Regulation of Gluconeogenesis and Lipogenesis

THE REGULATION OF MITOCHONDRIAL PYRUVATE METABOLISM IN GUINEA-PIG LIVER SYNTHESIZING PRECURSORS FOR GLUCONEOGENESIS

By ETHEL W. SOMBERG AND MYRON A. MEHLMAN*

Biochemistry Laboratory, Department of Zoology and Physiology, Newark College of Arts and Sciences, Rutgers University, Newark, N.J. 07102, and Institute for Enzyme Research, Department of Biochemistry, University of Wisconsin, Madison, Wis. 53706, U.S.A.

(Received 29 October 1968)

1. The carboxylation of pyruvate to oxaloacetate by pyruvate carboxylase in guinea-pig liver mitochondria was determined by measuring the amount of ^{14}C from $\text{H}^{14}\text{CO}_3^-$ fixed into organic acids in the presence of pyruvate, ATP, Mg^{2+} and P_i . The main products of pyruvate carboxylation were malate, fumarate and citrate. Pyruvate utilization, metabolite formation and incorporation of ^{14}C from $\text{H}^{14}\text{CO}_3^-$ into these metabolites in the presence and the absence of ATP were examined. The synthesis of phosphoenolpyruvate from pyruvate and bicarbonate is minimal during continued oxidation of pyruvate. Larger amounts of phosphoenolpyruvate are formed from α -oxoglutarate than from pyruvate. Addition of glutamate, α -oxoglutarate or fumarate did not appreciably increase formation of phosphoenolpyruvate when pyruvate was used as substrate. With α -oxoglutarate as substrate addition of fumarate resulted in increased formation of phosphoenolpyruvate, whereas addition of succinate inhibited phosphoenolpyruvate formation. In the presence of added oxaloacetate guinea-pig liver mitochondria synthesized phosphoenolpyruvate in amount sufficiently high to play an appreciable role in gluconeogenesis. 2. Addition of fatty acids of increasing carbon chain length caused a strong inhibition of pyruvate oxidation and phosphoenolpyruvate formation, and greatly promoted carbon dioxide fixation and malate, citrate and acetoacetate accumulation. The incorporation of ^{14}C from $\text{H}^{14}\text{CO}_3^-$, $[1\text{-}^{14}\text{C}]\text{pyruvate}$ and $[2\text{-}^{14}\text{C}]\text{pyruvate}$ into organic acids formed was examined. 3. It is concluded that guinea-pig liver pyruvate carboxylase contributes significantly to gluconeogenesis and that fatty acids and metabolites play an important role in its regulation.

In guinea-pig liver PEP $\dagger$ carboxykinase is located both in the cytosol and in the mitochondria. The use of mitochondria from guinea-pig liver therefore offers a system for studying both pyruvate carboxylase and PEP carboxykinase reactions within the same cellular compartment. The major studies of carbon dioxide fixation and conversion of pyruvate into PEP in the synthesis of precursors for gluconeogenesis has been intensively investigated in rat liver and kidney mitochondria (Krebs & Eggleston, 1940; Bartley, 1954; Bartley & Avidor, 1955; Nordlie & Lardy, 1963*a,b*; Henning, Seiffert & Seubert, 1963; Wagle, 1964; Freedman & Kohn, 1964; Haynes, 1965; Lardy, Paetkau &

Walter, 1965; Walter, Paetkau & Lardy, 1966; Mehlmán, Walter & Lardy, 1967*a,b*; Mehlmán, 1968*a,b*; Mehlmán & Walter, 1968).

It was found that in the rat pyruvate carboxylase, which catalyses the conversion of pyruvate into dicarboxylic acids, is a key enzyme in the pathway of gluconeogenesis (Haynes, 1965; Lardy *et al.* 1965; Walter *et al.* 1966; Mehlmán *et al.* 1967*a,b*; Mehlmán, 1968*b*). These studies *in vitro* showed that the oxaloacetate synthesized inside the mitochondria by carboxylation of pyruvate was not directly available to the cytosol for the synthesis of PEP, but was instead converted into malate, aspartate and citrate, which diffused into the cytosol, where they were reconverted into oxaloacetate (Lardy *et al.* 1965; Haynes, 1965; Mehlmán *et al.* 1967*a*).

Malate, in addition to supplying carbon atoms

*Present address: Department of Biochemistry, University of Nebraska College of Medicine, Omaha, Nebr. 68105, U.S.A.

$\dagger$ Abbreviation: PEP, phosphoenolpyruvate.

for the synthesis of glucose, may also be important in transporting reducing equivalents from inside the mitochondria to the cytosol, where they are needed for conversion of 1,3-diphosphoglycerate into glyceraldehyde 3-phosphate in the pathway of glucose formation (Walter *et al.* 1966; Mehlmán *et al.* 1967a; Lardy, 1965; Schimke, 1962, 1963).

Since these reactions have a regulatory role in the pathway of gluconeogenesis, it was the purpose of the present investigation to examine the ability of guinea-pig liver mitochondria to synthesize precursors for glucose from pyruvate and bicarbonate, where the location of the two enzymes offers an opportunity to study these complex reactions within the same compartment. A detailed study of the $^{14}\text{CO}_2$ incorporation capabilities of mitochondria and the regulatory effects of pH, Mg^{2+} , P_i , pyruvate, ATP, bicarbonate, mitochondrial nitrogen, fatty acids, organic acids and the fate of ^{14}C from $\text{H}^{14}\text{CO}_3^-$, $[1\text{-}^{14}\text{C}]\text{pyruvate}$ and $[2\text{-}^{14}\text{C}]\text{pyruvate}$ and synthesis of PEP were examined during these experiments.

METHODS AND MATERIALS

Experimental procedure. Male albino guinea pigs weighing 350–450 g. were used in all experiments. All experiments were performed with intact liver mitochondria, isolated by the method of Schneider (1948), except that mitochondria were centrifuged at 15000g for 5 min. as reported by Johnson & Lardy (1967). The liver mitochondria were washed twice in 0.25 M-sucrose by suspending them in 10 vol. of 0.25 M-sucrose/g. original wet wt. of liver and centrifuged for 5 min. at 15000g. The washed mitochondria were suspended in 0.25 M-sucrose at concentrations corresponding to 0.7–1.2 g. original wet wt. of liver/ml. The mitochondrial N content was determined in a Coleman N analyser and by the biuret procedure.

All incubations were carried out in stoppered thick-walled 25 ml. Erlenmeyer flasks, which were shaken in a water bath at 37°. The reagents were added in approximately iso-osmotic mixtures. The final volume of the reaction mixture (composition described in the legends to tables and figures) was brought to 3 ml. by the addition of 1.1–1.7 ml. of 0.25 M-sucrose. As in previous studies (Walter *et al.* 1966; Mehlmán *et al.* 1967a; Mehlmán, 1968b) all reactions were initiated by adding 0.5 ml. of mitochondrial suspension after a temperature equilibration period of 2 min. After appropriate incubation periods, the reactions were stopped by adding 3 ml. of 0.66 M- HClO_4 (final concn. 0.33 M). The precipitated proteins were removed by centrifugation. The clear deproteinized samples were adjusted to pH 4.0 with 2 M-KOH and the KClO_4 was removed by centrifugation. Both the protein and KClO_4 precipitates were rinsed with water and washed solutions were combined with respective supernatant samples to a designated volume. Pyruvate, α -oxoglutarate, malate, citrate, acetoacetate, aspartate and PEP were determined by methods previously cited (Lardy *et al.* 1965; Walter *et al.* 1966).

A sample (0.5 ml.) of acid mixture was spotted on a

Whatman no. 3MM paper strip (5 cm. $\times$ 125 cm.) at 24–25 cm. from one end. The strips were carefully wetted with 2 M-acetic acid buffer, pH 3.0 (adjusted with pyridine), and were placed in the electrophoresis tank (model D high-voltage Electrophorator) containing 2 M-acetic acid buffer, pH 3.0, which was 10 cm. below the spotted acid sample on the paper. The tank was filled with Varsol (Savant Instrument Inc., Hicksville, N.Y., U.S.A.) as cooling liquid to the top.

The organic acids were separated at 4500 v, which yields a current of about 10 ma./strip. The radioactive acids were located on the dried strips with a Vanguard model 880 paper chromatography scanner. The spots were identified by comparing their migration speeds with those of known radioactive acids, as described by Walter *et al.* (1966).

Radioactivity measurements. The radioactive zones of the paper strips were cut into 1 cm. $\times$ 5 cm. strips and put into counting bottles filled with 18 ml. of toluene containing 4 g. of 2,5-diphenyloxazole/l. and 50 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l. The total amount of $^{14}\text{CO}_2$ incorporated into organic acids was measured by spotting a 0.03 ml. sample of the HClO_4 -free mixtures on 1 cm. $\times$ 5 cm. Whatman no. 3MM paper. The strips were counted in Nuclear-Chicago and Beckman CPM-100 scintillation counters. The counting efficiency on paper for all acids was 40% for Nuclear-Chicago and 73% for Beckman CPM-100 scintillation counters. The disintegrations/min. values of the organic acid mixtures (malate, citrate, aspartate, fumarate and succinate) were determined by adding 0.05 ml. samples to 6 ml. of ethanol and 12 ml. of the scintillation counting solution described above, and $[^{14}\text{C}]\text{toluene}$ was used as an internal standard.

Materials. $[1\text{-}^{14}\text{C}]\text{pyruvate}$ and $[2\text{-}^{14}\text{C}]\text{pyruvate}$ were obtained from Tracerlab (Waltham, Mass., U.S.A.); $\text{NaH}^{14}\text{CO}_3$ was obtained from Volk Radiochemical Co. (Skokie, Ill., U.S.A.) and was diluted with unlabelled KHCO_3 to give a final concentration of 0.2–0.7 $\mu\text{C}/\mu\text{mole}$ of HCO_3^- . The exact specific radioactivity of the solution used was determined as described earlier (Walter *et al.* 1966; Mehlmán *et al.* 1967a). Fatty acids (99.8% pure) were purchased from Applied Science Laboratories Inc. (State College, Pa., U.S.A.). All other reagents were of the highest purity commercially obtainable.

RESULTS

Organic acids formed from pyruvate and bicarbonate by liver mitochondria. The rates of formation of organic acids (malate, fumarate and citrate) and of the incorporation of ^{14}C from $\text{H}^{14}\text{CO}_3^-$ by liver mitochondria in the presence of pyruvate, $^{14}\text{CO}_2$, ATP, P_i and magnesium sulphate are summarized in Fig. 1. The terms ' ^{14}C -labelled products', ' $^{14}\text{CO}_2$ incorporated' or 'organic acids formed' are used below to describe the total μmoles of ^{14}C from $\text{H}^{14}\text{CO}_3^-$ retained in non-volatile compounds. The data on the incorporation of $^{14}\text{CO}_2$ represent minimal values because they are based on the specific radioactivity of the added bicarbonate and because $^{14}\text{CO}_2$ that has been fixed is lost again during metabolism of the tricarboxylic acid cycle. It is also important to remember that the $^{14}\text{CO}_2$

fixed by the pyruvate carboxylase reaction will not be found in the succinate, fumarate or malate produced via the forward oxidation of the tri-carboxylic acid cycle. All of the radioactivity found in malate and fumarate will come from the carboxylation of pyruvate followed by reduction of the formed oxaloacetate.

The radioactivity in fumarate was found to be about 19% of that of malate. The total amount of fumarate produced, which was not determined, may be calculated by assuming that the specific radioactivities of these two acids are identical. The radioactivity in succinate was found to be about 10% of that of malate in experiments where $[2-^{14}\text{C}]$ pyruvate instead of $\text{H}^{14}\text{CO}_3^-$ was used. Thus the total amount of succinate produced may be

calculated by assuming that the specific radioactivities of these two acids are identical. As in rat liver (Walter *et al.* 1966) and kidney (Mehlman *et al.* 1967a) mitochondria, no radioactive succinate could be detected from $\text{H}^{14}\text{CO}_3^-$. Therefore it can be concluded that succinate dehydrogenase appears not to be catalysing the reduction of fumarate under our experimental conditions. In these experiments we did not analyse for isocitrate and oxaloacetate. A more accurate measure of the pyruvate carboxylase activity can be made by summing all of the measured metabolites that were synthesized by carboxylation of pyruvate to oxaloacetate in a manner similar to that used in our studies of rat liver and kidney mitochondria (Mehlman *et al.* 1967a). This procedure (see Fig. 1) gives a total of $12.47\mu\text{moles}$ of pyruvate carboxylated, compared with $9.1\mu\text{moles}$ of $^{14}\text{CO}_2$ incorporated after 20 min. of incubation, which is an increase of about 37% in the pyruvate carboxylase activity over that

Fig. 1. Formation of organic acids from pyruvate and bicarbonate by liver mitochondria. The reaction mixture contained 4mm-ATP, 10mm-MgSO₄, 16.7mm-potassium phosphate buffer, pH 7.0, 10mm-potassium triethanolamine buffer, pH 7.0, 6.6mm-pyruvate and 25mm-KH¹⁴CO₃. The final volume was 3ml., made up with iso-osmotic sucrose solution. Mitochondria from 0.5g. of guinea-pig liver contained 1.9mg. of N. The incubation time was varied as indicated. (a) Substrate used: ○, pyruvate. (b) Metabolites found: ●, malate; ■, citrate; ●, ¹⁴CO₂ incorporated. (c) H¹⁴CO₃⁻ incorporated into metabolites: ●, malate; ○, citrate; ○, fumarate.

Fig. 2. Effect of mitochondrial nitrogen concentration on pyruvate utilization, ¹⁴CO₂ incorporation and malate formation. The reaction mixture contained 4mm-ATP, 10mm-MgSO₄, 16.7mm-potassium phosphate buffer, pH 7.0, 10mm-potassium triethanolamine buffer, pH 7.0, 6.6mm-pyruvate and 25mm-KH¹⁴CO₃. The final volume was 3ml., made up with iso-osmotic sucrose. Mitochondria were added as indicated. The incubation time was 10min. Substrate used: ○, pyruvate. Metabolites found: ●, ¹⁴CO₂ incorporated; ○, malate.

Fig. 3. Effect of pH on pyruvate utilization and formation of ^{14}C -labelled products in liver mitochondria. The reaction mixture contained 4mM-ATP, 10mM- MgSO_4 , 6.7mM-potassium phosphate buffer, 10mM-potassium triethanolamine buffer, 6.6mM-pyruvate and 25mM- $\text{KH}^{14}\text{CO}_3$. Potassium phosphate and triethanolamine buffers of indicated pH were added. Mitochondria from 0.5g. of liver contained 1.65mg. of N. The incubation time was 10min. Substrate used: $\circ$, pyruvate. Metabolites found: $\bullet$, $^{14}\text{CO}_2$ incorporated; $\circ$, malate.

estimated by the amount of $\text{H}^{14}\text{CO}_3^-$ incorporated. This is still a minimal value for pyruvate carboxylase activity, because of continual metabolism of the intermediates formed and because some of the minor products such as isocitrate are not included.

When different amounts of mitochondria were added, it was found that malate formation and total ^{14}C incorporation were approximately proportional to the amount of liver mitochondria added in the range 0.95–2.80mg. of nitrogen/3ml. (Fig. 2). The amounts of mitochondrial nitrogen used in the experiments reported in this paper are all within this range. No attempt was made to separate mitochondria quantitatively from the homogenates; the average yield of isolated mitochondrial nitrogen was 3.2–5.0mg./g. of liver.

Pyruvate utilization and $^{14}\text{CO}_2$ fixation by mitochondrial pyruvate carboxylase were maximal at pH 7.0–7.4 (Fig. 3). Fig. 4 shows that optimum Mg^{2+} concentration for utilization of pyruvate and metabolite formation in liver mitochondria is 10mM. Fig. 5 shows that pyruvate utilization and $^{14}\text{CO}_2$ incorporation continued to increase as pyruvate concentration increased from 5 to 27mM. It is important to remember that these substrates,

Fig. 4. Effect of Mg^{2+} concentration on pyruvate utilization and formation of ^{14}C -labelled products in liver mitochondria. The reaction mixture contained 4mM-ATP, 16.7mM-potassium phosphate buffer, pH 7.0, 10mM-potassium triethanolamine buffer, pH 7.0, 6.6mM-pyruvate and 25mM- $\text{KH}^{14}\text{CO}_3$. The concentration of MgSO_4 was varied as indicated. Mitochondria from 0.5g. of liver contained 2.4mg. of N. The incubation time was 10min. Substrate used: $\circ$, pyruvate. Metabolites found: $\bullet$, $^{14}\text{CO}_2$ incorporated; $\circ$, malate.

especially pyruvate, are used in concentrations considerably higher than occur in tissues under physiological conditions.

Influence of P_i , ATP and bicarbonate concentrations on pyruvate metabolism and production of ^{14}C -labelled products. Keech & Utter (1963) showed that ADP is an inhibitor of purified pyruvate carboxylase isolated from chicken livers. With rat liver (Walter *et al.* 1966) and kidney (Mehlman *et al.* 1967a) mitochondria the omission of P_i or the addition of oligomycin to the mitochondrial incubation mixture strongly inhibited pyruvate carboxylation. This inhibition of $^{14}\text{CO}_2$ incorporation was attributed to accumulation of ADP. It was demonstrated (cf. Mehlman *et al.* 1967a) that intact mitochondria may be able to concentrate ADP as indicated by a lower apparent K_m for ADP (Bygrave & Lehninger, 1967). As with rat liver mitochondria, the omission of P_i in the presence of 4mM-ATP with guinea-pig liver mitochondria strongly inhibited pyruvate utilization and metabolite formation (Fig. 6). This inhibition of $^{14}\text{CO}_2$ incorporation could be overcome

Fig. 5. Effect of pyruvate concentration on pyruvate utilization and formation of ¹⁴C-labelled products in liver mitochondria. The reaction mixture contained 4mm-ATP, 10mm-MgSO₄, 16.7mm-potassium phosphate buffer, pH7.0, 10mm-potassium triethanolamine buffer, pH7.0, and 25mm-KH¹⁴CO₃. The pyruvate concentration was varied as indicated. Mitochondria from 0.5g. of liver contained 2.4mg. of N. The incubation time was 10min. Substrate used: ○, pyruvate. Metabolites found: ○, ¹⁴CO₂ incorporated; ●, malate.

by addition of P_i. Fig. 6 shows that the optimum concentrations of P_i for ¹⁴CO₂ fixation and metabolite formation were between 13 and 18mm at a constant ATP concentration of 4mm. The relationship between substrate utilization and P_i concentration has been intensively investigated (Van DenBergh & Slater, 1960; Chance, 1959; Chappell & Greville, 1961; Charles, Tager & Slater, 1963; Borst & Slater, 1961; Tager, 1964). The effect of P_i on PEP formation may be due to 'phosphorylation potential' (Gevers, 1967; Klingenberg, 1964) or it may influence the flow of PEP to and from mitochondria as suggested by Chappell (1966) for various organic acids. Fig. 7 shows that the optimum ratio of pyruvate used to ¹⁴CO₂ incorporated in the presence of a constant amount of P_i (16.6mm) was obtained in the absence of added ATP. At 4mm-ATP, pyruvate utilization was optimum and ¹⁴CO₂ incorporation was slightly decreased.

The effect of increased bicarbonate concentrations on pyruvate utilization and metabolite changes is shown in Fig. 8. Pyruvate utilization and meta-

Fig. 6. Effect of phosphate concentration on pyruvate utilization and formation of ¹⁴C-labelled products in liver mitochondria. The reaction mixture contained 4mm-ATP, 10mm-potassium triethanolamine buffer, pH7.0, 10mm-MgSO₄, 6.6mm-pyruvate and 25mm-KH¹⁴CO₃. The concentration of potassium phosphate buffer, pH7.0, was varied as indicated. Mitochondria from 0.5g. of liver contained 1.65mg. of N. The incubation time was 10min. Substrate used: ○, pyruvate. Metabolites found: ○, ¹⁴CO₂ incorporated; ●, malate.

Fig. 7. Effect of ATP concentration on pyruvate utilization and formation of ¹⁴C-labelled products in liver mitochondria. The reaction mixture contained 10mm-MgSO₄, 16.7mm-potassium phosphate buffer, pH7.0, 10mm-potassium triethanolamine buffer, pH7.0, 6.6mm-pyruvate and 25mm-KH¹⁴CO₃. The ATP concentration was varied as indicated. Mitochondria from 0.5g. of liver contained 2.4mg. of N. The incubation time was 10min. Substrate used: ○, pyruvate. Metabolites found: ○, ¹⁴CO₂ incorporated; ●, malate.

bolite accumulation reached their maximum at 25mm-bicarbonate. At higher concentration there was considerably less pyruvate used, ¹⁴CO₂ incorporated and malate found. Citrate formation

Fig. 8. Effect of bicarbonate concentration on pyruvate utilization and formation of ^{14}C -labelled products in liver mitochondria. The reaction mixture contained 4 mM-ATP, 10 mM-MgSO₄, 6.6 mM-potassium phosphate buffer, pH 7.4, 6.6 mM-potassium triethanolamine buffer, pH 7.4, and 6.6 mM-pyruvate. The concentration of $\text{KH}^{14}\text{CO}_3$ was varied as indicated. Mitochondria from 0.5 g. of liver contained 1.85 mg. of N. The incubation time was 10 min. Substrate used: ●, pyruvate. Metabolites found: ○, $^{14}\text{CO}_2$ incorporated; ○, malate; ▲, citrate; ■, acetoacetate.

decreased only slightly whereas acetoacetate formation began to rise.

Distribution of ^{14}C from $\text{H}^{14}\text{CO}_3^-$, $[1-^{14}\text{C}]$ pyruvate and $[2-^{14}\text{C}]$ pyruvate in organic acids. The carbon dioxide formed during oxidation of one turn of the tricarboxylic acid cycle is derived almost exclusively from oxaloacetate. To obtain a better understanding of the path of $^{14}\text{CO}_2$ and the distribution of radioactive carbon atoms in the organic acids formed, the incorporation of ^{14}C from $\text{H}^{14}\text{CO}_3^-$, $[1-^{14}\text{C}]$ pyruvate and $[2-^{14}\text{C}]$ pyruvate into organic acids was examined. Table 1 shows utilization of pyruvate and the formation of organic acids, the incorporation of ^{14}C into organic acids formed and the percentage specific radioactivity of the organic acids formed from $\text{H}^{14}\text{CO}_3^-$, $[1-^{14}\text{C}]$ pyruvate and $[2-^{14}\text{C}]$ pyruvate. When $\text{H}^{14}\text{CO}_3^-$ was used (Table 1, Expt. 1) 12.1 μmoles of pyruvate were utilized and 4.76 μmoles of ^{14}C could be accounted for in the organic acids found. The percentage specific radioactivity in malate was 86% and in citrate 59%. In these reactions 100% of the organic acids are synthesized from pyruvate and bicarbonate; thus

the percentage of specific radioactivity of malate and citrate show that some of the $\text{H}^{14}\text{CO}_3^-$ that is incorporated into pyruvate is lost during forward metabolism of the tricarboxylic acid cycle. These determinations of the percentage of specific radioactivity are minimal because they are based on the specific radioactivity of the original $\text{KH}^{14}\text{CO}_3$ and they do not take into account the dilution of $\text{H}^{14}\text{CO}_3^-$ by non-radioactive carbon dioxide produced from oxidation of pyruvate. In Expt. 2, where $[1-^{14}\text{C}]$ pyruvate was used, it was possible to account for about 95% of the ^{14}C . On the other hand, in Expt. 1, only 40% of ^{14}C from $\text{H}^{14}\text{CO}_3^-$ can be accounted for in the organic acids, 60% having been reconverted into $^{14}\text{CO}_2$. In Expt. 2 as much as 48% of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ pyruvate is trapped from the oxidative decarboxylation of pyruvate, isocitrate, α -oxoglutarate and oxaloacetate. The percentage specific radioactivity of citrate in guinea-pig liver mitochondria from $[1-^{14}\text{C}]$ pyruvate is lower than in rat kidney mitochondria (Mehlman, 1968b) and rat liver mitochondria (M. A. Mehlman, unpublished work). The reverse reductive carboxylation of α -oxoglutarate to citrate by guinea-pig liver mitochondria (Table 4) is approximately two- to three-fold that found in rat kidney and rat liver mitochondria (Mehlman, 1968c). This is consistent with the results obtained in this study, since the carboxylation of α -oxoglutarate will occur from the unlabelled carbon dioxide in the system. In Expt. 3, with $[2-^{14}\text{C}]$ pyruvate, it was again possible to account for 95% of ^{14}C from pyruvate. The percentage specific radioactivities of malate and citrate were 124 and 220% respectively. This increase of 24% above 100% specific radioactivity in malate would be expected because ^{14}C is entering via $[1-^{14}\text{C}]$ acetyl-CoA and $[2,3-^{14}\text{C}]$ oxaloacetate from $[2-^{14}\text{C}]$ pyruvate and the continued metabolism of the tricarboxylic acid cycle. It is possible to conclude, on the basis of the percentage specific radioactivities of malate and citrate obtained with $\text{H}^{14}\text{CO}_3^-$, that oxidative decarboxylations contribute significantly to the lowering of specific radioactivity of tricarboxylic acid-cycle intermediates labelled with $\text{H}^{14}\text{CO}_3^-$ during the forward metabolism of the tricarboxylic acid cycle. Table 2 shows the incorporation of ^{14}C from $[1-^{14}\text{C}]$ pyruvate into organic acids in the presence of 3.3 mM-glutamate. The aspartate formed by glutamate transamination with oxaloacetate had the same percentage specific radioactivity as that of malate. In the presence of glutamate, the increased incorporation of total ^{14}C from $[1-^{14}\text{C}]$ pyruvate into organic acids by carboxylation through oxaloacetate is found primarily in aspartate, without major changes in the amounts of ^{14}C found in malate and citrate. Thus it is possible to conclude that the increase in

Table 1. *Incorporation of ^{14}C into organic acids from various labelled substrates*

The reaction mixtures contained 4 mM-ATP, 10 mM-MgSO₄, 16.6 mM-potassium phosphate and 10 mM-potassium triethanolamine buffers, pH 7.2, 6.6 mM-pyruvate and 21 mM-KH¹⁴CO₃ for Expt. 1. For Expt. 2 [1-¹⁴C]pyruvate (specific radioactivity 279 000 d.p.m./μmole) and for Expt. 3 [2-¹⁴C]pyruvate (specific radioactivity 287 500 d.p.m./μmole) instead of bicarbonate were labelled. The final volume was 3 ml. containing a total of 1.2–1.7 ml. of iso-osmotic sucrose solution. Mitochondria from 0.5 g. of liver suspended in 0.5 ml. of iso-osmotic sucrose solution were added to each incubation flask and contained 2.4 mg. of N for Expt. 1, 1.8 mg. of N for Expt. 2 and 1.5 mg. of N for Expt. 3. The incubation time was 10 min. for all experiments at 37°. Percentage specific radioactivity is defined as 100 × (the amount of ¹⁴C incorporated into organic acid divided by the amount of organic acid found). ¹⁴CO₂ trapped is the amount of radioactive ¹⁴CO₂ trapped from oxidative decarboxylation of [¹⁴C]pyruvate and oxidative decarboxylation reactions of the tricarboxylic acid cycle.

Labelled substrate ...	Expt. 1	Expt. 2	Expt. 3
	H ¹⁴ CO ₃ ⁻	[1- ¹⁴ C]Pyruvate	[2- ¹⁴ C]Pyruvate
	Metabolite changes (μmoles/3 ml.)		
Pyruvate used	12.1	9.7	7.9
¹⁴ CO ₂ incorporated	5.5	—	—
¹⁴ CO ₂ trapped	—	4.7	0.08
Malate found	3.5	2.6	1.9
Citrate found	2.2	1.8	1.4
α-Oxoglutarate found	1.3	1.0	0.8
	¹⁴ C incorporated into organic acids (μmoles)		
Malate	3.0	2.7	2.37
Citrate	1.3	0.87	3.06
Fumarate	0.46	0.48	0.46
Succinate	—	—	0.34
	Specific radioactivity (%)		
Malate	86	103	124
Citrate	59	48	220

the incorporation of ¹⁴C from [1-¹⁴C]pyruvate into organic acids with added glutamate is regulated by the ability of the cell to remove the oxaloacetate formed by carboxylation of pyruvate via transamination with glutamate to form aspartate.

Effect of fatty acids on pyruvate and α-oxoglutarate metabolism. Fatty acids have been reported to inhibit oxidation of pyruvate in mitochondria from liver (Bremer, 1966; Walter *et al.* 1966) and kidney (Mehlman *et al.* 1967a). The oxidation of fatty acids in mitochondria produces acetyl-CoA, which is an allosteric activator of pyruvate carboxylase (Utter & Keech, 1963). It was decided to investigate whether the addition of fatty acids to our system would lead to any alterations in product pattern. Fig. 9 shows the degree of inhibition of pyruvate utilization in the presence of various fatty acids. With increasing fatty acid chain length, the inhibition of pyruvate utilization becomes more pronounced. It was apparent that in the presence of 0.66 mM-fatty acids there was a large decrease in the ratios of pyruvate used to ¹⁴CO₂ incorporated, because the amount of ¹⁴CO₂ fixed was elevated by about 30–35% in the presence of all fatty acids tested. Formation of malate, acetoacetate and, to a smaller extent, citrate was increased with increasing chain length of fatty acids added. In the system with 0.66 mM-octanoate (Fig. 9)

9.2 μmoles of pyruvate were used and 8.1 μmoles of ¹⁴CO₂ were incorporated. This corresponds to about 88% of pyruvate carboxylated and only 12% of pyruvate oxidized. With the standard system, in the absence of fatty acids, 57% of pyruvate was carboxylated and 43% was oxidized. It is shown that fatty acids strongly inhibit pyruvate decarboxylation.

The ability of guinea-pig liver mitochondria to carboxylate α-oxoglutarate in the presence of fatty acids of various chain lengths was examined (Fig. 10). Under these conditions considerably less α-oxoglutarate was used compared with pyruvate. This may be due to a restricted permeability to this keto acid in mitochondria. Additions of fatty acids of various chain lengths led to a large increase in acetoacetate, citrate and ¹⁴CO₂ incorporation. The formation of malate remained essentially unchanged.

Effect of tricarboxylic acid-cycle intermediates on synthesis of PEP from pyruvate and bicarbonate or α-oxoglutarate and bicarbonate. Addition of 10 mM-fumarate and 10 mM-glutamate (Table 3, Expt. 1A) in the presence of 10 mM-KH¹⁴CO₃ leads to a small increase in the total ¹⁴CO₂ incorporation and PEP formation. An increase in KH¹⁴CO₃ concentration from 10 to 30 mM (Table 3, Expt. 1B) leads to an increase in the total ¹⁴CO₂ incorporation and in

Table 2. Incorporation of ^{14}C into organic acids from $[1\text{-}^{14}\text{C}]\text{pyruvate}$ in the presence of glutamate

The reaction mixture contained 4mM-ATP, 10mM- MgSO_4 , 16.6mM-potassium phosphate and 10mM-potassium triethanolamine buffers, pH 7.0, 3.3mM-L-glutamate, 6.6mM-pyruvate and 21mM- KHCO_3 . Instead of bicarbonate, $[1\text{-}^{14}\text{C}]\text{pyruvate}$ (specific radioactivity 279000 d.p.m./ μmole) was labelled. Mitochondria from 0.5g. of liver contained 1.8mg. of N. The incubation time was 10min. Percentage specific radioactivity is defined as $100 \times (\text{the amount of } ^{14}\text{C} \text{ incorporated into organic acid divided by the amount of organic acid found})$. $^{14}\text{CO}_2$ trapped is the amount of radioactive $^{14}\text{CO}_2$ trapped from oxidative decarboxylation of $[1\text{-}^{14}\text{C}]\text{pyruvate}$ and oxidative decarboxylation reactions of the tricarboxylic acid cycle.

Metabolite changes ($\mu\text{moles}/3\text{ml.}$)

Pyruvate used	9.9
$^{14}\text{CO}_2$ trapped	2.7
Malate found	2.1
Aspartate found	2.3
Citrate found	1.8
α -Oxoglutarate found	2.1

^{14}C incorporated into organic acids (μmoles)

Malate	1.95
Aspartate	2.15
Citrate	0.88
Fumarate	0.34
Glutamate	0.31

Specific radioactivity (%)

Malate	93
Aspartate	94
Citrate	49

pyruvate utilization. This is evident from the decrease in the ratio of pyruvate used to $^{14}\text{CO}_2$ incorporated. In the presence of glutamate PEP formation was increased. In the presence of 10mM-succinate (Table 3, Expt. 1B) the total $^{14}\text{CO}_2$ fixed was larger than the amount of total pyruvate used. This indicates that in guinea-pig liver mitochondria some of the added succinate led to the formation of pyruvate, which was then carboxylated by pyruvate carboxylase. In the presence of added succinate PEP formation was inhibited.

The addition of 3.3mM-isocitrate, 3.3mM- α -oxoglutarate or 3.3mM-fumarate resulted in an increase (by 0.2–1.3 μmoles) in $^{14}\text{CO}_2$ incorporation and an increase in malate formation (Table 3, Expt. 2). Addition of 3.3mM-oxaloacetate in the same experiment resulted in an increased malate formation and decrease in $^{14}\text{CO}_2$ incorporation. PEP formation was increased from 0.39 to 0.85 μmole in the presence of α -oxoglutarate. A marked increase in PEP formation was observed in the presence of oxaloacetate (from 0.39 to 1.8 μmoles).

When pyruvate was used as the substrate the

Fig. 9. Effect of carbon chain length of fatty acids on pyruvate utilization and formation of ^{14}C -labelled products in liver mitochondria. The reaction mixture contained 4mM-ATP, 10mM- MgSO_4 , 13.3mM-potassium phosphate buffer, pH 7.0, 10mM-potassium triethanolamine buffer, pH 7.0, 6.6mM-pyruvate, 25mM- $\text{KH}^{14}\text{CO}_3$ and 5mg. of fatty acid-poor albumin/ml. Fatty acids of different carbon chain lengths were added (0.66mM) as indicated. Mitochondria from 0.5g. of liver contained 2.2mg. of N. The incubation time was 10min. (a) Substrate used: $\circ$, pyruvate. (b) Metabolites found: $\square$, $^{14}\text{CO}_2$ incorporated; $\bullet$, malate; $\circ$, citrate; $\odot$, acetoacetate.

synthesis of PEP was small. Since α -oxoglutarate stimulated PEP formation in the presence of pyruvate (Table 3) the effects of malonate, fluorocitrate, succinate, fumarate and glutamate on PEP synthesis from α -oxoglutarate were examined (Table 4). Inhibitors of the tricarboxylic cycle, malonate and fluorocitrate decreased carboxylation of α -oxoglutarate and PEP formation. Addition of 10mM-succinate or 10mM-fumarate, but not 10mM-glutamate, greatly stimulated $^{14}\text{CO}_2$ incorporation. The formation of PEP was considerably greater from α -oxoglutarate than from pyruvate. In the presence of succinate PEP formation was markedly inhibited, whereas in the presence of fumarate PEP formation was greatly increased. Both succinate and fumarate stimulated incorporation of ^{14}C from $\text{H}^{14}\text{CO}_3^-$ into malate and citrate, with succinate producing more pronounced stimulation. Expt. 2 shows that addition of 1.7mM-GTP did not effect synthesis of PEP. Expt. 3 in Table 4 shows the effect of adding various concentrations of ATP on

PEP synthesis. There is a large increase in PEP formation caused by the addition of exogenous ATP.

Effect of octanoate on synthesis of PEP from pyruvate and bicarbonate. The synthesis of PEP from pyruvate or from α -oxoglutarate is inhibited in the presence of succinate. In mitochondria succinate is known to increase the NADH/NAD⁺ ratio by reverse electron flow (Chance & Hollunger, 1961), thereby preventing conversion of malate into oxaloacetate. The oxidation of fatty acids in mitochondria is known to be associated with increased production of NADH (Bode & Klingenberg, 1965). Table 5 shows that fatty acids greatly stimulate ¹⁴CO₂ incorporation and malate formation. In Expt. 1 (Table 5) addition of 1.7mm-octanoate resulted in approx. 56% increase in ¹⁴CO₂ incorporation and 124% malate formation.

On the other hand PEP formation is almost completely inhibited. Similar results were obtained in Expt. 3 (Table 5). Results in Expt. 2 (Table 5) show that when the incubations were carried out when substrate (pyruvate) was limiting the synthesis of PEP was increased from 0.40 μ mole (Expt. 1) to 1.2 μ moles (Expt. 2). Increase in the concentrations of octanoate from 0.33 to 1.7mm resulted in about threefold decrease in the formation of PEP. There was an increase in ¹⁴CO₂ incorporation (62%), malate (142%) and citrate (127%) formation in the presence of 1.7mm-octanoate (Expt. 3). The ratio of pyruvate used to ¹⁴CO₂ fixed was decreased from 2.0 to 1.2.

DISCUSSION

The pathways of intermediary metabolism are known to be regulated by the intercompartmental distribution of metabolites, substrates, enzymes,

Fig. 10. Effect of carbon chain length of fatty acids on α -oxoglutarate utilization and formation of ¹⁴C-labelled products in liver mitochondria. The reaction mixture contained 4mm-ATP, 10mm-MgSO₄, 13.3mm-potassium phosphate buffer, pH 7.0, 10mm-potassium triethanolamine buffer, pH 7.0, 6.6mm- α -oxoglutarate, 25mm-KH¹⁴CO₃ and 5mg. of fatty acid-poor albumin/ml. Fatty acids of different carbon chain lengths were added (0.66mm) as indicated. Mitochondria from 0.5g. of liver contained 2.2mg. of N. The incubation time was 10min. Substrate used: ○, α -oxoglutarate. Metabolites found: ●, citrate, ●, ¹⁴CO₂ incorporated; ▲, acetoacetate; □, malate.

Table 3. *Effect of tricarboxylic acid-cycle intermediates on the synthesis of PEP from pyruvate and bicarbonate*

The same basic reaction mixture was used for all experiments as described for Fig. 1, except that KH¹⁴CO₃ for Expt. 1A was 10mm, for Expt. 1B 30mm and for Expt. 2 25mm. Mitochondria from 0.5g. of liver were added to each incubation, which contained 2.4mg. of N for Expts. 1A and 1B and 1.6mg. of N for Expt. 2. The incubation time for all experiments was 10min.

Expt. no.	Addition to system	Metabolite changes (μ moles/3ml.)				Ratio pyruvate/ ¹⁴ CO ₂ incorporation
		Pyruvate used	¹⁴ CO ₂ incorporation	Malate found	PEP found	
1A	None	10.1	4.8	1.6	0.34	2.1
	Fumarate (10mm)	8.2	5.4	8.1	0.43	1.5
	Glutamate (10mm)	11.6	5.3	1.6	0.63	2.2
1B	None	10.5	6.8	2.0	0.30	1.6
	Fumarate (10mm)	10.3	8.3	7.7	0.48	1.2
	Succinate (10mm)	8.2	9.1	20.0	0.05	0.9
	Glutamate (10mm)	13.2	9.4	1.6	0.96	1.4
2	None	5.9	2.8	1.3	0.39	2.1
	Isocitrate (3.3mm)	5.0	3.0	1.7	0.53	1.7
	α -Oxoglutarate (3.3mm)	4.8	3.2	1.7	0.85	1.5
	Fumarate (3.3mm)	5.1	4.1	7.8	0.39	1.2
	Oxaloacetate (3.3mm)	—	2.4	2.2	1.80	—

Table 4. *Effect of organic acids, malonate and fluorocitrate on α -oxoglutarate metabolism and formation of PEP*

The reaction mixture contained 4mm-ATP (except in Expt. 3), 10mm-MgSO₄, 13.3mm-phosphate and 10mm-triethanolamine buffers, pH7.2, 20mm-KH¹⁴CO₃ and 6.7mm- α -oxoglutarate. Mitochondria from 0.5g. of liver contained 1.7mg. of N for Expt. 1, 2.1mg. of N for Expt. 2 and 2.8mg. of N for Expt. 3. The incubation time was 30min. for all experiments.

Expt. no.	Addition to system	Metabolite changes (μ moles/3ml.)						Ratio α -oxo-glutarate/ ¹⁴ C incorporation
		α -Oxoglutarate used	¹⁴ CO ₂ incorporation	Malate found	Malate incorporation	Citrate ¹⁴ C incorporation	PEP found	
1	None	7.3	3.5	2.4	0.74	2.04	1.7	2.1
	Malonate (6.6mm)	6.6	1.0	0.4			0.4	6.6
	Fluorocitrate (0.133mm)	4.3	1.0	2.3			1.0	4.3
	Succinate (10mm)	6.1	8.2	18.6	2.02	4.74	0.5	0.8
	Fumarate (10mm)	8.4	5.9	13.8	1.78	2.50	2.8	1.4
	Glutamate (3.3mm)	6.2	3.1	1.7	1.10	1.50	1.4	2.0
2	None	9.2	4.5	2.9			2.7	
	GTP (1.7mm)	8.9	4.4	3.1			2.6	
3	ATP (0)	9.1	4.5	2.7			1.2	
	ATP (2mm)	10.8	3.7	3.3			2.6	
	ATP (4mm)	9.4	3.4	3.1			3.1	

Table 5. *Effect of octanoate on the synthesis of PEP from pyruvate and bicarbonate*

The reaction mixture contained for all experiments 4mm-ATP, 10mm-MgSO₄, 16.7mm-potassium phosphate and 10mm-potassium triethanolamine buffers, pH7.0. Expts. 1 and 2 contained 20mm-KH¹⁴CO₃ and 10mg of albumin/incubation flask. Expt. 3 contained 25mm-KH¹⁴CO₃ and 8.8mm-pyruvate. Expt. 2 contained 3.7mm-pyruvate. Mitochondria from 0.5g. of liver were added to each incubation, which contained 2.3mg. of N for Expt. 1, 2.4mg. of N for Expt. 2 and 2mg. of N for Expt. 3. The incubation time was 10min. for all experiments.

Expt. no.	Addition to system	Metabolite changes (μ moles/3ml.)					Ratio pyruvate/ ¹⁴ CO ₂ incorporation
		Pyruvate used	¹⁴ CO ₂ incorporation	Malate found	Citrate found	PEP found	
1	None	11.4	5.2	1.7		0.40	2.2
	Octanoate (1.0mm)	10.3	7.3	3.1		0.05	1.4
	Octanoate (1.7mm)	10.1	8.1	3.8		0.05	1.2
	Octanoylcarnitine (0.66mm)	9.1	7.3	3.3			1.3
2	None	11.3	5.8	1.2	0.84	1.2	2.0
	Octanoate (0.33mm)	11.3	6.4	1.5	0.90	1.0	1.8
	Octanoate (1.0mm)	11.3	8.1	2.2	1.5	0.7	1.4
	Octanoate (1.7mm)	11.3	9.4	2.9	1.90	0.4	1.2
3	None	8.0	3.6	1.9		0.65	2.2
	Octanoate (0.66mm)	7.1	5.8	2.7		0.02	1.2

cofactors and the movement of these substances between the intramitochondrial and extramitochondrial compartments of the cell. In guinea-pig liver, pyruvate carboxylase and PEP carboxykinase are present in the same compartment of the cell (Lardy, 1966). It is therefore worthwhile to compare the results of a previous study (Mehlman *et al.* 1967a) performed with rat liver and kidney mitochondria, where these two enzymes are located in separate compartments, with the results presented in this paper.

The synthesis of PEP by guinea-pig liver mitochondria from pyruvate and bicarbonate under the conditions of these experiments is not sufficiently high (0.2 μ mole of PEP/min./g. wet wt. of liver) to play an important role in gluconeogenesis, since a minimum of 1 μ mole/min./g. wet wt. of tissue is required (Solomon, Vennesland, Klemperer, Buchanan & Hasting, 1941) to contribute significantly. This low rate of synthesis of PEP is probably due to the rapid conversion of oxaloacetate into malate and citrate, thus decreasing the avail-

ability of oxaloacetate for the formation of PEP. This is demonstrated by adding excess of oxaloacetate, which results in an increased formation of PEP (0.75 μ mole of PEP/min./g. wet wt. of liver; Table 3). Addition of octanoate is known to increase the NADH/NAD⁺ ratio (Bode & Klingenberg, 1965; Chance & Hollunger, 1960) and in our experiments resulted in a nearly complete inhibition of PEP formation (Table 5, Expts. 1 and 3) due to the conversion of oxaloacetate into malate, as was evident by the increase in pyruvate carboxylation and malate formation (Table 5). In Expt. 2 (Table 5) where pyruvate was limiting for oxidation the synthesis of PEP was two to three times that when pyruvate was present in excess. This indicates that NADH produced from the oxidation of pyruvate or fatty acids is readily available for the conversion of oxaloacetate into malate. An additional explanation for the low PEP value may be that the oxaloacetate may be converted into PEP at a different mitochondrial site and during the production of NADH from fatty acids or pyruvate oxidation the oxaloacetate formed is not available to the site of PEP synthesis. This hypothesis is partially supported by the results obtained in Table 4, where it is shown that more PEP is formed when α -oxoglutarate is used as a substrate instead of pyruvate. Thus the oxaloacetate used for the synthesis of PEP is derived from malate produced from the metabolism of α -oxoglutarate. In the presence of malonate, which is known to inhibit oxidation of succinate, PEP synthesis is greatly decreased (Table 4).

The synthesis of PEP from oxaloacetate is catalysed by GTP. In mitochondria GTP is synthesized by substrate-level phosphorylation during oxidation of α -oxoglutarate (Hager, 1962; Heldt & Klingenberg, 1965). The mitochondrial activity of nucleoside diphosphate kinase, an enzyme that catalyses the formation of ATP from GTP and ADP, is very low in guinea pig and very high in rat and pigeon liver (Ishihara & Kikuchi, 1968). This low activity of nucleoside diphosphate kinase in guinea-pig liver mitochondria as contrasted with rat liver mitochondria may contribute to the relative importance of these two organs in synthesizing PEP. PEP synthesis in guinea-pig liver mitochondria is high and is almost negligible in rat liver mitochondria. Thus GTP formed by substrate-level phosphorylation during α -oxoglutarate oxidation may be directed for synthesis of PEP in guinea-pig liver mitochondria and in the rat GTP may be directed primarily toward ATP synthesis, owing to the high activity of nucleoside diphosphate kinase. The inability of exogenously added GTP to stimulate PEP synthesis was previously reported by Ishihara & Kikuchi (1968). In our systems we have also found that addition of 1.7 mM-GTP (Table 4)

did not stimulate PEP synthesis. The inability of exogenous GTP to stimulate PEP synthesis is probably due to a mitochondrial permeability barrier to guanine nucleotide (Heldt, 1966; Klingenberg & Pfaff, 1966). In contrast to the findings of Ishihara & Kikuchi (1968) we have found that exogenous ATP markedly stimulates the synthesis of PEP from α -oxoglutarate in guinea-pig liver mitochondria. Gevers (1967) reported similar increases in PEP formation with addition of ATP in pigeon liver. On the basis of these findings it may be suggested that exogenous ATP interacts with mitochondrial GDP, thus facilitating synthesis of PEP. Mitochondrial synthesis of PEP may be of physiological importance both as a carbon source and as a means of utilization of mitochondrial high-energy compounds for synthesis of glucose.

The decrease in the total amount of $^{14}\text{CO}_2$ fixed from 3.5 to 1.0 μ moles in the presence of malonate suggests that the oxidation of succinate may contribute to the reverse carboxylation of α -oxoglutarate. In the same experiment in the presence of succinate the $^{14}\text{CO}_2$ fixation is greatly increased from 3.5 to 8.2 μ moles. There was also a large increase in the incorporation of ^{14}C from $\text{H}^{14}\text{CO}_3^-$ into both malate and citrate. Thus it is possible to suggest that succinate oxidation leads to stimulation of reverse electron flow, thereby providing increased amounts of reducing equivalents needed for reductive carboxylation of α -oxoglutarate to citrate. The decreased formation of PEP in the presence of succinate and the increase in the total amount of $^{14}\text{CO}_2$ fixed above the total amount of α -oxoglutarate used is due to the conversion of succinate into pyruvate, which is then carboxylated by pyruvate carboxylase to dicarboxylic acids. This is evident by the increased incorporation of ^{14}C from $\text{H}^{14}\text{CO}_3^-$ into malate. The incorporation of 0.74 μ mole of ^{14}C from $\text{H}^{14}\text{CO}_3^-$ into malate in the control experiment is due to pyruvate carboxylase. Thus the measured amounts of PEP in these experiments represent minimal values, because of possible PEP metabolism in the mitochondria. Since oxidation of fumarate is known not to be associated with increased NADH production, the addition of fumarate resulted in a large increase in PEP formation.

There was an increase (from 2.04 to 4.74 μ moles) in the amount of $\text{H}^{14}\text{CO}_3^-$ fixed (Table 4) into citrate in the presence of succinate. This indicates the important role that the stimulation of the reduced nicotinamide nucleotides may play on reverse electron flow. The total $^{14}\text{CO}_2$ fixation was greater in the presence of succinate and fumarate (Table 4) than control values. The decrease in α -oxoglutarate utilization in the presence of fluorocitrate is associated with a decrease in $^{14}\text{CO}_2$ fixation, which is a result of inhibition of α -oxoglutarate carboxylation.

However, the decrease in the PEP formation from 1.7 to 1.0 μ moles in the presence of fluorocitrate is difficult to explain. It is possible that fluorocitrate in addition to inhibiting aconitase may also inhibit at other sites (Mehlman, 1968a).

Previous studies with kidney (Mehlman *et al.* 1968), liver (Walter *et al.* 1966) and heart (Bremer, 1966) mitochondria showed that fatty acids, acetyl-CoA or acylcarnitines inhibit oxidation of pyruvate. Thus this competition for mitochondrial CoA by fatty acid-oxidizing and pyruvate oxidase pathways is responsible for greatly decreased pyruvate oxidation, thereby permitting the use of pyruvate for synthesis of sugars. The effect of fatty acids in sparing the oxidation of pyruvate may be of major significance in the cell, especially under physiological conditions where the production of pyruvate may be limited. In fact, the mobilization of fatty acids from adipose tissue is known to be greatly increased in a variety of conditions such as starvation, heavy muscular work and diabetes, which are known to be associated with conditions of greatly increased gluconeogenesis. It is unlikely that acyl-CoA inhibits citrate-condensing enzyme in this study, since the formation of citrate (Fig. 9) was slightly increased in the presence of fatty acids of increasing chain lengths. Srere (1965) has also concluded that, on the basis of present available evidence where increased concentrations of palmitoyl-CoA have been observed, increased citrate concentrations have also been detected, and thus it is unlikely that under physiological conditions palmitoyl-CoA would inhibit citrate-condensing enzyme.

In contrast with rat liver mitochondria, where the addition of fatty acids (0.66 mM) did not greatly increase $^{14}\text{CO}_2$ fixation (Walter *et al.* 1966), a large increase in the pyruvate carboxylation was observed in this study with guinea-pig liver mitochondria in the presence of fatty acids (Table 5 and Fig. 9). Addition of fatty acids to rat kidney slices (Krebs, Speake & Hems, 1965) greatly stimulated gluconeogenesis from lactate even at high pyruvate concentrations. From this study with guinea-pig liver mitochondria it can therefore be concluded that increased $^{14}\text{CO}_2$ fixation is probably not the result of increased acetyl-CoA production but is due to the removal of the oxaloacetate from the system. Oxaloacetate is known to be involved in inhibition of pyruvate carboxylation (Mehlman, 1968b).

On the basis of these studies it may be possible to suggest that increased mobilization of fatty acids under conditions associated with enhanced gluconeogenesis results in their utilization for the production of energy. They may also play a key role in 'turning on' or 'turning off' these metabolic processes by inhibiting pyruvate oxidation, mitochondrial PEP formation and by stimulating production of malate. Malate is used in the cytosol

both as a carbon source and more importantly as a mechanism for the transport of intramitochondrial reducing equivalents for the synthesis of hexoses.

It can be concluded that guinea-pig liver mitochondria do have the capacity to synthesize PEP in quantities that may be significant for gluconeogenesis and that the synthesis of PEP is subject to highly sensitive controls by the concentrations of metabolites, ATP/ADP, GTP/GDP and NADH/NAD⁺ ratios and by fatty acids.

The authors express their sincerest appreciation to Dr Henry A. Lardy of the Institute for Enzyme Research for his help with initial aspects of this work. They also thank Miss S. Friedman for her excellent help with some experiments. The authors are grateful to Mrs Anne Wolf for typing this manuscript. This work was supported by grants from the National Institutes of Health (AM12780) and the National Science Foundation (GB-3002).

REFERENCES

- Bartley, W. (1954). *Biochem. J.* **56**, 387.
- Bartley, W. & Avi-Dor, Y. (1955). *Biochem. J.* **59**, 194.
- Bode, C. & Klingenberg, M. (1965). *Biochem. Z.* **341**, 271.
- Borst, P. & Slater, E. C. (1961). *Biochim. biophys. Acta*, **48**, 362.
- Bremer, J. (1966). *Biochim. biophys. Acta*, **116**, 1.
- Bygrave, F. L. & Lehninger, A. L. (1967). *Proc. nat. Acad. Sci., Wash.*, **57**, 1409.
- Chance, B. (1959). In *Ciba Found. Symp.: Regulation of Cell Metabolism*, p. 91. Ed. by Chance, B. London: J. and A. Churchill Ltd.
- Chance, B. & Hollunger, G. (1960). *Nature, Lond.*, **185**, 666.
- Chance, B. & Hollunger, G. (1961). *J. biol. Chem.* **236**, 1534.
- Chappell, J. B. (1966). *Biochem. J.* **100**, 43P.
- Chappell, J. B. & Greville, G. D. (1961). *Nature, Lond.*, **190**, 502.
- Charles, R., Tager, J. M. & Slater, E. C. (1963). *Biochim. biophys. Acta*, **74**, 33.
- Freedman, A. C. & Kohn, L. (1964). *Science*, **145**, 58.
- Gevers, W. (1967). *Biochem. J.* **103**, 141.
- Hager, L. P. (1962). In *The Enzymes*, vol. 6, p. 387. Ed. by Boyer, P. D., Lardy, H. A. & Myrback, K. New York: Academic Press Inc.
- Haynes, R. C., jun. (1965). *J. biol. Chem.* **240**, 4103.
- Heldt, H. W. (1966). *Biochim. biophys. Acta Library Vol. 7: Regulation of Metabolic Processes in Mitochondria*, p. 51. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Heldt, H. W. & Klingenberg, M. (1965). *Biochem. Z.* **343**, 433.
- Henning, H.-V., Seiffert, I. & Seubert, W. (1963). *Biochim. biophys. Acta*, **77**, 345.
- Ishihara, N. & Kikuchi, G. (1968). *Biochim. biophys. Acta*, **153**, 733.
- Johnson, D. & Lardy, H. A. (1967). *Methods in Enzymology*, vol. 10, p. 94. Ed. by Estabrook, R. W. & Pullman, M. E. New York: Academic Press Inc.
- Keech, D. B. & Utter, M. F. (1963). *J. biol. Chem.* **238**, 2609.
- Klingenberg, M. (1964). *Angew. Chem. (int. Ed.)*, **3**, 54.

- Klingenberg, M. & Pfaff, E. (1966). In *Biochim. biophys. Acta Library Vol. 7: Regulation of Metabolic Processes in Mitochondria*, p. 180. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 1383.
- Krebs, H. A., Speake, R. N. & Hems, R. (1965). *Biochem. J.* **94**, 712.
- Lardy, H. A. (1965). In *A Symposium on Control of Energy Metabolism*, p. 245. Ed. by Chance, B. New York: Academic Press Inc.
- Lardy, H. A. (1966). *Harvey Lect.* no. 60, p. 261.
- Lardy, H. A., Paetkau, V. & Walter, P. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1410.
- Mehlman, M. A. (1968a). *J. biol. Chem.* **243**, 1919.
- Mehlman, M. A. (1968b). *J. biol. Chem.* **243**, 3289.
- Mehlman, M. A. (1968c). *N.J. Acad. Sci. Bull.* **13**, 9.
- Mehlman, M. A. & Walter, P. (1968). *Arch. Biochem. Biophys.* **127**, 590.
- Mehlman, M. A., Walter, P. & Lardy, H. A. (1967a). *J. biol. Chem.* **242**, 4594.
- Mehlman, M. A., Walter, P. & Lardy, H. A. (1967b). *Abstr. Pap. Amer. chem. Soc. 154th Meet., Chicago*, p. 199c.
- Nordlie, R. C. & Lardy, H. A. (1963a). *J. biol. Chem.* **238**, 2259.
- Nordlie, R. C. & Lardy, H. A. (1963b). *Biochem. Z.* **338**, 356.
- Schimke, R. T. (1962). *J. biol. Chem.* **237**, 1921.
- Schimke, R. T. (1963). *J. biol. Chem.* **238**, 1012.
- Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.
- Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M. & Hasting, A. B. (1941). *J. biol. Chem.* **140**, 171.
- Srere, P. A. (1965). *Biochim. biophys. Acta*, **106**, 445.
- Tager, J. M. (1964). *Abstr. 6th int. Congr. Biochem., New York*, **10**, 793.
- Utter, M. F. & Keech, D. B. (1963). *J. biol. Chem.* **238**, 2603.
- Van DenBergh, S. G. & Slater, E. C. (1960). *Biochim. biophys. Acta*, **40**, 176.
- Wagle, S. R. (1964). *Biochem. biophys. Res. Commun.* **14**, 533.
- Walter, P., Paetkau, V. & Lardy, H. A. (1966). *J. biol. Chem.* **241**, 2523.