyruvate significantly inhibited lactate production from pyruvate (Table IV), indicating that pyruvate carboxylase is involved in the transfer of reducing equivalents from the mitochondria to the cytosol (see Fig. 1).

* *p*-Chlorophenylpyruvate was chosen rather than phenylpyruvate (cf. ref. 36) since at concentrations of 5 mM or higher the latter compound occasionally depressed ATP levels (not shown).

DISCUSSION

Experimental evidence in favor of malate as carrier of reducing equivalents from the mitochondria to the cytosol is given by the observation that gluconeogenesis from pyruvate is inhibited by butylmalonate [5], an inhibitor of the dicarboxylate translocator in mitochondria [37], and that the reduction of pyruvate to lactate (in the absence of a cytosolic hydrogen donor) is inhibited by fluoromalate [10] or butylmalonate [38]. In the cytosol malate is oxidized to oxaloacetate. The question arises of whether oxaloacetate itself is transported back to the mitochondria or some product formed from it. It cannot be aspartate since this compound is not readily transported into the mitochondria [16, 17].

Berry [29] was the first to observe that rotenone, uncouples and inhibitors of the energy-conserving system, inhibit the reduction of pyruvate to lactate in isolated liver cells. He concluded that the transport of reducing equivalents from mitochondria to cytosol may be energy dependent.

We have found that addition of high concentrations of Amytal to cells incubated with pyruvate leads to decreased lactate production. However, this inhibition was accompanied by partial disintegration of the cells as shown by leakage of lactate dehydrogenase into the medium and by a decrease in the NAD and NADP pools. In other experiments we observed a similar disintegration of the cells when they were incubated in the presence of rotenone or arsenite. It is important to note that high concentrations of Amytal have been found to inhibit Na^+ and K^+ transport in rat liver slices [39]. It is conceivable that a disturbed cation balance across the plasma membrane due to energy deficiency initiates structural changes eventually leading to disruption of the cell.

When oleate was present to protect against cell damage, it was found that low Amytal concentrations depressed the oxaloacetate concentration by a factor of more than 10, but decreased lactate production only slightly. We consider this result to be inconsistent with the malate–oxaloacetate cycle as an important mechanism for hydrogen transfer from mitochondria to cytosol under these conditions.

Addition of quinolinate led to inhibition of transport of reducing equivalents from mitochondria to cytosol, leading us to postulate the pathway of hydrogen transport depicted in Fig. 1.

Recycling of carbon via pyruvate carboxylase, phosphoenolpyruvate carboxylase and pyruvate kinase can be considerable in kidney [35] and liver ([40], depending on the nutritional conditions [40]. Friedman et al. [40] found that when livers from fasted rats were perfused with [2-^{14}C]pyruvate an amount of lactate equal to that of the glucose formed was due to operation of this recycling process. Since in our experiments (like in those of Friedman et al. [40]). lactate production was 2-3 times the glucose formed, it may be estimated that about 40 % of the lactate produced under these conditions is due to the activity of this cycle, which is in good agreement with the 30–40 % inhibition of lactate production obtained with quinolinate and with *p*-chlorophenylpyruvate. It must be stressed, however, that the inhibitors certainly did not completely inhibit all phosphoenolpyruvate carboxylase or pyruvate carboxylase activity, respectively, as evidenced by the fact that inhibition of glucose production was only partial. Thus under our experimental conditions, the contribution of the recycling process to the transport of reducing equivalents from mitochondria to cytosol not directly connected to gluconeogenesis, may be even greater than 40 %.

It should be pointed out that the recycling process of Fig. 1 also involves malate dehydrogenase and the transport of malate across the mitochondrial membrane. Therefore, the inhibition of lactate production from pyruvate by fluoromalate, an inhibitor of malate dehydrogenase and of the dicarboxylate translocator of the mitochondrial inner membrane [10], cannot be taken as evidence in favor of the malate–oxaloacetate cycle. Similarly, butyl malonate, an inhibitor of both the malate–phosphate [41] (an essential step in the recycling process; see Fig. 1) and malate–oxaloacetate [15] exchanges across the mitochondrial inner membrane, cannot be used to distinguish the two pathways of hydrogen transfer. In several experiments (see ref. 38), we found an average inhibition of about 45 % of lactate production from pyruvate by 5 mM butylmalonate. This percentage inhibition agrees rather well with the 36 % inhibition by fluoromalate observed by Berry and Kun [10], and could be taken as evidence of both the recycling process and the malate–oxaloacetate cycle acting together. However, from the fact that the percentage inhibition by butyl malonate or fluororomalate is so close to that observed with either quinolinate (Table III) or *p*-chlorophenylpyruvate (Table IV), it may be concluded that the activity of the malate–oxaloacetate cycle is negligible under our experimental conditions.

It has been found recently [42] that in pigeon liver, pyruvate in contrast to lactate [43], is a rather poor substrate for gluconeogenesis. Since in this species phosphenolpyruvate carboxylase is mainly mitochondrial [44], transport of reducing equivalents together with the transport of phosphoenolpyruvate out of the mitochondria is obligatory when pyruvate is the substrate. The fact that lactate is a good gluconeogenic substrate in pigeon liver, whereas pyruvate is not, shows that hydrogen transfer out of the mitochondria must be defective in this tissue. An explanation for this phenomenon may be that the cycle depicted in Fig. 1 cannot be used in pigeon liver to replenish the cytosol with reducing equivalents because of the low activity of cytosolic phosphoenolpyruvate carboxylase. Apparently in pigeon liver no mechanisms other than that depicted in Fig. 1 are available to bring about a net transfer of reducing equivalents from mitochondria to cytosol.

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REFERENCES

1 Lehninger, A. L. (1951) J. Biol. Chem. 190, 345–359
2 Borst, P. (1963) in Functionelle und Morphologische Organisation der Zelle (Karlson, P., ed.), pp. 137–162, Springer-Verlag, Berlin
3 Greville, G. D. (1969) in Citric Acid Cycle Control and Compartmentation (Lowenstein, J. M., ed.), pp. 1–136, Marcel Dekker, New York
4 Krebs, H. A., Gascoyne, T. and Notton, B. M. (1967) Biochem. J. 102, 275–282
5 Williamson, J. R., Anderson, J. and Browning, E. T. (1970) J. Biol. Chem. 245, 1717–1726

6 Anderson, J. H., Nicklas, W. J., Blank, B., Refino, C. and Williamson, J. R. (1971) in Regulation of Gluconeogenesis: 9th Conference of the Gesellschaft für Biologische Chemie (Söling, H.-D. and Willms, B., eds), pp. 293–315, Thieme-Verlag, Stuttgart and Academic Press, New York, London
7 Safer, B. and Williamson, J. R. (1972) in Recent Advances in Studies on Cardiac Structure and Metabolism (Bajusz, E. and Rona, G., eds), Vol. 1, 'Myocardiology', pp. 34–43, Universtiy Park Press, Baltimore
8 Rognstad, R. and Katz, J. (1970) Biochem. J. 116, 483–491
9 Ylikahri, R. H., Hassinen, I. and Kähönen, M. T. (1970) Biochem. Biophys. Res. Commun. 44, 150–156
10 Berry, M. N. and Kun, E. (1972) Eur. J. Biochem. 27, 395–400
11 Arinze, I. J., Garber, A. J. and Hanson, R. W. (1973) J. Biol. Chem. 248, 2266–2274
12 Bücher, Th. and Klingenberg. M. (1958) Angew. Chem. 70, 552–570
13 Chappell, J. B. (1961) in Biological Structure and Function (Goodwin, T. W. and Lindberg, O., eds), Vol. 2, pp. 71–83, Academic Press, London
14 Haslam, J. M. and Krebs, H. A. (1968) Biochem. J. 107, 659–667
15 Gimpel, J. A., De Haan, E. J. and Tager, J. M. (1973) Biochim. Biophys. Acta 292, 582–591
16 LaNoue, K. F. and Williamson, J. R. (1971) Metabolism 20, 119–140
17 LaNoue, K. F., Wałajtys, E. I. and Williamson, J. R. (1973) J. Biol. Chem. 248, 7171–7183
18 Safer, B., Smith, C. and Williamson, J. R. (1971) J. Mol. Cell. Cardiol. 2, 111–124
19 Williamson, J. R. (1969) in Energy Level and Metabolic Control in Mitochondria (Papa, S., Tager, J. M., Quagliariello, E. and Slater, E. C., eds), pp. 385–400, Adriatica Editrice, Bari
20 Scholz, R. and Bücher, Th. (1965) in Control of Energy Metabolism (Chance, B., Estabrook, R. W. and Williamson, J. R., eds), pp. 393–414, Academic Press, New York and London
21 Veneziale, C. M., Walter, P. Kneer, N. and Lardy, H. A. (1967) Biochemistry 6, 2129–2138
22 Berry, M. N. and Friend, D. S. (1969) J. Cell. Biol. 43, 506–520
23 Johnson, M. E. M., Das, N. M., Butcher, F. R. and Fain, J. N. (1972) J. Biol. Chem. 247, 3229–3235
24 Williamson, J. R., Browning, E. T. and Scholz, R. (1969) J. Biol. Chem. 244, 4607–4616
25 Meijer, A. J. (1971) Anion Translocation in Mitochondria, Ph. D. Thesis, University of Amsterdam, Academic Service, Amsterdam
26 Williamson, J. R. and Corkey, B. E. (1969) Methods in Enzymol. 13, 434–513
27 Bergmeyer, H. U. (1970) Methoden der Enzymatischen Analyse, Verlag Chemie, Weinheim
28 Vogel, A. I. (1961) A Textbook of Practical Organic Chemistry, 3rd edn, pp. 488–489, Spottiswoode, Ballantyne and Comp., Ltd, London
29 Berry, M. N. (1971) Biochem. Biophys. Res. Commun., 44, 1449–1456
30 Veneziale, C. M., Gabrielli, F. and Lardy, H. A. (1970) Biochemistry 9, 3960–3970
31 Klahr, S. and Schoolwerth, A. C. (1972) Biochim. Biophys. Acta 279, 157–162
32 Papa, S., Francavilla, A., Paradies, G. and Meduri, B. (1971) FEBS Lett. 12, 285–288
33 Brouwer, A., Smits, G. G., Tas, J., Meijer, A. J. and Tager, J. M. (1973) Biochimie, 55, 717–725
34 Zahlten, R. N., Hochberg, A. A., Stratman, F. W. and Lardy, H. A. (1972) FEBS Lett. 21, 11–13
35 Rognstad, R. and Katz, J. (1972) J. Biol. Chem. 247, 6047–6054
36 Weiss, G., Ohly, B., Brod, H. and Seubert, W. (1971) in Regulation of Gluconeogenesis: 9th Conference of the Gesellschaft für Biologische Chemie (Söling, H.-D. and Willms, B., eds). pp. 29–41, Thieme-Verlag, Stuttgart and Academic Press, New York and London
37 Robinson, B. H. and Chappell, J. B. (1967) Biochem. Biophys. Res. Commun. 28, 249–255
38 Williamson, J. R., Meijer, A. J. and Ohkawa, K. (1973) in Regulation of Hepatic Metabolism: Alfred Benzon Symp. VI (Lundquist, F., Tygstrup, N. and Thaysen, J. H., eds), Munksgaard, Copenhagen, in the press
39 Van Rossum, G. D. V. (1972) Biochem. J. 129, 427–438
40 Friedman, B., Goodman, E. H., Saunders, H. L., Kostos, V. and Weinhouse, S. (1971) Arch. Biochem. Biophys. 143, 566–578
41 Meijer, A. J. and Tager, J. M. (1969) Biochim. Biophys. Acta 189, 136–139
42 Söling, H.-D. (1973) in Regulation of Hepatic Metabolism: Alfred Benzon Symp. VI (Lundquist, F., Tygstrup, N. and Thaysen, J. H., eds), Munksgaard, Copenhagen, in the press
43 Söling, H.-D., Willms, B., Kleineke, J. and Gehlhoff, M. (1970) Eur. J. Biochem. 16, 289–302
44 Lardy, H. A. (1966) Harvey Lectures 60, 261–278