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Metabolism of Exogenous Oxaloacetate in Isolated Hepatocytes (41195)

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Abstract. The utilization of exogenous oxaloacetate (OAA) was studied in isolated rat liver parenchymal cells. Phenylpyruvate and α-cyanohydroxycinnamate, which inhibit pyruvate carboxylase (EC 6.4.1.1) and pyruvate transport across the mitochondrial membrane, respectively, were equally effective in decreasing glucose formation from pyruvate and OAA. Additionally, the specific activity of glucose formed in cells incubated with sodium [^{14}C]bicarbonate and either pyruvate or OAA was the same. The specific activity of glucose formed from asparagine plus sodium [^{14}C]bicarbonate was approximately 25% of that observed when pyruvate or OAA served as the carbon sources. The results suggest that OAA is decarboxylated to pyruvate prior to its metabolism.

The cellular membrane of the liver parenchymal cell is selectively permeable to a number of physiologically important compounds. Lactate, pyruvate, and many amino acids are readily converted to glucose in the perfused liver, indicating that they are able to pass freely through the plasma membrane (1). Based on the low rates of gluconeogenesis from malate, aspartate, and glutamate, the plasma membrane of the liver parenchymal cell has generally been considered impermeable to dicarboxylic acids (1, 2).

Glucose formation from oxaloacetate was equal to that seen from pyruvate in the perfused liver (1, 3), suggesting that the liver cell membrane is permeable to that dicarboxylic acid. However, Exton and Park (3) observed that lactate and malate accumulation from oxaloacetate was approximately the same as that observed from pyruvate. Exton and Park (3) maintained that if oxaloacetate was entering the cell intact, then lactate production would be lower, and malate accumulation greater than that observed from pyruvate. Consequently, they concluded that oxaloacetate was not entering the parenchymal cell as such, but was metabolized in the cell only after decarboxylation to pyruvate. It is not clear whether this decarboxylation was presumed to occur extracellularly or at the cell membrane.

Recent studies have cast doubt on the conclusions of Exton and Park (3). The high levels of lactate formation in livers perfused with oxaloacetate could be accounted for by the recycling of the phosphoenolypyruvate derived from oxaloacetate to pyruvate, which could then be converted to oxaloacetate within the mitochondria (4, 5). Subsequent malate efflux would provide reducing equivalents for both glucose and lactate production (5, 6). It is apparent that if the permeability of the cellular membrane to oxaloacetate is low, then malate accumulation during oxaloacetate metabolism would be unlikely. The reducing equivalents provided by mitochondrially derived malate would be utilized for glucose and lactate production.

It is therefore possible that oxaloacetate is metabolized as such by isolated hepatocytes after first crossing the cellular membrane. The object of this study was to investigate this possibility further.

Materials and Methods. Male Sprague–Dawley rats (300–350 g), fed a commercial laboratory chow *ad libitum,* were used throughout the study. Feed was withheld from the animals 48 hr prior to experimentation.

Isolation of hepatocytes. Rat liver parenchymal cells were isolated by the method of Berry and Friend as modified by Krebs *et al.* (7, 8). Cells were resuspended in 2.5% bovine serum albumin at a 1:17 dilution (w/v). The hepatocytes were incubated with the indicated substrates in 25-ml Ehrlenmyer flasks at 37° in a total volume of 4 ml.

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Incubations were terminated with 0.3 ml 60% $HClO_4$, and the denatured protein was pelleted by centrifugation. The supernatants were neutralized with 0.6 ml 3.0 M K_2CO_3:0.5 M triethanolamine. When substrates included sodium [^{14}C]bicarbonate, the supernatants were neutralized with approximately 0.6 ml 30% KOH. The neutralized supernatants were utilized for the various assays.

Metabolite determinations. Glucose production was measured by the method of Krebs *et al.* (9). Pyruvate and oxaloacetate were measured spectrophotometrically by following the decrease in absorbance at 340 nm after the addition of approximately 10 U of either lactate dehydrogenase (EC 1.1.1.27) or malate dehydrogenase (E.C. 1.1.1.37) and 0.3 mg/ml NADH in a 3-ml cuvette.

The rate of glucose formation is expressed as per milligram of DNA, which was measured in the cellular suspensions by the method of Burton (10). There were 0.18 to 0.20 mg of DNA per flask, which is equivalent to 0.032 to 0.036 g wet weight of liver (11).

Metabolic inhibitors. Some experiments included the use of either 5 mM α-cyanohydroxycinnamate or 10 mM phenylpyruvate. α-Cyanohydroxycinnamate (Aldrich Chemical Co.) inhibits pyruvate transport across the mitochondrial membrane (12), and phenylpyruvate inhibits pyruvate carboxylase (E.C. 6.4.1.1) (13). Higher concentrations of pyruvate and oxaloacetate (10 mM) were used in these experiments in order to increase glucose production to levels at which the effects of the inhibitors would be more apparent.

Radioactive isotope assays. The incorporation of $^{14}CO_2$ into glucose was determined essentially by the method of Rognstad and Katz (5). Each flask contained 2 mM pyruvate, oxaloacetate, or asparagine. Ethanol (2 mM) was included in flasks containing either pyruvate or oxaloacetate in order to decrease the recycling of phosphoenolpyruvate to pyruvate (5) and to provide sufficient cytosolic NADH for the formation of glucose and lactate.

At the beginning of the incubation period, sodium [^{14}C]bicarbonate was added to each flask (50 μCi per flask). The flasks were gassed for 1 min and then stoppered. The reactions were stopped after 45 min and the samples neutralized as described previously.

A 3.0-ml aliquot of each sample was poured over a 1.5 × 5-cm ion exchange column of Dowex AG50 W-X8 H^+ (200–400 mesh). Glucose was eluted off the columns with 35 ml double-distilled water. A 2.0-ml aliquot was added to 15 ml of Aquasol scintillation fluid (New England Nuclear), and the samples were counted on a scintillation counter. The efficiency was corrected for by the channels ratio method.

The sodium [^{14}C]bicarbonate and Aquasol were purchased from New England Nuclear. Unless otherwise indicated all other chemicals and enzymes were purchased from Sigma Chemical Company.

Results and Discussion. Glucose production from 2 mM pyruvate, oxaloacetate, and malate is listed in Table I. Rates of gluconeogenesis from oxaleacetate and pyruvate were similar, as previously demonstrated by Ross *et al.* (1), utilizing the perfused liver system. The rate of glucose formation from malate was approximately 20% of that from pyruvate, which is also similar to the results obtained by Ross *et al.* (1)

If oxaloacetate entered the cell intact, its conversion to glucose would be initiated in the cytosolic compartment by phosphoenolpyruvate carboxykinase (EC 4.1.1.38). It is probable that a portion of the phosphoenolpyruvate generated by this reaction would be converted to pyruvate via pyruvate kinase (EC 2.7.1.40), which could subsequently be transported into the mitochondria to provide reducing equivalents for the process of glucose and lactate formation. In isolated hepatocytes from starved rats, 40 to 50% of the phosphoenolpyruvate derived from exogenous pyruvate is recycled back to pyruvate ((5), unpublished observations), and it is likely that an equivalent proportion of the phosphoenolpyruvate produced from oxaloacetate is recycled via pyruvate kinase. Therefore, α-cyanohydroxycinnamate, which inhibits pyruvate transport across the mitochondrial mem-

TABLE I. EFFECTS OF α-CYANOHYDROXYCINNAMATE AND PHENYLPYRUVATE ON GLUCOSE FORMATION FROM PYRUVATE AND OXALOACETATE

Expt	Substrate	Conc. (mM)	Inhibitor		Glucose formation (μmole/min/mg of DNA)
			CHC	PP	
A	Pyruvate	2	−	−	0.230
	Oxaloacetate	2	−	−	0.221
	Malate	2	−	−	0.086
B	Pyruvate	10	−	−	0.409
		10	+	−	0.117
	Oxaloacetate	10	−	−	0.305
		10	+	−	0.073
C	Pyruvate	10	−	−	0.427
		10	−	+	0.074
	Oxaloacetate	10	−	−	0.387
		10	−	+	0.049

Note. Hepatocytes were incubated for 45 min at 37° with indicated substrates and inhibitors. Inhibitor concentrations: α-cyanohydroxycinnamate (CHC), 5 mM; phenylpyruvate (PP), 10 mM. Representative experiments are shown. The endogenous rate of glucose formation for the three experiments was 0.043 ± 0.019 μmole/min/mg of DNA (mean ± SEM).

brane (12), and phenylpyruvate, which inhibits the pyruvate carboxylase reaction (13), should be effective in decreasing a portion of the rate of gluconeogenesis from oxaloacetate. Since the production of glucose from pyruvate is initiated within the mitochondria by pyruvate carboxylase, the inhibitors should effectively abolish gluconeogenesis from exogenous pyruvate. As expected, α-cyanohydroxycinnamate and phenylpyruvate substantially reduced gluconeogenesis from 10 mM pyruvate (Table I). However, the inhibitors were equally effective in decreasing glucose production from 10 mM oxaloacetate, indicating that little exogenous oxaloacetate is entering the cytosolic compartment of the isolated hepatocytes.

Since metabolic inhibitors can have nonspecific effects which could lead to erroneous conclusions, additional evidence was obtained utilizing the incorporation of $^{14}CO_2$ into glucose produced from pyruvate or oxaloacetate to substantiate the route of gluconeogenesis from oxaloacetate. $^{14}CO_2$ was rapidly incorporated into glucose in cells incubated with oxaloacetate (Table II). Furthermore, the specific activity of glucose from oxaloacetate was equal to that formed from pyruvate. This suggests that glucose formation from oxaloacetate is as dependent on pyruvate carboxylase as is glucose formation from pyruvate, a finding which would be highly unlikely if oxaloacetate was entering the cytoplasmic compartment intact.

The low specific activity of [^{14}C]glucose produced in cells incubated with asparagine

TABLE II. SPECIFIC ACTIVITY OF GLUCOSE PRODUCED FROM A VARIETY OF SUBSTRATES IN ISOLATED HEPATOCYTES INCUBATED WITH SODIUM [^{14}C]BICARBONATE

Substrate	Glucose formation (μmole/mg DNA)	^{14}C in glucose (dpm × 10^{-5})	Specific activity (dpm/μmole glucose)
Pyruvate + ethanol	5.58	0.222	13700
Oxaloacetate + ethanol	4.41	0.169	13300
Asparagine	2.48	0.026	3500

Note. Hepatocytes were incubated for 45 min at 37° with indicated substrate concentrations: Pyruvate, 2 mM; oxaloacetate, 2 mM; asparagine, 2 mM; ethanol, 2 mM. Glucose formation and isotopic yields represent total values for the 45-min incubation period.

TABLE III. PRODUCTION OF PYRUVATE FROM OXALOACETATE IN THE ABSENCE OF ISOLATED HEPATOCYTES

Incubation time (min)	μmoles/3 ml cuvette		pyruvate
	OAA	Pyruvate	OAA + pyruvate
10	0.084	0.005	0.05
20	0.082	0.008	0.08
30	0.072	0.011	0.13
45	0.074	0.015	0.17

Note. Oxaloacetate (OAA) (~0.09 μmole/3 ml cuvette) was incubated at 37° in Krebs–Henseleit buffer with 2.5% dialyzed bovine serum albumin and 0.3 mg/ml NADH. Malate dehydrogenase (~10 U) was added to each cuvette at the indicated times. After 20 min, the change in absorbance at 340 nm was recorded, lactate dehydrogenase (~10 U) was added to the cuvettes, and the additional decrease in absorbance was recorded.

(Table II) provides evidence that glucose formation from four-carbon compounds which can penetrate the cytoplasmic membrane does not heavily involve pyruvate carboxylase activity. The metabolism of asparagine begins in the mitochondria with its deamination to aspartate. The aspartate can exit the mitochondria and be converted to phosphoenolpyruvate via aspartate aminotransferase (EC 2.6.1.1) and phosphoenolpyruvate carboxykinase. Alternatively, the aspartate can enter the urea cycle within the cytosolic compartment and be converted to malate, which can then be oxidized to oxaloacetate and subsequently converted to phosphoenolpyruvate. Therefore, unless the phosphoenolpyruvate produced from asparagine was recycled to provide reducing equivalents, glucose formation from asparagine should not involve pyruvate carboxylase activity. Correspondingly, the specific activity of glucose produced from asparagine was markedly lower than that from pyruvate or oxaloacetate.

If oxaloacetate was entering the cell before being degraded to pyruvate, then the results presented in Tables I and II would be unlikely, since at least some of the phosphoenolpyruvate produced from oxaloacetate would be converted to glucose without first being recycled via pyruvate kinase. It therefore appears that oxaloacetate is decarboxylated either spontaneously in the incubation medium or at the cellular membrane.

The stability of oxaloacetate in the incubation medium was determined in 3-ml cuvettes in which isolated hepatocytes were omitted (Table III). Approximately 17% of the oxaloacetate initially present in the cuvettes had degraded to pyruvate after 45 min of incubation at 37°. This would be equivalent to the formation of 1.4 and 6.8 μmole of pyruvate from 2 and 10 mM oxaloacetate, respectively, in a 4-ml incubation medium. At an average of 0.2 mg DNA per flask, and assuming that 2 μmole of pyruvate produce 1 μmole glucose, this rate of oxaloacetate degradation would provide enough pyruvate to account for rates of glucose formation of 0.08 and 0.38 μmole/min/mg of DNA. It therefore appears that the extracellular breakdown of oxaloacetate to pyruvate could account for at least a portion of the rate of glucose formation observed from oxaloacetate. However, this does not rule out the possibility that some decarboxylation of oxaloacetate occurs at the cell membrane.

In summary, the results obtained with inhibitors of pyruvate transport into the mitochondria and pyruvate carboxylase activity, as well as $^{14}CO_2$ incorporation into glucose, provide the most convincing evidence to date that oxaloacetate is first degraded to pyruvate prior to its metabolism in isolated hepatocytes. These findings would appear to rule out the possibility that oxaloacetate is entering the cell intact.

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1. Ross, B. D., Hems, R., and Krebs, H. A., Biochem. J. **102**, 942 (1967).
2. Ross, B. D., Hems, R., Freedland, R. A., and Krebs, H. A., Biochem. J **105**, 869 (1967).
3. Exton, J. H., and Park, C. R., J. Biol. Chem. **243**, 4189 (1968).
4. Rognstad, R., and Katz, J., J. Biol. Chem. **247**, 6047 (1972).
5. Rognstad, R., Int. J. Biochem. **7**, 403 (1976).
6. Krebs, H. A., Gascoyne, T., and Notten, B. M., Biochem. J. **102**, 275 (1967).
7. Berry, M. N., and Friend, D. S., J. Cell Biol. **43**, 506 (1969).

8. Krebs, H. A., Cornell, N. W., Lund, P., and Hems, R., *in* "Regulation of Hepatic Metabolism" (F. Lundquist and N. Tystrup, eds.), pp. 726–750. Academic Press, New York/London (1974).
9. Krebs, H. A., Bennett, A. M., DeGarquet, P., Gascoyne, T., and Yoshida, T., Biochem. J. **86**, 22 (1963).
10. Burton, K., Biochem. J. **62**, 315 (1956).
11. Story, D. L., O'Donnell, J. A., Dong, F. M., and Freedland, R. A., *Biochem. Biophys. Res. Commun.* **73**, 799 (1976).
12. Halestrap, A. P., *Biochem. J.* **148**, 85 (1975).
13. Pilkis, S. J., Rious, J. P., and Claus, R. H., *J. Biol. Chem.* **251**, 7841 (1976).

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