Regulation of Pyruvate Carboxylase Activity by Calcium in Intact Rat Liver Mitochondria

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SUMMARY

Low concentrations of calcium (<100 μM) cause a marked inhibition of 14C-carbon dioxide production from carboxyl-labeled pyruvate by isolated rat liver mitochondria. This effect disappears in the presence of agents which preclude pyruvate carboxylase activity such as dinitrophenol plus oligomycin. Adding ATP in combination with these agents restores carboxylase activity and the inhibitory effect of calcium returns. If malate is added, the system again becomes less dependent on pyruvate carboxylase activity and calcium loses its effectiveness. Malate can also overcome inhibition caused by dinitrophenol.

Pyruvate carboxylase activity can be monitored more directly by measuring the net accumulation of Krebs cycle intermediates formed from pyruvate or incorporation of pyruvate-carboxyl-14C to the cycle intermediates. Calcium (100 μM) inhibited both of these processes by as much as 75%. Studies with partially purified carboxylase confirm the concept that calcium is an effective inhibitor, and indicate that the effect may be due to competition with magnesium ion.

Mitochondria from vitamin D-deficient or cortisone-treated animals exhibited higher carboxylase activities than those of control animals as measured by the indirect 14CO2 assay method. These changes are consistent with the known effects of those agents on calcium metabolism in vivo. Results are discussed in terms of the possibility that calcium may play an important role in regulating the initial steps of gluconeogenesis.

It is now generally recognized that the pathway of carbon from lactate or pyruvate to P-enolpyruvate during gluconeogenesis does not occur by a simple reversal of pyruvate kinase, but instead involves a cycle of carboxylation and decarboxylation (1–5). Most current data are consistent with a carboxylation of pyruvate to oxaloacetate by pyruvate carboxylase as first described by Utter and Keech (6), followed by decarboxylation of the 4-carbon compound to P-enolpyruvate catalyzed by P-enolpyruvate carboxykinase (7). Pyruvate therefore represents an important branch point metabolite. It may either be oxidized to acetyl-CoA and used for subsequent energy production and lipid biosynthesis or it may be carboxylated to initiate the process of gluconeogenesis. Thus it is logical to expect that the metabolism of pyruvate will be efficiently regulated in common with that of other major branch point metabolites (8).

One means of regulating pyruvate metabolism, not heretofore considered, is that regulation which might be directed by changes in ion distribution within the cell. This possibility is especially intriguing considering that pyruvate metabolism is a mitochondrial function and in light of the efficient ion transport machinery of these organelles. Also, both pyruvate carboxylase and dehydrogenase are ion-activated enzymes (5, 9), and might be expected to exhibit differences in activity depending on the nature of the mitochondrial ion content. The carboxylase is especially interesting in this respect in that magnesium functions not only in the Mg-ATP substrate complex, but also as an allosteric activator (10). In view of the known inhibitory effects of calcium on many magnesium-activated enzymes (11), and the active calcium-accumulating process of mitochondria (12), we decided to assess the effects of calcium on pyruvate metabolism in intact rat liver mitochondria.

METHODS

All experiments were performed with mitochondria prepared from the livers or kidneys of adult male white rats of the Wistar strain. The isolation procedure used was a modification of the method of Schneider (13). Sucrose, 0.37 M, was used in place of isotonic sucrose, and 100 μM ethylene glycol biss(β-aminoethyl ether)-N,N'-tetraacetic acid was included initially to bind calcium released during homogenization resulting from calcium carried over in the vascular space of the tissue. Lipid on the walls of the centrifuge tube was removed after each spin by absorbing it on cleansing tissue. The mitochondria were suspended...
in 0.37 M sucrose after preperation (approximately 30 mg of protein per ml), and used as soon as possible thereafter. Protein concentration was determined by the biuret method (14).

Production of $^{14}$C-carbon dioxide from pyruvate-$1^{-14}$C was followed by incubating the mitochondria in the appropriate medium in stopped 25-ml Erlenmeyer flasks containing a center well. A small glass vial with 0.2 ml of hydroxide of Hyamine (Packard) was placed in each center well and the incubation was continued for 10 to 20 min at 37° in a thermostated Dubnoff type shaker bath. At the conclusion of the desired incubation time, 0.1 ml of 2 N H$_2$SO$_4$ was injected into the medium through the rubber stopper, and shaking was continued for 1 hour to allow time for complete diffusion of the CO$_2$ to the Hyamine. The glass vials were then removed and dropped directly into scintillation vials filled with 10 ml of toluene-based scintillation fluid containing 5 g per liter of 2,5-diphenyloxazole (POPOP) and 50 mg per liter of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP). In most instances the scintillation fluid also contained 37.5% (v/v) methyl-Cellosolve to allow accommodation of water vapor which had condensed on the outer surface of the center well vials. The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer with gain and window settings appropriate for $^{14}$C.

When $^{14}$C-labeled Krebs cycle intermediates were separated from pyruvate-$1^{-14}$C, the method of von Korff (15) was used. Mitochondria were incubated for 10 min in the appropriate medium and the reaction was stopped by adding perchloric acid to a final concentration of 5%. Contents of the flask were transferred immediately to glass centrifuge tubes and kept chilled at 0° for 15 min. Following removal of protein by centrifugation, the supernatants were adjusted to pH 6.5 with 0.5 M triethanolamine-HCl and centrifuged, and the two extracts combined. Calcium and magnesium were then determined by atomic absorption spectroscopy on a Beckman flame photometer, again by comparison with standards of known identity. Potassium, magnesium, and calcium as follows. Concentrated perchloric acid was added to 2 ml of the undiluted mitochondrial suspension to a final concentration of 6%, the mixture was kept on ice for 10 min with occasional stirring, and the denatured protein was centrifuged off. The supernatant was decanted and saved, and the protein pellet was dispersed and re-extracted in an additional 2 ml of 6% perchloric acid. Again the mixture was centrifuged, and the two extracts combined. Calcium in magnesium were then determined by atomic absorption spectrophotometry of the properly diluted extracts and comparison with standards of known concentration. Potassium was determined on a Beckman flame photometer, again by comparison with known potassium standards.

Pyruvate carboxylase was partially purified from lyophilized rat liver mitochondria according to the method of Scrutton and Utter (16). The procedure was followed up to the column chromatography step and represented a 15-fold purification. The preparation was free of malic and lactic acid dehydrogenase activities. Assays of each fraction were performed by coupling the carboxylase with malate dehydrogenase and monitoring oxidation of DPNH on a Beckman DB recording spectrophotometer at 340 m$\mu$ (17). In the early stages of purification, two assays of each fraction were performed, one without acetyl-CoA and malate dehydrogenase in order to correct for the lactic dehydrogenase activity which was present. Values obtained in this assay were subtracted from those for the complete system to obtain net carboxylase activity.

In some experiments, Krebs cycle intermediates were determined by direct fluorimetric assay of triethanolamine-CO$_2$-neutralized perchloric acid extracts of mitochondria. These assays were performed on fluorimeters designed and constructed at the Johnson Foundation electronics shop of the University of Pennsylvania, and equipped with Esterline-Angus recorders. Malate (18), pyruvate (17), isocitrate (19), ATP (20), ADP (21), and oxaloacetate (22) were determined essentially according to the methods cited. Citrate was determined by the citrate lyase method as described by Williamson (23).

Vitamin D-deficient rats were produced by maintaining the animals on a vitamin D-deficient diet obtained from General Biochemicals Corporation, Chagrin Falls, Ohio (GMI No. 180604). The animals were used 1 week or more after cessation of weight gain (24).

Some animals were given 2.5 mg of cortisone once a day for 4 days by subcutaneous injection. Mitochondria from the liver of these animals were prepared 4 hours after the last injection.

All $^{14}$C-labeled intermediates were purchased from New England Nuclear except for malate (Nuclear-Chicago). Pyruvate-$1^{-14}$C was dissolved in a stoichiometric amount of HCl and stored at -20° to prevent spontaneous degradation (15). Enzymes used for fluorimetric assays were obtained from Calbiochem with the exception of malate dehydrogenase (Worthington). All other reagents were obtained from commercial suppliers and of the highest purity available.

RESULTS

Fig. 1 illustrates the alternative pathways of pyruvate utilization possible in isolated rat liver mitochondria. It shows that pyruvate may either be decarboxylated to acetyl-CoA by the pyruvate dehydrogenase enzyme complex, with concomitant formation of DPNH and carbon dioxide, or that it may be carboxylated via pyruvate carboxylase to oxaloacetate. The latter enzyme requires ATP and bicarbonate as substrates. The dashed lines indicate the requirements of the enzyme for the magnesium and acetyl-CoA cofactors (6). Of course acetyl-CoA and oxaloacetate generated by these enzymes may then condense to form citrate and the other intermediates of the Krebs cycle. Alternatively, because the equilibrium constant for malate dehydrogenase is very much in favor of malate formation (18), considerable amounts of oxaloacetate may be reduced directly to malate and fumarate without the necessity of moving carbon in the normal oxidative direction of the cycle. Malate may then leave the mitochondrion to provide carbon for gluconeogenesis or the urea cycle (1).

A careful consideration of Fig. 1 allows several predictions to...
be made about the characteristics of pyruvate metabolism. For example, if mitochondria are oxidizing pyruvate-1-14C, the rate of 14C-carbon dioxide production should be dependent not only on pyruvate dehydrogenase activity which forms 14CO2 directly, but also on the rate at which oxaloacetate is supplied to the system to draw acetyl-CoA into the Krebs cycle, i.e. on pyruvate carboxylase activity. This is true for two reasons: first, acetyl-CoA is a potent inhibitor of pyruvate dehydrogenase activity (25); and second, the limited pool of free coenzyme A available in isolated mitochondria is quickly depleted as the acetyl-CoA pool increases (26). Free coenzyme A is required for dehydrogenase activity, and is liberated when oxaloacetate condenses with acetyl-CoA to form citrate. Thus, the oxaloacetate functions not only by removing an inhibitor of pyruvate dehydrogenase, but also by providing a substrate.

Because of these facts, agents which affect the activity of either pyruvate dehydrogenase or carboxylase should change the rate of 14C-carbon dioxide production from carboxyl-labeled pyruvate. However, if mitochondrial ATP pools are depleted by the addition of uncoupler, then pyruvate carboxylase should be rendered inactive because of lack of a substrate. True uncoupler should cause an inhibition of 14CO2 production, and under these conditions agents affecting the carboxylase should lose their effectiveness, while those acting on pyruvate dehydrogenase would retain their action. Finally, if an alternative source of oxaloacetate, such as malate, is supplied to the system, the rate of 14C-carbon dioxide production should again become independent of pyruvate carboxylase activity, and agents which alter the activity of that enzyme would lose their effectiveness.

Fig. 2 shows the amount of 14CO2 produced by rat liver mitochondria from carboxyl-labeled pyruvate under several different conditions. With the criteria given above, the data show that calcium was a potent and relatively selective inhibitor of pyruvate carboxylase. Note that in Experiment I, the uncoupler dinitrophenol caused a striking inhibition of 14CO2 production, reflecting its postulated effect of precluding pyruvate carboxylase activity. Calcium, 50 μM, also caused a significant inhibition, but more importantly when uncoupler and calcium were added together there was no further decrease in carboxylase activity caused by calcium beyond that caused by dinitrophenol alone. This indicated that the effect of calcium was at the carboxylase locus rather than at the dehydrogenase. Experiment II shows that catalytic amounts of malate added to the control system caused no change in 14CO2 production. Thus the carboxylase was sufficiently active under these conditions to form adequate amounts of oxaloacetate for acetyl-CoA removal. The same amount of malate was able to overcome the inhibitory effects of dinitrophenol completely, which is consistent with the concept that uncoupler inhibition is due to precusion of oxaloacetate formation. Malate was also able to overcome the inhibitory effects of calcium, again indicating that calcium acts on the carboxylase. The third experiment (III) of Fig. 2 illustrates that, if a labeled Krebs cycle intermediate is used as substrate, then dinitrophenol caused a marked and calcium a slight stimulation of 14CO2 production, in contrast to the results observed with pyruvate.

Table 1 shows the effect of calcium concentration on 14CO2 production from carboxyl-labeled pyruvate. Note that as little as 5 μM calcium caused a significant inhibition, that there was a progressive inhi-
bition with increasing calcium concentration, and that again the effects of calcium disappeared in the presence of dinitrophenol.

It was possible that the lack of a response to calcium in the presence of dinitrophenol was a result of inability of the mitochondria to accumulate calcium under these conditions. However, it seemed that enough calcium might reach intramitochondrial sites by diffusion to cause at least a partial inhibition if a calcium-sensitive site were operative. To examine this possibility, we attempted to restore carboxylase activity in dinitrophenol-treated mitochondria by supplementing the system with ATP and oligomycin. Oligomycin was included to inhibit dinitrophenol-stimulated ATPase activity (27). Data are shown in Fig. 3. Note the gradual restoration of $^{14}$CO$_2$ production to control levels as the ATP concentration was increased. As soon as some carboxylase activity was restored, the system again became sensitive to calcium, indicating that sufficient calcium had penetrated by diffusion (no ATP-supported calcium accumulation is possible because of the presence of oligomycin) to exert detectable control. In the absence of carboxylase activity (no ATP), calcium caused no inhibition beyond that due to uncoupler.

Mildvan, Scrutton, and Utter (28) have recently reported that oxamate is an effective inhibitor of purified pyruvate carboxylase. This suggested an additional method for testing the reliability of the $^{14}$CO$_2$ assay technique, this time with an inhibitor not actively accumulated by the mitochondrion. Data are shown in Fig. 4, and indicate that, as with calcium, oxamate caused significant inhibition in the absence of dinitrophenol, but not in its presence. This agent thus seemed relatively specific for pyruvate carboxylase in intact mitochondria, although much less efficient than calcium. The limited effectiveness could be due to low permeability of the mitochondrial membrane, a lack of an active accumulation process for oxamate, or both.

On the basis of the experiments described above, it appeared that calcium was a potent and relatively selective inhibitor of pyruvate carboxylase. It was, however, desirable to have a more direct method of estimating carboxylase activity in order to strengthen this concept further. The method chosen was based

### Table I

<table>
<thead>
<tr>
<th>Component added</th>
<th>Activity</th>
<th>Activity</th>
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<tbody>
<tr>
<td>Calcium</td>
<td>Dinitrophenol</td>
<td>cpm $^{14}$CO$_2$</td>
</tr>
<tr>
<td>2.5</td>
<td>5</td>
<td>4855</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>5019</td>
</tr>
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<td>5</td>
<td>3400</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>3358</td>
</tr>
</tbody>
</table>

FIG. 3. Restoration of $^{14}$C-carbon dioxide-producing capability (pyruvate carboxylase activity) and calcium sensitivity in dinitrophenol-treated mitochondria by ATP. The incubation medium and conditions were the same as described for Fig. 2, except that 2.5 μg per ml of oligomycin (OLIGO) and 50 μM dinitrophenol (DNP) were also included. Calcium chloride, 50 μM, was added to the indicated group. The point labeled CONTROL indicates the amount of $^{14}$C-carbon dioxide formed when only oligomycin and no dinitrophenol was present. Mitochondrial protein concentration, 2.5 mg per ml.

FIG. 4. Effect of sodium oxamate on $^{14}$CO$_2$ production from pyruvate-1-14C in the presence and absence of dinitrophenol (DNP). Conditions and medium used for the incubation were the same as those described for Fig. 2. $^{14}$CO$_2$ production in the absence of oxamate was taken as the control value in each case. Mitochondrial protein, 2.1 mg per ml, was used. Conc., concentration.
on one first suggested and used by Haslam and Krebs (20) in which carboxylase activity is estimated by the increment in total 14C-Krebs cycle intermediates formed from pyruvate-1-14C. Any carboxyl-labeled pyruvate oxidized via pyruvate dehydrogenase will lose all of its 14C as 14CO2, while that which is carboxylated retains 14C and contributes it to oxaloacetate, and hence to the other Krebs cycle intermediates. Extracts of mitochondria which had oxidized pyruvate-1-14C were chromatographed as described under "Methods" to separate unutilized pyruvate from labeled Krebs cycle components. A typical separation of known labeled intermediates is shown in Fig. 5, and is the same as that reported by von Korff (15). Identity of the components corresponding to each peak was done by comparing the Rf value with those obtained from columns loaded with a single radioactive species. Alanine, which was not included in this experiment, elutes with Fractions 6 to 8, and is thus well separated from those shown. α-Ketoglutarate, also not shown, elutes in a low broad peak between Fractions 120 to 140. Acetate and the ketone bodies elute between Fractions 20 to 30, but any of these intermediates formed from pyruvate-1-14C would not contain 14C, and thus not interfere with elution patterns determined by radioactivity measurements.

Fig. 5 depicts the elution patterns obtained from extracts of mitochondria which had been oxidizing pyruvate-1-14C over a 10-min interval in the presence or absence of 100 μM calcium. The exact amount of 14C-citrate and pyruvate present in the twin peak was determined as described under "Methods." Citrate and malate normally constituted about 90% of the intermediates detected, but in this case considerable amounts of aspartate were also generated. Fumarate made up the bulk of the remainder. When 100 μM calcium was included in the incubation medium the amount of each intermediate fell dramatically, and consequently unutilized pyruvate increased as illustrated. The amount of each intermediate formed was estimated assuming that the specific activity of each was equal to that of the added pyruvate (see "Discussion"). This calculation indicated a 75% inhibition of carboxylase activity by 100 μM calcium.

Carboxylase activity was also evaluated by assaying each Krebs cycle intermediate fluorimetrically before and after pyruvate oxidation. Data are shown in Table II. Again note the significant decrease in total intermediates generated when 100 μM calcium was included in the system. It is especially significant that control levels of ATP were maintained in the presence of 100 μM calcium. Incubation medium and conditions were the same as those described for Fig. 2, except that 1.0 μC of pyruvate-1-14C was used. Preparation of extracts was described under "Methods." Mitochondrial protein concentration, 6 mg per ml.
of calcium. This is a further indication that the effect of calcium is due to a direct inhibition of enzyme activity rather than depletion of a critical substrate.

Partial purified pyruvate carboxylase was also inhibited by calcium. Experiment I of Table III indicates that the extent of calcium dehydrogenase and monitoring oxidation of DNPH at 340 nm. The complete reaction medium contained 100 mM Tris-Cl (pH 7.4), 6.7 mM MgCl₂, 20 mM KHCO₃, 10 mM sodium pyruvate, 3.3 mM sodium ATP, 0.2 mM acetyl-CoA, 0.1 mM DPNH, and 5 μg of malate dehydrogenase. The reaction rate was constant for more than the indicated 3-min interval. Concentrations are given as millimolar.

<table>
<thead>
<tr>
<th>Intermediate</th>
<th>Endogenous*</th>
<th>Control</th>
<th>Control minus endogenous</th>
<th>Calcium</th>
<th>Calcium treated minus endogenous</th>
<th>Dinitrophenol</th>
<th>Dinitrophenol-treated minus endogenous</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>11.7</td>
<td>11.3</td>
<td>55</td>
<td>10.4</td>
<td>37</td>
<td>1.05</td>
<td>3</td>
</tr>
<tr>
<td>Malate</td>
<td>6</td>
<td>61</td>
<td>43</td>
<td>18</td>
<td>10</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Citrate</td>
<td>8</td>
<td>62.5</td>
<td>4.7</td>
<td>4.0</td>
<td>2.6</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
<td>L-o-Leucinitrate</td>
<td>0.6</td>
<td>1.2</td>
<td>5.8</td>
<td>4.4</td>
<td>2.6</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
<td>a-Ketoglutarate</td>
<td>1.4</td>
<td>5.8</td>
<td>4.4</td>
<td>4.4</td>
<td>2.6</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
<td>Total Krebs cycle intermediates</td>
<td>15.9</td>
<td>130.5</td>
<td>114.6</td>
<td>65.0</td>
<td>49.6</td>
<td>26</td>
<td>10.6</td>
</tr>
</tbody>
</table>

* Endogenous content of Krebs cycle intermediates.

**TABLE III**

**Inhibition of partially purified pyruvate carboxylase by calcium**

The enzyme was partially purified by the method of Scrutton and Utter (16) from lyophilized rat liver mitochondria. Assays were performed by coupling the carboxylase reaction with malate dehydrogenase and monitoring oxidation of DNPH at 340 nm. The complete reaction medium contained 100 mM Tris-Cl (pH 7.4), 6.7 mM MgCl₂, 20 mM KHCO₃, 10 mM sodium pyruvate, 3.3 mM sodium ATP, 0.2 mM acetyl-CoA, 0.1 mM DPNH, and 5 μg of malate dehydrogenase. The reaction rate was constant for more than the indicated 3-min interval. Concentrations are given as millimolar.
Pyruvate Carboxylase and Calcium

**FIG. 7.** Effects of calcium and dinitrophenol (DNP) on \(^{14}\)CO\(_2\) production from pyruvate-\(^{14}\)C by mitochondria from livers and kidneys of normal (+D) or vitamin D-deficient (−D) rats. The standard incubation medium and conditions were used, with calcium and 50 μM dinitrophenol added where indicated. All data are expressed per mg of mitochondrial protein.

**TABLE V**

<table>
<thead>
<tr>
<th>Cation content of freshly isolated rat liver and kidney mitochondria obtained from vitamin D-fed and vitamin D-deficient rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cation</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
</tr>
<tr>
<td>Calcium</td>
</tr>
<tr>
<td>Potassium</td>
</tr>
</tbody>
</table>

Mitochondria were extracted twice with 6% perchloric acid and assays for each cation performed as described in the text.

**Fig. 8.** Effect of calcium and dinitrophenol (DNP) on \(^{14}\)CO\(_2\) production from pyruvate-\(^{14}\)C by mitochondria from livers of normal or cortisone-treated rats. Incubation medium and conditions were the same as those described for Fig. 2. Dinitrophenol concentration, 50 μM. Results are expressed per mg of mitochondrial protein in every case.

mitochondria obtained from these two types of animals. In this case there is a very striking decrease in the calcium content of the kidney mitochondria, but the variation in content of the liver was greater than the mean difference so that a significant difference in calcium content of the mitochondria from the two different types of animals was not seen. Similarly, because of the same variability it was not possible to show a significant difference in the calcium content of liver mitochondria isolated from control and cortisone-treated animals.

Fig. 8 shows the results of a \(^{14}\)CO\(_2\) assay with mitochondria from animals previously treated with cortisone as compared with those from untreated controls. The greater amount of \(^{14}\)C-carbon dioxide formed and higher sensitivity of dinitrophenol in the cortisone-treated group indicated that these mitochondria also had an enhanced pyruvate carboxylase activity. At any given level of added calcium, the activity of the system was greater in the cortisone-treated group.

**DISCUSSION**

As mentioned at the outset, pyruvate carboxylase is thought to play an important role in the process of gluconeogenesis. The carboxylase-carboxykinase carbon shunt from pyruvate to P-enolpyruvate seems to explain satisfactorily the known incorporation of \(^{14}\)C-bicarbonate to glucose which occurs with lactate or pyruvate as substrate and the observed distribution of \(^{14}\)C in glucose synthesized from specifically labeled pyruvate (32). Also, the carboxylase has been shown to have sufficient activity per mg of liver tissue to account for observed rates of glucose production (2). Moreover, tissues unable to perform gluconeogenesis, such as brain, heart, and spleen, are devoid of pyruvate carboxylase, while such highly gluconeogenic tissues as liver and kidney are rich in the enzyme (33). Finally, the proposed carbon shunt effectively circumvents pyruvate kinase, the reversal of which is thermodynamically and kinetically unfavorable under physiological conditions (34).

Several mechanisms for regulating pyruvate utilization have been postulated. Most emphasize the contrasting requirements of pyruvate dehydrogenase and pyruvate carboxylase. For instance, Serutton, Keech, and Utter (35) have shown that the latter enzyme consists of two half-reactions, a CO\(_2\)-capturing step during which an enzyme-biotin-CO\(_2\) complex is formed and the actual carboxylation when the complex donates its carboxyl group to pyruvate, thereby forming the product oxaloacetate. The first of these steps utilizes ATP as an energy source and also shows an absolute requirement for acetyl-CoA as an allosteric activator (35). Acetyl-CoA is, of course, the product of pyruvate dehydrogenase, while the DPNH produced by the dehydrogenase can be used to generate ATP. Thus, dehydrogenase activity is linked to the formation of two components necessary for carboxylase activity. Moreover, the dehydrogenase is self-limiting in that two of its products, acetyl-CoA and DPNH, have been shown to inhibit the reaction severely (25). DPNH also shunts oxaloacetate, the product of pyruvate carboxylase, to-
ward malate formation. This may favor transfer of carbon from mitochondria to cytosol, a necessity for effective gluconeogenesis in those species in which P-enolpyruvate carboxykinase is extramitochondrial (1). It has therefore been postulated that acetyl-CoA, ATP, and DPNH are important in shifting utilization of pyruvate away from oxidation and energy production in favor of carboxylation and gluconeogenesis (36).

In keeping with this proposal is the fact that these same three components are all products of fatty acid oxidation, and that this process is markedly enhanced in virtually every circumstance in which enhanced gluconeogenesis is observed (36). This had led to the suggestion that fat mobilization and oxidation are of prime importance in initiating gluconeogenesis by producing the agents responsible for regulating pyruvate metabolism (37). However, there are several drawbacks to this interpretation. First of all, the $K_c$ of acetyl-CoA for pyruvate carboxylase is extremely low (<20 $\mu M$) (5), while mitochondrial levels of this metabolite are probably 200 $\mu M$ or higher (26). It is therefore doubtful that significant regulation of carboxylase can normally be exerted by changes in the concentration of acetyl-CoA unless different acetyl-CoA compartments exist, as Fritz has proposed (39). Furthermore, ATP levels in livers perfused with fatty acids do not rise as expected, but instead actually fall to some extent (37), again negating effective activation of carboxylase activity. Also, Walter, Paetkau, and Lardy (2) and Haynes (39) have shown that mitochondria oxidizing pyruvate do not show an increase in carboxylating activity when a fatty acid is added to the system, although there is a marked increase in mitochondrial oxidation reduction potential and a fall in pyruvate dehydrogenase activity. Thus, it appears that acetyl-CoA, ATP, and DPNH may be more important as determinants of pyruvate dehydrogenase than of pyruvate carboxylase activity.

Finally, one further requirement for efficient gluconeogenesis, in addition to rapid carboxylation and carboxykinase activities, is restricted activity of pyruvate kinase. This is necessary in order to prevent recycling of carbon from P-enolpyruvate back to pyruvate. It has been known for some time that calcium is an effective inhibitor of the kinase (40), and there is some evidence that calcium might regulate glycolytic flux by inhibiting at this point (41, 42). This led Gevers and Krebs (43) to suggest that mitochondrial loss of calcium may play a role in regulating gluconeogenesis by effectively raising cytoplasmic calcium levels and turning off pyruvate kinase. The kinase is a cytoplasmic enzyme (40).

Pyruvate carboxylase, on the other hand, is primarily mitochondrial in distribution (33). Gevers and Krebs (43) thus proposed that calcium loss from mitochondria might activate pyruvate carboxylase and exert a dual stimulation of gluconeogenesis. This second prediction appears to have been purely speculation, as there was no literature report of carboxylase control by calcium at that time. It may have been based on the known requirements of the enzyme for ATP and magnesium, and the fact that calcium is known to inhibit many magnesium-activated enzymes. The present data comprise the first indication that intramitochondrial carboxylase is inhibited by calcium, although there is one recent report of calcium inhibition of isolated yeast carboxylase (44).

More important than simple observations of carboxylase inhibition by calcium are the facts that this inhibition can be shown in intact mitochondria and in some situations requires extremely minute amounts of calcium. In the experiment reported in Table I, an amount of calcium representing only 10$^\circ$ of the endogenous pool was added at the lowest level, and yet it produced a significant (17$^\circ$) inhibition of $^{14}$C-carbon dioxide production. It should be pointed out that not all mitochondrial preparations exhibited such a high degree of sensitivity toward calcium. This is undoubtedly due to the nature of the $^{14}$CO$_2$ test system which is a function of oxaloacetate generation as described earlier. Those mitochondria able to generate oxaloacetate from other sources such as endogenous pools of aspartate or malate, or both, would be expected to show less sensitivity to calcium. This is true because carboxylase activity is not as closely matched to pyruvate dehydrogenase activity under these circumstances. Thus while several preparations showed the degree of sensitivity indicated in Table I most were less sensitive. In 20 more typical experiments 50 $\mu M$ calcium caused a mean inhibition of 25$^\circ$, while 100 $\mu M$ calcium inhibited an average of 35$^\circ$. If the degree of inhibition observed with dinitrophenol (approximately 50$^\circ$) represents 100$^\circ$ inhibition of carboxylase activity, then these values indicate about 50 and 70$^\circ$ inhibition of carboxylase activity at 50 and 100 $\mu M$ calcium, respectively. It is important that with any given preparation of mitochondria the amount of $^{14}$CO$_2$ produced in replicate experiments showed less than 5$^\circ$ variation. Increasing calcium concentration always caused a corresponding increase in the degree of inhibition. For these reasons comparisons made within a single experiment are more reliable than those between several different experiments.

Other data indicate that the actual carboxylase sensitivity may not be as great as the $^{14}$CO$_2$ experiments indicate. The incorporation of $^{14}$C into Krebs cycle intermediates, which represents a more direct assay, was not significantly lowered until added calcium reached a concentration of 25 $\mu M$. Also, $^{14}$C-carboxylic acid experiments often indicated greater than 70$^\circ$ inhibition of carboxylase at 100 $\mu M$ calcium, while the column experiments and fluorometric assays indicated about 50$^\circ$ inhibition at this concentration. Thus, there may be other calcium-sensitive sites contributing to the observed decreases in $^{14}$C-carboxylic acid production. One possibility is at the level of the pyruvate dehydrogenase enzyme complex. However, calcium has been reported to stimulate partially purified preparations of this enzyme (9). Also, calcium often caused a slight stimulation of $^{14}$C-carboxylic acid production in the dinitrophenol-treated mitochondria, which would suggest enhanced pyruvate dehydrogenase activity. Perhaps most important is the observation that 50 $\mu M$ Ca++ inhibits $^{14}$CO$_2$ production by 40$^\circ$ in the A1'-oligomycin experiment, while it is only slightly more effective (50$^\circ$) in the coupled system of the same experiment.

This indicates that carboxylase function is a necessity for any inhibitory effect of calcium. Furthermore, it indicates a high degree of sensitivity of the enzyme to calcium because under these conditions (dinitrophenol plus oligomycin) it is known that active calcium transport is inhibited.

Part of the apparent discrepancy between the results obtained by assessing carboxylase activity by $^{14}$CO$_2$ evolution and by measurement of labeled Krebs cycle intermediates is undoubtedly a result of the fact that the assumption was made that each labeled intermediate had the same specific radioactivity as that of the added pyruvate. This was certainly a good approximation when dealing with the control situation (Table II) in which case total endogenous mitochondrial intermediates were only 10$^\circ$ of that seen after a 10-min control incubation. However, in in-
hibited states (Ca++ or dinitrophenol added) the endogenous intermediates represented 25 to 40% of the total. Thus, depending upon the degree of isotope exchange, the estimation of substrate concentration by the method of von Korff (15) may have been too high with a consequent underestimation of the degree of carboxylase inhibition. Also, the amount of mitochondrial protein used in experiments for determining the labeled and unlabeled Krebs cycle intermediates was usually double that used for the $^{14}$C02 experiments. If the effects of Ca++ are not normalized to the same mitochondrial protein concentration and the endogenous substrate concentration subtracted from the total, then the data in Tables I and II are quite comparable. In the experiment shown in Table I 50 $\mu$M calcium or 33.3 $\mu$moles per mg of mitochondrial protein led to a 59% inhibition of the control rate of decarboxylation, whereas in the case of the experiment recorded in Table II 100 $\mu$M or 35.5 $\mu$moles per mg of protein led to an inhibition of 57% of the control activity. Thus, when appropriate corrections for differences in protein content and endogenous substrate are used there is a good agreement between the two methods, further emphasizing the validity of the $^{14}$C02 method as an indirect measure of carboxylase activity.

Other possible sites of calcium sensitivity, which would be reflected in the rate of $^{14}$CO2 production, are any of the Krebs cycle reactions. This is true not only because the cycle forms CO2 directly at the isocitrate dehydrogenase and α-ketoglutarate dehydrogenase loci, but also because the rate of cycle activity is another parameter of oxaloacetate generation, an important regulatory factor as discussed earlier. Furthermore, at least one site in the cycle (isocitrate dehydrogenase) (42, 45) has previously been reported to be calcium-sensitive. However, calcium at the highest concentrations used in our experiments actually caused a slight increase in $^{14}$CO2 produced from uniformly labeled malate-$^{14}$C as shown in Fig. 2. This suggests that isocitrate dehydrogenase was not an important determinent of calcium inhibition under our conditions. Moreover, it emphasizes the degree of control that pyruvate carboxylase activity exerts on pyruvate dehydrogenase activity, since in the presence of calcium the Krebs cycle may actually be turning slightly more rapidly, yet $^{14}$CO2 from carbonyl-labeled pyruvate decreases as a result of inhibition of carboxylation. Also, dinitrophenol, which markedly enhances cycle activity, still causes an inhibition of $^{14}$CO2 production by preceding carboxylase action. It is equally clear that the inhibitory effects of calcium cannot be attributed to a fall in ATP levels as emphasized by the data in Table II. This conclusion is reinforced by the observation that 50 to 100 $\mu$M ADP does not inhibit $^{14}$CO2 production, although it would be expected to impose the same energy drain as an equivalent amount of calcium. Dinitrophenol, on the other hand, exerts its inhibitory effects by depleting mitochondrial ATP stores (Table II).

The fact that partially purified pyruvate carboxylase seems less sensitive to calcium than does the enzyme in intact mitochondria is perhaps significant. It may reflect the amplification of control achieved by an active cation transport system or the fact that free magnesium concentration within the mitochondrion is significantly less than total magnesium. The latter condition would effectively raise the Ca++:free Mg++ ratio, a situation which would allow more effective inhibition by calcium. Of course, the discrepancy may also indicate that the apparent carboxylase inhibition in intact mitochondria involves an indirect mechanism, a possibility which cannot be fully evaluated at this time.

The observation that malate, fumarate, and citrate account for practically all of the products of pyruvate carboxylase activity in isolated mitochondria is in good agreement with the data of Walter et al. (2), Stuart and Williams (46), and Haynes (39). It is also consistent with the extremely small pools of mitochondrial and cellular oxaloacetate which have been shown. Even though oxaloacetate is the immediate product of pyruvate carboxylase, it must either condense with acetyl-CoA immediately to form citrate or be reduced to malate. This suggests that the mitochondrial pyridine nucleotide oxidation-reduction state may be the most important of the three factors mentioned earlier in shunting carbon toward gluconeogenesis. Such control has been indicated by the work of Stuart and Williams (46). Also, Williamson et al. (47) have shown that the carbon flux from malate to citrate was decreased by increases in oxidation-reduction potential brought about by fatty acid oxidation. We have shown a similar response by monitoring pyruvate-$^{1-14}$C incorporation to Krebs cycle intermediates when oxaloacetate and carnitine are added to the medium. These components caused an increased malate to citrate ratio with no change in total carboxylation. Furthermore, Williamson, Kreisberg, and Felts (37) have shown a 2-fold increase in oxidation-reduction potential of liver pyridine nucleotides following fatty acid perfusion. These observations all suggest that the stimulatory effect of fatty acid oxidation on gluconeogenesis may be due more to an increased mitochondrial oxidation-reduction potential than to an increase in acetyl-CoA or ATP. However, this form of control does not represent control of pyruvate carboxylase, but rather control of product utilization. The effects of calcium described in this paper, on the other hand, represent a direct control of pyruvate carboxylase activity.

The enhancement of pyruvate carboxylation noted during vitamin D deficiency and following cortisone treatment is particularly interesting. Both vitamin D and cortisone are known to alter calcium metabolism in several ways which are in opposition to one another, and it is possible that the observed changes in carboxylase activity reflect a change in cellular calcium distribution. For example, it has been known for many years that vitamin D enhances intestinal transport of calcium (48, 49), and DeLuca et al. (31) have shown impaired mitochondrial calcium accumulation during vitamin D deficiency. Vitamin D added in vitro causes a restoration of mitochondrial calcium transport, although a number of other steroids do not (31). Harrison and Williams (48), Williams et al. (49), and Kimberg, Schacter, and Schenker (50) have all noted an antagonistic effect of cortisone on vitamin D-stimulated gut transport of calcium. Kimberg and Goldstein (51) have also reported that mitochondria isolated from animals treated with cortisone or other glucocorticoids were less able to bind calcium than were those from untreated controls. Cortisone has been used clinically to correct hypercalcemia due to vitamin D overdosage (49). Our observations that mitochondria from either vitamin D-deficient rats or cortisone-treated rats show enhanced carboxylase activity may represent another example of vitamin D-cortisone antagonism. However, it is also obvious that there is a striking difference in the response of liver and kidney mitochondria to vitamin D deficiency, and this difference is correlated with the difference in their content of calcium (Table V).
The present observations thus raise the possibility that a change in the intracellular distribution of calcium ion may play a role in regulating the activities of key enzymes in cytosol and mitochondria, and could possibly be of significance in the hepatic parenchymal cell when this cell changes from glycolytic to gluconeogenic activity. However, considerably more work must be carried out to substantiate such a hypothesis.

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REFERENCES
Regulation of Pyruvate Carboxylase Activity by Calcium in Intact Rat Liver Mitochondria

George A. Kimmich and Howard Rasmussen

J. Biol. Chem. 1969, 244:190-199.

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