Regulation by Calcium Ions of Pyruvate Carboxylation, Pyruvate Transport, and Adenine Nucleotide Transport in Isolated Rat Liver Mitochondria

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The activity of pyruvate carboxylase in isolated, tightly coupled, rat liver mitochondria was estimated by measuring the incorporation of H\(^{14}\)CO\(_3\) into acid-stable metabolites in the presence of pyruvate and ATP. The addition of a given amount of Ca\(^{2+}\) (10 to 100 nmol/mg of protein) to isolated mitochondria catalyzing the incorporation of H\(^{14}\)CO\(_3\) into acid-stable intermediates at a steady state rate caused a rapid decrease in the rate of H\(^{14}\)CO\(_3\) incorporation, and the establishment of a new inhibited steady state rate. This effect corresponded with the uptake of Ca\(^{2+}\) (monitored with the use of \[^{45}\]Ca) from the incubation medium by the mitochondria. When the steady state rate of H\(^{14}\)CO\(_3\) incorporation was measured in the presence of various fixed concentrations of added Ca\(^{2+}\), it was found to decrease as the concentration of intramitochondrial Ca\(^{2+}\) increased from 21 (no added Ca\(^{2+}\)) to 120 nmol/mg of protein (100 nmol/mg of added Ca\(^{2+}\)). Inhibition by Ca\(^{2+}\) was more pronounced at low concentrations of pyruvate. Thus a 50% inhibition of the rate of H\(^{14}\)CO\(_3\) incorporation was observed after the addition of either 25 or 10 nmol/mg of exogenous Ca\(^{2+}\) at 7.5 and 1 mm pyruvate, respectively. Inhibition of H\(^{14}\)CO\(_3\) incorporation by Ca\(^{2+}\) could be completely reversed by the subsequent addition of Mn\(^{2+}\) (25 to 100 nmol/mg). An increase in intramitochondrial Ca\(^{2+}\) (from 20 to 50 nmol/mg of protein) was accompanied by 25% increase in the activity of active form of pyruvate dehydrogenase.

Elevated concentrations of intramitochondrial Ca\(^{2+}\) (50 nmol/mg of protein) were associated with (a) an 80% inhibition of the rate of mitochondrial ATP transport and a decrease of 50% in the pool of intramitochondrial adenine nucleotides available for exchange with exogenous \([^{14}\text{C}]\text{ATP}\) (measured at 0\(^{\circ}\)); (b) a 40% inhibition of the rate of mitochondrial pyruvate transport measured at 6\(^{\circ}\) in the presence of rotenone and antimycin A; (c) a 20% decrease in the intramitochondrial pyruvate concentration; (d) a small increase in the intramitochondrial concentration of acetyl-CoA; and (e) no significant change in the intramitochondrial concentrations of total ATP and ADP. A control experiment showed that an increase in intramitochondrial Ca\(^{2+}\) from 20 to 65 nmol/mg of protein caused a small stimulation of the activity of the citric acid cycle when this was estimated by measuring the release of \(^{14}\)CO\(_2\) in the presence of \([\text{U-^{14}C}]\text{malate}\).

It is concluded that (a) inhibition by Ca\(^{2+}\) of H\(^{14}\)CO\(_3\) incorporation is the result of inhibition of the pyruvate carboxylase enzyme by CaATP\(^{2+}\) and Ca\(^{2+}\) present in the mitochondrial matrix as well as a decrease in the concentration of intramitochondrial pyruvate; (b) the data are consistent with the proposal that changes in the intramitochondrial concentrations of Ca\(^{2+}\) can contribute to the regulation of the activity of pyruvate carboxylase in the liver; and (c) under some conditions, changes in the mitochondrial Ca\(^{2+}\) concentrations may regulate cellular metabolism through their effects on mitochondrial ATP and pyruvate transport.

Calcium ions are known to influence a number of intracellular reactions, and it has been proposed that these ions play an important part in metabolic regulation (for review, see Refs. 1, 2). Recent data have provided evidence that mitochondria contribute to alterations in the intracellular distribution of Ca\(^{2+}\) in cells of a number of tissues including the liver, kidney, and heart (1-3). Recently, we have shown that the administration of insulin to rats by intraperitoneal injection is associated with an increase in the initial rate of Ca\(^{2+}\) uptake, and an enhancement of the retention of Ca\(^{2+}\) by mitochondria subsequently isolated from the liver (4). It was suggested that Ca\(^{2+}\) may mediate some of the intracellular effects of insulin on the liver, including effects on carbohydrate and lipid metabolism (4). This suggestion is supported by data from other laboratories which indicate that alterations in intracellular Ca\(^{2+}\) distribution may mediate, in part, the effects of insulin on lipolysis in adipose tissue (5) and glucagon on gluconeogenesis in the liver (6, 7). These observations suggest that hormones such as glucagon and insulin may induce significant fluctuations in the concentration of intramitochondrial Ca\(^{2+}\). Therefore, we have re-examined the effect of Ca\(^{2+}\) on the activity of an important enzyme of the gluconeogenic and lipogenic pathways, pyruvate carboxylase, in isolated liver mitochondria.

Although it is clear that the activity of isolated or purified pyruvate carboxylase is inhibited by Ca\(^{2+}\) (8-10), the results of previous studies of the effect of Ca\(^{2+}\) on the activity of this enzyme in isolated mitochondria have led to conflicting conclusions on the role of Ca\(^{2+}\) as an effector of pyruvate carboxylase in vivo (11, 12). In these studies (11, 12), relatively large concentrations of Ca\(^{2+}\) were employed, the mitochondria were often incubated in the presence of Ca\(^{2+}\) under conditions where they may have become partially uncoupled, and little care was taken to ensure that steady state rates of pyruvate carboxylase activity were being measured. This report describes the results of experiments in which the effect of low concentrations...
Calcium and Pyruvate Carboxylation

Isolation of Mitochondria — Male hooded Wistar rats (Institute of Medical and Veterinary Science, Adelaide, South Australia) weighing 250 g were starved for 15 h prior to the isolation of liver mitochondria. Isolation of the mitochondria was performed by homogenization of the liver in 250 mM sucrose which contained 2 mM Hepes and 0.5 mM EGTA, adjusted to pH 7.4 at 0° with KOH, followed by differential centrifugation (15). The mitochondria were washed twice in the sucrose/Hepes/EGTA medium and finally resuspended in 100 mM sucrose, 2 mM Hepes (pH 7.4) — 0.2 mM EGTA. EGTA was included in the wash medium in order to reduce the amount of Ca** taken up from the medium by the mitochondria. Mitochondrial protein and acceptor control ratios were measured as described by Reed and Bygrave (14). A YSI model 53 bioluminescence monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) was used to measure oxygen uptake. The mitochondria were tightly coupled as judged by the measurement of their acceptor control ratios which, in the presence of 1.25 mM succinate, were found to be 4.5 ± 0.4 for 12 different preparations of mitochondria. For a given mitochondrial preparation, the acceptor control ratio did not change significantly after four successive additions of ADP to the reaction mixture in the oxygen electrode chamber (0.5 μmol of ADP/4 mg of mitochondrial protein).

Pyruvate Carboxylation — The rate of pyruvate carboxylation was estimated by measuring the rate of H**14CO3 incorporation into acid-stable metabolites (15, 16) at 30° in a water-jacketed vessel arranged so that the incubation mixture could be stirred continuously with a magnetic stirrer. The incubation medium contained, in a final volume of 2 ml, adjusted to pH 7.4: 100 mM KCl, 50 mM Hepes, 5 mM potassium phosphate, 5 mM MgCl2, 7.5 mM sodium pyruvate, 3 mM ATP, 10 mM KH**14CO3, (5 μCi). Ca** at the concentrations indicated, and 3 mg/ml of mitochondrial protein. After equilibration of the mitochondria with the medium for 2½ min, the reaction was initiated by the addition of a mixture of ATP and KH**14CO3. At approximately three times, an aliquot (200 μl) of the incubation medium was withdrawn, mixed with 48 μl of 24% (w/v) HClO4 at 0°, and then centrifuged at 7000 × g (Eppendorf Microfuge 3200) for 2 min in order to remove the precipitated protein. The supernatant was placed in a clean tube, adjusted to pH 3 by the addition of 2 M K2CO3 (about 25 μl), the mixture allowed to stand for 15 min at room temperature, then cooled to 0°, and centrifuged for 2 min at 7000 × g to remove the KCIO4 precipitate. An aliquot of the supernatant (50 μl) was mixed with 10 ml of a scintillation mixture which consisted of 2.5 diphenyl oxazol (PPO) (7 g/liter) and 1.4 bis(2-5-phenyloxazolyl)benzene (POP0) (50 mg/liter) dissolved in a mixture of toluene 60% (w/v) and methoxyethanol 40% (v/v). The amount of radioactivity was determined by means of an Isocap-300 (Searle Analytic, Inc.) liquid scintillation counter.

The specific activity of the KH**14CO3 was determined by measuring the radioactivity of an aliquot of the KH**14CO3 solution dissolved in 100 μl of phenethanolamine. The rate of H**14CO3 incorporation was estimated from the slope of a plot of H**14CO3 incorporation as a function of time. The value of the slope and its associated standard error were determined by linear regression analysis. Control experiments showed that (a) H**14CO3 incorporation is completely dependent upon the addition of exogenous pyruvate and (b) maximum steady state rates of H**14CO3 incorporation can only be obtained in the presence of exogenous ATP.

In some experiments, the rate of pyruvate carboxylation was measured in the mannitol-sucrose medium described by Morikofer-Zwez et al. (12). This contained, in a final volume of 3 ml, adjusted to pH 7.4: 50 mM sucrose, 175 mM mannitol, 6.8 mM triethanolamine/ HCl, 25 mM potassium phosphate, 5 mM MgCl2, 5 mM pyruvate, 10 mM potassium bicarbonate, 2 mM ATP, and 3 mg/ml of mitochondrial protein. The addition of substrates to the reaction medium, and the measurement of pyruvate carboxylase activity were performed as described above.

Active Form of Pyruvate Dehydrogenase — The activity of the active form of the pyruvate dehydrogenase complex in liver mitochondria was estimated by means of a modification of the method described for its measurement in heart mitochondria by Schuster and Olson (17). Mitochondria (1.5 mg/ml) were preincubated at 30° in 25 ml Erlenmeyer flasks in a medium which contained, in a final volume of 12 ml: 100 mM KCl, 50 mM Hepes/KOH and 5 mM potassium phosphate (pH 7.0), 5 mM MgCl2, 5 mM succinate, 0.4 mM thiamin pyrophosphate, 0.5 mM dithioerythritol, and Ca** when present, at the concentrations indicated. After 3 min, the flasks were cooled to 0° and the activity of the active form of pyruvate dehydrogenase measured at 30° by following the decarboxylation of [1-**14C]pyruvate as described by Schuster and Olson (17). The reactions were performed in 2-ml volumes (0.06 μCi of [1-**14C]pyruvate) in 25-ml Erlenmeyer flasks with incubation times of 3, 6, and 9 min. The formation of **14CO2 was found to be a linear function of time over this period, and the rate of the reaction was estimated from the slopes of a plot of the amount of **14CO2 formed as a function of time, by means of linear regression analysis. In liver mitochondria, the presence of pyruvate carboxylase could interfere with the assay for pyruvate dehydrogenase by providing an alternative pathway for the conversion of CO2 from **14CO2 to pyruvate. Hence conditions used to measure the activity of pyruvate dehydrogenase, pyruvate carboxylase activity was found to be less than 20% of that measured in intact mitochondria, presumably as a result of a decrease in the concentration of acetyl-CoA. Therefore, it was considered that possible interference by pyruvate carboxylase would be small.

**14CO2 Release from [U-**14C]Malate — Rates of **14CO2 release from [U-**14C]malate were measured by means of the same procedure as that employed for the measurement of **14CO2 release from [1-**14C]pyruvate. All reaction components were mixed at 0°, and the reaction was initiated by raising the temperature from 0 to 30°.

Adenine Nucleotide, Pyruvate, and Acetyl-CoA — Mitochondria were separated from the incubation medium by centrifugation through a layer of silicone oil as described by LaNeve et al. (18). ADP and ATP present in neutralized perchoric acid extracts of the intra- and extramitochondrial fractions were measured spectrophotometrically using the procedures described by Williamson and Corkey (19). The amounts of pyruvate present in perchloric acid extracts of the mitochondrial pellets or incubation media were measured by the method of von Korff (20) immediately after preparation of the extracts. In order to determine the amount of pyruvate present in the mitochondrial matrix, pyruvate trapped in the extramitochondrial space (the sucrose-permeable space) was subtracted from the measured amount of total pyruvate associated with the mitochondrial pellet. The volumes of the sucrose-permeable and H2O-permeable spaces were determined with the use of [U-**14C]sucrose and H2O as described by Harris and van Dam (21).

The amounts of acetyl-CoA present in perchloric acid extracts of mitochondria after separation of the organelles from the incubation medium by centrifugation through a layer of silicone oil were estimated by a modification of the method of Piii et al. (22). The amount of **14C citrate formed after the condensation of excess (**14C)oxaloacetate and acetyl-CoA in the presence of citrate synthase was determined after separation of the (**14C)citrate from other components of the reaction mixture by ion exchange chromatography (23).

Calcium — Total intramitochondrial calcium was measured by atomic absorption spectroscopy. An aliquot of the incubation medium (about 5 to 10 mg of mitochondrial protein) was removed, neutralized with 25 μl of 1 M KOH, and centrifuged at 7000 × g (Eppendorf Microfuge 3200) for 2 min. The supernatant was removed and the mitochondrial pellet extracted twice with 1 ml of 5% (w/v) trichloroacetic acid. The trichloroacetic acid extracts were combined, and the amount of calcium present was measured with a model AA6 atomic absorption spectrometer (Varian, Springvale, Victoria, Australia). The calcium concentration were prepared from a standard solution of 0.1 M CaCl2 (Ajax Chemicals Ltd., Sydney, Australia) diluted with 5% (w/v) of Ca** on the steady state rate of pyruvate carboxylation in isolated rat liver mitochondria has been investigated under conditions where the mitochondria remain tightly coupled, and the intramitochondrial Ca** concentration is monitored. The results indicate that pyruvate carboxylation is inhibited by low concentrations of Ca** which are within the same range as those which activate pyruvate dehydrogenase. The data are consistent with (a) an effect of intramitochondrial Ca** on mitochondrial pyruvate transport; and (b) an effect of Ca** (and possibly Ca++ directly on the pyruvate carboxylation enzyme.

EXPERIMENTAL PROCEDURES

Calcium and Pyruvate Carboxylation

Calcium — Total intramitochondrial calcium was measured by atomic absorption spectroscopy. An aliquot of the incubation medium (about 5 to 10 mg of mitochondrial protein) was removed, neutralized with 25 μl of 1 M KOH, and centrifuged at 7000 × g (Eppendorf Microfuge 3200) for 2 min. The supernatant was removed and the mitochondrial pellet extracted twice with 1 ml of 5% (w/v) trichloroacetic acid. The trichloroacetic acid extracts were combined, and the amount of calcium present was measured with a model AA6 atomic absorption spectrometer (Varian, Springvale, Victoria, Australia). The calcium concentration were prepared from a standard solution of 0.1 M CaCl2 (Ajax Chemicals Ltd., Sydney, Australia) diluted with 5% (w/v).
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v) trichloroacetic acid. The standard deviation of values obtained for intramitochondrial calcium was determined from data obtained for 12 determinations of the calcium content of trichloroacetic acid extracts prepared in an identical manner from the same preparation of mitochondria.

The uptake and retention of "Ca by mitochondria were measured by the method of Reed and Bygrave (24).

Mitochondrial Transport of ATP and Pyruvate - ATP transport was measured using (U-14C)ATP in the "forward exchange" procedure, as described by Spencer and Bygrave (25). The inhibitor-stop method developed by Halesktr (26), which employs [3-14C]pyruvate and α-cyano-3-hydroxycinnamate as the transport inhibitor was used to measure pyruvate transport. The components of the various incubation media and temperatures used are described in the legend of the figures and in the text. The amounts of [14C]ATP or [14C]pyruvate transported into the mitochondria were calculated from the measured radioactivity of the mitochondrial pellet and the specific activity of the added [14C]ATP or [14C]pyruvate.

Materials - Phenethylamine was obtained from the Packard Instrument Co., Inc., Downers Grove, Ill., "Ca, NaH14CO3, sodium [1-14C]pyruvate, [3-14C]pyruvate, [U-14C]ATP, [U-14C]sucrose, and H2O from the Radiochemical Centre, Amersham, Bucks., U.K., dL-[4-14C]aspartic acid from New England Nuclear, atractylside and EGTA from the Sigma Chemical Co., St. Louis, Mo., and silicone oils No. 550 and 200 from Dow-Corning Australia Pty Ltd., Blacktown, New South Wales. Lactate dehydrogenase, pyruvate kinase, glucose-6-phosphate dehydrogenase, hexokinase, citrate synthetase, and glutamate oxaloacetate transaminase were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). α-Cyano-3-hydroxycinnamate (Aldrich Chemical Co., Gillingham, U. K.) was kindly provided by Drs. J. Clark, the Medical College of St. Bartholomew's Hospital, London, and A. P. Halestrap, University of Bristol Medical School, Bristol. All other reagents were of analytical grade.

RESULTS

Estimation of Initial Rate of Pyruvate Carboxylation in Isolated Mitochondria - The incorporation of H14CO3- into acid-stable metabolites has been used previously to measure the activity of pyruvate carboxylase in mitochondria isolated from a number of sources (15, 16, 23, 27). It has been shown that rates of pyruvate carboxylation obtained using this assay method are comparable with those measured by other procedures (15). When the incorporation of H14CO3- into acid-stable metabolites in isolated rat liver mitochondria is measured as a function of time, a significant lag period is observed when the time of preincubation of the mitochondria with all components of the medium with the exception of ATP and H14CO3- is short, e.g., 1/2 min (Fig. 1). A lag period is also observed at preincubation times of 1 and 1 1/2 min (data not shown) but is essentially abolished when the time of preincubation is increased to 2 1/2 min (Fig. 1). Other experiments (data not shown) have shown that the extent of the lag period is markedly dependent on the temperature and composition of the incubation medium. Thus the lag period is most pronounced at 37°C or when the KCl component of the incubation medium is replaced by 180 mM sucrose.

All subsequent experiments were performed under the conditions described under "Experimental Procedures" with a preincubation time of 2 1/2 min. The rate of H14CO3- incorporation, determined from the slope of the plot of the amount of H14CO3- incorporated as a function of time, was used to estimate the rate of pyruvate carboxylation.

Effect of Calcium on Rate of Pyruvate Carboxylation - The addition of calcium (50 nmol/mg of mitochondrial protein) to isolated mitochondria in the presence of pyruvate (7.5 mM) and HCO3- causes a rapid inhibition of the rate of H14CO3- incorporation into acid-stable metabolites (Fig. 2A). When 45Ca was used to monitor mitochondrial Ca++ uptake, the inhibition of pyruvate carboxylation was found to coincide with the entry of Ca++ into the mitochondria (Fig. 2A). In the presence of Ca++, the inhibited rate of H14CO3- incorporation remains essentially constant for at least 10 min (Fig. 2A). Inhibition by Ca++ is more marked when H14CO3- incorporation is measured at lower pyruvate concentrations. Thus in the presence of an initial concentration of 1 mM pyruvate, the rate of H14CO3- incorporation in the presence of 50 nmol/mg of Ca++ is 11% of the control rate (Fig. 2B). A reduction in the pyruvate concentration from 7.5 to 1.0 mM also leads to a decrease in the rate of H14CO3- incorporation in the absence of Ca++ (Fig. 2).

![Fig. 1 (left). Effect of the time of the preincubation on the initial rate of H14CO3- incorporation by isolated mitochondria. The complete reaction mixture contained, in a final volume of 4 ml: 100 mM KCl, 50 mM Hepes and 5 mM potassium phosphate (pH 7.0), 5 mM MgCl2, 7.5 mM sodium pyruvate, 3 mM ATP, 10 mM KH2CO3 (10 μCi mitochondrial membrane (3 mg of protein/ml). Prior to the addition of ATP and KH2CO3, the mitochondria were preincubated with the other components of the medium for 0.5 (○) or 2.5 (□) min. The incorporation of H14CO3- into acid-stable intermediates was initiated by the addition of a mixture of ATP and KH2CO3, and the amount of H14CO3- incorporated was measured as described under "Experimental Procedures."](image1)

![Fig. 2 (right). Effect of Ca++ on the steady state rate of H14CO3- incorporation by isolated mitochondria at 7.5 mM (A) and 1.0 mM (B) initial concentrations of pyruvate. The incorporation of H14CO3- into acid-stable intermediates in the presence (●) and absence (○) of Ca++ (50 nmol/mg) was measured as described under "Experimental Procedures" in a medium of the same composition as that described in the legend of Fig. 1. The mitochondria were preincubated for 2 1/2 min at 30°C with all components of the medium with the exception of ATP and KH2CO3. The incorporation of H14CO3- was initiated by the addition of a mixture of ATP and KH2CO3. At 3 min (indicated by the arrows), Ca++ was added. The amount of Ca++ taken up by the mitochondria (△) was measured by using 45Ca (1 μCi/ml) incubation medium as described under "Experimental Procedures" in an identical experiment in which KH2CO3 was replaced by unlabeled KHCO3.](image2)
The effect of varying the concentration of added Ca\(^{2+}\) on the rate of HCO\(_3^−\) incorporation was investigated. At each concentration of Ca\(^{2+}\) tested, a rapid inhibition by Ca\(^{2+}\) of H\(^{14}\)CO\(_3^−\) incorporation, similar to that shown in Fig. 2, was observed (data not shown). When plotted as a function of intramitochondrial Ca\(^{2+}\) concentration, the rate of H\(^{14}\)CO\(_3^−\) incorporation measured in the presence of 7.5 mM pyruvate was found to decrease as the intramitochondrial Ca\(^{2+}\) concentration increased (Fig. 3). The effects of Ca\(^{2+}\) at high and low pyruvate concentrations are compared in Table I. It can be seen that whereas at 7.5 mM pyruvate, 50% inhibition of the rate of H\(^{14}\)CO\(_3^−\) incorporation was observed at about 25 nmol/mg of added Ca\(^{2+}\), this degree of inhibition is achieved by the addition of only 10 nmol/mg of Ca\(^{2+}\) at 1.0 mM pyruvate. The data indicate that relatively low concentrations of added Ca\(^{2+}\) (e.g. 5 to 10 nmol/mg of protein) are sufficient to cause a significant inhibition in the rate of H\(^{14}\)CO\(_3^−\) incorporation.

The effect of Ca\(^{2+}\) on H\(^{14}\)CO\(_3^−\) incorporation was also investigated in the 50 mM sucrose, 175 mM mannitol medium used by Morikofer-Zwez et al. (12). When measured at 37° by means of a procedure identical with that employed for the experiment described in Fig. 2, the degree of inhibition of H\(^{14}\)CO\(_3^−\) incorporation by Ca\(^{2+}\) (50 nmol/mg) was found to be 49%. This compares with values of 70% for the data of Fig. 2A obtained at 30° in a KCl medium and about 5% for the data reported by Morikofer-Zwez et al. (12).

A control experiment was performed in order to test the possibility that inhibition by Ca\(^{2+}\) of the rate of H\(^{14}\)CO\(_3^−\) incorporation may be due to a decrease in the rate of utilization of oxaloacetate (as a result of inhibition by Ca\(^{2+}\) of one or more reactions of the citric acid cycle). This was tested by measuring the effect of Ca\(^{2+}\) on the rate of \(^{14}\)CO\(_2\) release from [\(^{14}\)C]malate. The data show that an increase in intramito-

![Graph 1](image1)

**Fig. 3 (left).** Effect of increasing intramitochondrial calcium concentrations on the rate of pyruvate carboxylation and the activity of the active form of the pyruvate dehydrogenase complex in isolated mitochondria. The rate of pyruvate carboxylation (\(\mathcal{O}\)) was estimated by measuring the incorporation of H\(^{14}\)CO\(_3^−\) into acid-stable metabolites as described under "Experimental Procedures" under the conditions described in the legend of Fig. 2A. Varying concentrations of Ca\(^{2+}\) (0, 10, 25, 50, and 100 nmol/mg of mitochondrial protein) were added 3 min after H\(^{14}\)CO\(_3^−\) incorporation had been initiated, and the steady state rate of H\(^{14}\)CO\(_3^−\) incorporation in the presence of Ca\(^{2+}\) measured (cf. Fig. 2) and plotted as a function of the amount of intramitochondrial Ca\(^{2+}\) present at 10 min after Ca\(^{2+}\) addition. The values shown for the rates of H\(^{14}\)CO\(_3^−\) incorporation are derived from the slope, and its associated standard error, obtained by linear regression analysis of the data for H\(^{14}\)CO\(_3^−\) incorporation as a function of time, as described under "Experimental Procedures." The data shown were obtained from one of five similar experiments which gave identical results. The activity of the active form of the pyruvate dehydrogenase complex (\(\mathcal{O}\)) was measured as described under "Experimental Procedures" after the mitochondria (1.5 mg/ml) had been preincubated for 3 min at 30° in a medium which contained, in a final volume of 12 ml: 100 mM KCl, 50 mM Heps, 5 mM MgCl\(_2\), 5 mM potassium phosphate, 0.4 mM thiamin pyrophosphate, 0.5 mM dihydrothiurylethyl, and exogenous Ca\(^{2+}\) at 0, 13, 28, and 53 nmol/mg of protein. The activity of the active form of pyruvate dehydrogenase is plotted as a function of the intramitochondrial Ca\(^{2+}\) concentration measured at the end of the 3-min preincubation period in an identical experiment. The data shown for the activity of pyruvate dehydrogenase are the pooled results of three similar experiments. Intramitochondrial Ca\(^{2+}\) was measured as described under "Experimental Procedures."
Chondrial Ca\(^{2+}\) from 21 to 64 nmol/mg of protein causes a small increase in the rate of \(^{14}\)CO\(_2\) release (Fig. 4). The average increase in a series of four experiments was 13 ± 5% (mean ± S.E.). By contrast, a significant inhibition of the rate of \(^{14}\)CO\(_2\) release is observed in the presence of arsenite, an inhibitor of the reactions catalyzed by the pyruvate and \(\alpha\)-ketoglutarate dehydrogenase complexes (28). These data indicate that inhibition of the rate of \(^{14}\)CO\(_2\) incorporation by Ca\(^{2+}\) is not the result of an inhibition of one or more reactions of the citric acid cycle.

Effect of Manganese on Inhibition by Calcium of Pyruvate Carboxylation — Manganese ions have been shown to activate pyruvate carboxylase enzymes isolated from chicken and rat liver (29, 30) and to stimulate pyruvate carboxylation in isolated rat liver mitochondria (30). Moreover, studies with the isolated enzyme indicate that the Mn\(^{2+}\) reduces the inhibition of pyruvate carboxylase activity by Ca\(^{2+}\) (30). The effect of Mn\(^{2+}\) on the inhibition of pyruvate carboxylation by Ca\(^{2+}\) in isolated mitochondria was investigated in order to determine whether the inhibitory effect of Ca\(^{2+}\) could be reversed. The results show that inhibition by Ca\(^{2+}\) (60 nmol/mg) can be completely reversed by the addition of 125 nmol/mg of Mn\(^{2+}\) (Fig. 5). The reactivation of pyruvate carboxylation by Mn\(^{2+}\) in the presence of Ca\(^{2+}\) appears to be a relatively slow process because the reaction rate continues to increase for a period of about 3 min after the addition of the Mn\(^{2+}\) (Fig. 5). Results similar to those shown in Fig. 5 were obtained when (a) the concentration of Mn\(^{2+}\) used to reactivate pyruvate carboxylation was reduced from 125 to 65 nmol/mg in the presence of 50 nmol/mg of Ca\(^{2+}\) and (b) inhibition by 25 nmol/mg of Ca\(^{2+}\) was reversed by 50 or 125 nmol/mg of Mn\(^{2+}\) (data not shown). In some experiments, Mn\(^{2+}\) caused a small increase in the rate of pyruvate carboxylation in the absence of exogenous Ca\(^{2+}\) However, in all cases, the final rate of pyruvate carboxylation achieved in the presence of Mn\(^{2+}\) was found to be the same in the presence and absence of Ca\(^{2+}\) (Fig. 5).

Pyruvate Dehydrogenase Activity — The effect of Ca\(^{2+}\) on pyruvate dehydrogenase activity in coupled liver mitochondria was studied in order to compare the range of Ca\(^{2+}\) concentrations which may influence the activity of this enzyme with that which inhibits the rate of pyruvate carboxylation. The activity of the active form of pyruvate dehydrogenase was measured after mitochondria had been incubated with 3 min in the presence of succinate and in the presence or absence of various concentrations of Ca\(^{2+}\). Succinate was included in the incubation medium in order to (a) provide an energy source for the uptake of Ca\(^{2+}\) (31) and (b) reduce the proportion of the pyruvate dehydrogenase complex which is initially present in the active form (32). When the intramitochondrial concentration of Ca\(^{2+}\) is increased from 20 to 50 nmol/mg of protein, the activity of the active form of pyruvate dehydrogenase is increased by about 25% (Fig. 3). The major part of this increase occurs at intramitochondrial Ca\(^{2+}\) concentrations below 30 nmol/mg.

Intramitochondrial Concentrations of ADP, ATP, Pyruvate, and Acetyl-CoA — One mechanism by which Ca\(^{2+}\) ions could alter the activities of pyruvate carboxylase or pyruvate dehydrogenase is through changes in the intramitochondrial concentrations of ATP and ADP (12, 33). The levels of these adenine nucleotides were measured after separation of the mitochondria from the incubation medium by centrifugation through a layer of silicone oil (18). As the intramitochondrial concentration of Ca\(^{2+}\) increased from 20 to 30 nmol/mg of protein, no significant change in the concentrations of ATP or ADP, or the ratio of ATP to ADP, was observed under the conditions used for studies of the effect of Ca\(^{2+}\) on the active form of pyruvate dehydrogenase (Table II). At higher concentrations of intramitochondrial Ca\(^{2+}\) (60 nmol/mg of protein), a decrease in the intramitochondrial concentration of ATP and in the corresponding ratio of ATP/ADP is observed (Table II). The amount of ATP or ADP present in the incubation medium outside the mitochondria was also measured. For each adenine nucleotide this was found to be 38 ± 4% (n = 6) of the sum of the amounts of adenine nucleotide present in the mitochondria and the medium.

Intramitochondrial concentrations of ATP and ADP are more difficult to determine accurately in the case of the conditions used for the assay of pyruvate carboxylation because the incubation medium contains 3 mM ATP. The amounts of ATP and ADP associated with the mitochondria after separation of the organelles from the incubation medium by centrifugation through silicone oil were measured directly without making a correction for adenine nucleotide associated with the inter- and extramembrane spaces. No significant change in the amount of ATP or ADP associated with the mitochondrial fraction was observed when the intramitochondrial Ca\(^{2+}\) concentration increased from 21 to 70 nmol/mg of protein (Table III). At a higher concentration of Ca\(^{2+}\) (120 nmol/mg of protein), a decrease in the amount of ATP and an increase in the amount of ADP were observed (Table III). Therefore, it is concluded that an increase in intramitochondrial Ca\(^{2+}\) concentration of up to 70 nmol/mg of protein does not cause a significant change in the intramitochondrial concentrations of ATP and ADP under the conditions used for measurement of the rate of pyruvate carboxylation.

Two other mechanisms by which Ca\(^{2+}\) could inhibit \(^{14}\)CO\(_2\) incorporation involve decreases in the intramitochondrial concentration of either pyruvate or acetyl-CoA, a substrate and activator of pyruvate carboxylase, respectively. At 1 mM pyruvate, the addition of Ca\(^{2+}\) (50 nmol/mg) was found to cause a small but significant decrease in the amount of intramitochondrial pyruvate (Table IV). Measurement of the volume of the sucrose-inaccessible space (i.e. the apparent volume of the mitochondrial matrix) in the same experiment showed that this decreased slightly in the presence of Ca\(^{2+}\) (Table IV).

### Table II

Intramitochondrial concentrations of ATP and ADP under conditions used for investigation of effect of Ca\(^{2+}\) on activity of pyruvate dehydrogenase

<table>
<thead>
<tr>
<th>Ca(^{2+})</th>
<th>Intramitochondrial adenine nucleotides</th>
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<tbody>
<tr>
<td>Added nmol/mg</td>
<td>ATP nmol/mg</td>
</tr>
<tr>
<td>0</td>
<td>20 ± 2</td>
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<tr>
<td>13</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>28</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>53</td>
<td>50 ± 6</td>
</tr>
</tbody>
</table>

Mitochondria were incubated with the indicated concentrations of exogenous Ca\(^{2+}\) under the conditions used for the preincubation prior to the determination of pyruvate dehydrogenase activity, as described in the legend of Fig. 3. Mitochondria were separated from the medium as described under ‘Experimental Procedures’ at 3 min after the beginning of the incubation. Intramitochondrial adenine nucleotides and Ca\(^{2+}\) concentrations were determined as described under ‘Experimental Procedures.’ The data are expressed as nanomoles of adenine nucleotide or Ca\(^{2+}\) per mg of mitochondrial protein (mean and S.E., n = 3 or 4) and represent the pooled results of two experiments.
The addition of Ca\(^{2+}\) (50 nmol/mg) causes an increase in the concentration of intramitochondrial acetyl-CoA (Table IV) at both 1 mM and 7.5 mM pyruvate. These results indicate that the inhibition by Ca\(^{2+}\) of H\(^{14}\)CO\(_3\)\(^{-}\) incorporation is not the result of depletion of intramitochondrial acetyl-CoA. This is supported by the observation that acetylcarbinitine (3 mM), a precursor of acetyl-CoA (34), has no effect on the rate of H\(^{14}\)CO\(_3\)\(^{-}\) incorporation in the presence of 50 nmol/mg of Ca\(^{2+}\) (data not shown). In a control experiment, in which pyruvate decarboxylation was inhibited by 2.5 mM arsenite, this concentration of acetylcarbinitine increased the rate of H\(^{14}\)CO\(_3\)\(^{-}\) incorporation from 0.25 to 2 nmol/min/mg.

**Adenine Nucleotide Transport**—In isolated mitochondria, the activity of pyruvate carboxylase is dependent on the transport of ATP, pyruvate, and H\(^{14}\)CO\(_3\)\(^{-}\) into the mitochondrial matrix. In order to determine whether the inhibition by Ca\(^{2+}\) of H\(^{14}\)CO\(_3\)\(^{-}\) incorporation may be due to an inhibition of the mitochondrial transport of ATP or pyruvate, the effects of Ca\(^{2+}\) on the transport of these metabolites were investigated. Since the inhibition of H\(^{14}\)CO\(_3\)\(^{-}\) incorporation is observed when Ca\(^{2+}\) has entered the mitochondrial matrix (Fig. 2A), effects of Ca\(^{2+}\) on mitochondrial anion transport were studied after the Ca\(^{2+}\) had been permitted to enter the mitochondria. Measurement of Ca\(^{2+}\) transport showed that under these conditions, the accumulated Ca\(^{2+}\) remained in the mitochondria, indicating that the organelles were not uncoupled.

Adenine nucleotide transport was measured in the same medium as that used for H\(^{14}\)CO\(_3\)\(^{-}\) incorporation studies. Initially, pyruvate and H\(^{14}\)CO\(_3\)\(^{-}\) were omitted from the reaction mixture, and the experiments conducted at 0\(^\circ\) in order to reduce the rates of adenine nucleotide transport to values which could conveniently be measured. In the absence of Mg\(^{2+}\), extramitochondrial [\(^{14}\)C]ATP rapidly equilibrates with a total of about 16 nmol/mg of adenine nucleotide in the mitochondrial matrix (Fig. 6A). The inclusion of 5 mM Mg\(^{2+}\) in the medium leads to a decrease in the maximum concentration of [\(^{14}\)C]-labeled adenine nucleotides in the mitochondrial matrix from 16 to 6 nmol/mg and a marked increase in the initial rate of [\(^{14}\)C]ATP transport into the matrix (Fig. 6A).

In the absence of Mg\(^{2+}\), Ca\(^{2+}\) decreases both the initial rate of [\(^{14}\)C]ATP transport (Fig. 6A) and the maximum concentration of intramitochondrial adenine nucleotides labeled with \(^{14}\)C from 16 to 11 nmol/mg (data not shown). In the presence of Mg\(^{2+}\), Ca\(^{2+}\) also causes a significant decrease in both the initial rate of [\(^{14}\)C]ATP transport and in the maximum concentration of intramitochondrial adenine nucleotides labeled with [\(^{14}\)C]ATP (Fig. 6B). The magnitude of the decrease in the pool of labeled intramitochondrial nucleotides is proportional to the amount of Ca\(^{2+}\) added (Fig. 6B).

Experiments using \(^{40}\)Ca showed that under the conditions used in the experiment described in Fig. 6B (preincubation of the mitochondria with 50 nmol/mg of Ca\(^{2+}\) for 4 min prior to the initiation of ATP transport), 44 nmol/mg of Ca\(^{2+}\) had been transported into mitochondria at the time [\(^{14}\)C]ATP transport was initiated. When Ca\(^{2+}\) is added to the mitochondria at the same time as the [\(^{14}\)C]ATP, its effects on the initial rate of [\(^{14}\)C]ATP transport and the extent of labeling of the intramitochondrial adenine nucleotides are reduced to about one-third of those seen when the mitochondria are permitted to accumulate Ca\(^{2+}\) prior to the addition of [\(^{14}\)C]ATP (cf. Fig. 6D). In this case, about 20 nmol/mg of Ca\(^{2+}\) were accumulated by the mitochondria at the time ATP transport was begun. These results are consistent with the idea that Ca\(^{2+}\) does not inhibit ATP transport, or reduce the pool of exchangeable intramito-

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**Table III**

Intramitochondrial concentrations of ATP and ADP under conditions used for investigation of effect of Ca\(^{2+}\) on activity of pyruvate carboxylase

Mitochondria were incubated with the indicated concentrations of Ca\(^{2+}\) under the conditions described in the legend of Fig. 2. Ca\(^{2+}\) was added 3 min after the initiation of H\(^{14}\)CO\(_3\)\(^{-}\) incorporation. Mitochondria were separated from the medium for adenine nucleotide determinations at 3 min after the addition of Ca\(^{2+}\), and aliquots of the incubation medium removed for the determination of intramitochondrial Ca\(^{2+}\) at 10 min after the addition of Ca\(^{2+}\). Intramitochondrial adenine nucleotide and Ca\(^{2+}\) concentrations were determined as described under "Experimental Procedures." The data are expressed as nanomoles of adenine nucleotides or Ca\(^{2+}\) per mg of mitochondrial protein (mean and S.E., n = 3 or 4) and represent the pooled results of two experiments.

<table>
<thead>
<tr>
<th>Ca(^{2+}) Added</th>
<th>Intramitochondrial adenine nucleotides</th>
<th>ATP</th>
<th>ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>21 ± 2</td>
<td>15.2 ± 0.9</td>
<td>5.8 ± 0.8</td>
</tr>
<tr>
<td>10</td>
<td>34 ± 4</td>
<td>14.8 ± 0.5</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td>25</td>
<td>46 ± 5</td>
<td>15.6 ± 0.5</td>
<td>6.9 ± 0.3</td>
</tr>
<tr>
<td>50</td>
<td>70 ± 8</td>
<td>15.4 ± 0.6</td>
<td>7.5 ± 0.6</td>
</tr>
<tr>
<td>100</td>
<td>120 ± 13</td>
<td>13.8 ± 0.9</td>
<td>10.1 ± 0.6</td>
</tr>
</tbody>
</table>

**Table IV**

Intramitochondrial concentrations of pyruvate and acetyl-CoA under conditions used for investigation of effect of Ca\(^{2+}\) on activity of pyruvate carboxylase

Mitochondria (6 mg of protein/ml) were incubated under the conditions described in the legend of Fig. 2 (final volume of 6 ml) in the presence of dextran 83,000, 80 mg/ml, and the indicated concentrations of Ca\(^{2+}\) and pyruvate. Ca\(^{2+}\) (when present) was added 2/3 min after the initiation of H\(^{14}\)CO\(_3\)\(^{-}\) incorporation. Eight aliquots (0.2 ml) were removed for the estimation of intramitochondrial pyruvate or acetyl-CoA between 2/3 and 6/3 min after the initiation of H\(^{14}\)CO\(_3\)\(^{-}\) incorporation. The amounts of pyruvate and acetyl-CoA present in perchloric acid extracts of the mitochondria after their separation from the medium by centrifugation through silicone oil were measured as described under "Experimental Procedures." At 7 min, H\(_2\)O (15 μCi) and [U-\(^{14}\)C]glucose (2 μCi) were added and aliquots (0.2 ml) of the incubation medium removed for estimation of the sucrose-impermeable and impermeable spaces as described under "Experimental Procedures." The results shown are the means ± S.E. of the pooled results of separate estimations (number given in parentheses) made in three different experiments.

<table>
<thead>
<tr>
<th>Concentration of pyruvate</th>
<th>Extraneous Ca(^{2+})</th>
<th>Intramitochondrial pyruvate</th>
<th>Intramitochondrial acetyl-CoA</th>
<th>Volume of sucrose-impermeable space</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>nmol/mg protein</td>
<td>μg/mg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>0</td>
<td>0.63 ± 0.05 (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>50</td>
<td>1.09 ± 0.00 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>1.58 ± 0.12 (8)</td>
<td>0.73 ± 0.07 (9)</td>
<td>0.74 ± 0.06 (8)</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>50</td>
<td>1.22 ± 0.08 (8)</td>
<td>0.91 ± 0.07 (11)</td>
<td>0.72 ± 0.08 (8)</td>
</tr>
</tbody>
</table>

Assuming that the volume of the matrix space which is accessible to pyruvate is equal to that of the sucrose-impermeable space (Table IV), these data indicate that Ca\(^{2+}\) causes a decrease in the concentration of intramitochondrial pyruvate from about 2.2 mM to 1.7 mM. It was not possible to measure the levels of intramitochondrial pyruvate accurately in the presence of 7.5 mM pyruvate because the amount of pyruvate present in the sucrose-accessible space is large in comparison to that present in the sucrose-impermeable space.
Calcium and Pyruvate Carboxylation

Pyruvate Transport - Since pyruvate is readily metabolized by isolated mitochondria, it was necessary to measure pyruvate transport under conditions in which its metabolism is reduced to a minimum. Therefore, the inhibitors antimycin A and rotenone were included in the incubation medium, and the experiments were performed at 6°C (cf. Halestrap (26)).

Estimation of the initial rate of [14C]ATP transport in the presence of Mg2+ and Ca2+ (50 nmol/mg) from the data of Fig. 6B yields a value of 18 nmol/min/mg at 0°C. It is likely that the rate of ATP transport increases at least 8-fold as the temperature increases from 0 to 30°C. Therefore, it is concluded that the inhibited rate of ATP transport is well in excess of the rate of pyruvate carboxylation at 30°C (Table 1).

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The omission of ATP from the medium resulted in the accumulation of the much lower levels of Ca2+ (11 nmol/mg) after the addition of 50 nmol/mg of exogenous Ca2+. However, the degree of inhibition of pyruvate transport was much greater than that observed in the presence of ATP. Thus the initial rate of [14C]pyruvate transport in the presence of Ca2+ was 37 ± 9% (n = 4) of that for control mitochondria. This result indicates that in contrast to the effect of Ca2+ on ATP transport, inhibition by Ca2+ of pyruvate transport is more marked when the cation is outside the mitochondrial matrix.

DISCUSSION

Effect of Ca2+ on Adenine Nucleotide and Pyruvate Transport - The measurement of ATP transport under conditions similar to those employed for the measurement of pyruvate carboxylation is complicated by the presence of Mg2+. It is likely that this cation alters the rate and extent of mitochondrial adenine nucleotide exchange through (a) activation of the enzymes nucleoside diphosphokinase, adenylate kinase, and Mg2+-stimulated ATPase which are located in the intermembrane space (35); (6) alteration of the properties of the mitochondrial pool of exchangeable adenine nucleotides; unless the cation is transported into the mitochondrial matrix.

The effect of Ca2+ on [14C]ATP transport was also studied at (a) 30°C in the same medium as that used in the experiment described in Fig. 6B; and (b) 0°C in the presence of 10 mM HCO3− and 7.5 mM pyruvate. In both situations, a result qualitatively similar to that shown in Fig. 6B, viz. a marked reduction in the rate of [14C]ATP transport and in the intramitochondrial pool of exchangeable adenine nucleotides was observed in the presence of Ca2+.

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Ca2+ has two major effects of ATP transport. It inhibits the rate of transport and decreases the size of the pool of intramito-
chondrial adenine nucleotides with which external ATP can exchange. Calculations (see "Results") have shown that at 30° the inhibited rate of ATP transport is not likely to be a rate-limiting step in the reactions involved in mitochondrial H\(^{14}\)CO\(_3\) \(-\) incorporation.

A decrease in the size of the pool of exchangeable intramitochondrial adenine nucleotides could result from the conversion of intramitochondrial ATP and ADP to AMP. The rate of exchange of the mononucleotide is much slower than that of the di- and trinucleotides (35). However, this explanation is considered unlikely because no significant decrease in the concentrations of total intramitochondrial ATP and ADP was observed in the presence of Ca\(^{2+}\). A second explanation for the decrease in the pool of exchangeable intramitochondrial adenine nucleotides is the formation of pools of CaATP\(^2-\) and CaADP\(^-\) in the mitochondrial matrix which do not readily exchange with exogenous ATP.

The inhibitory effect of Ca\(^{2+}\) on ATP transport reported here contrasts with an activation of adenine nucleotide translocation by Ca\(^{2+}\) reported by Spencer and Bygrave (25) and Meisner (36). This difference is most likely due to (a) the presence of phosphate ions, (b) the longer time of exposure of the mitochondria to Ca\(^{2+}\) before the addition of ATP, and (c) the higher concentration of ATP employed here. These conditions permitted most of the added Ca\(^{2+}\) to enter the mitochondria prior to the assay of ATP transport. In the experiments reported by Spencer and Bygrave (25) and Meisner (36), the added Ca\(^{2+}\) was most likely present on the outside of the inner mitochondrial membrane.

The inhibition by Ca\(^{2+}\) of mitochondrial ATP and pyruvate transport indicates that, under some conditions, changes in the cytoplasmic or mitochondrial Ca\(^{2+}\) concentration may contribute to the regulation of metabolism in these compartments through modification of the intracellular distribution of ATP and pyruvate.

Effect of Ca\(^{2+}\) on Pyruvate Carboxylase Activity — The data show that in isolated rat liver mitochondria, low concentrations of Ca\(^{2+}\) inhibit the rate of H\(^{14}\)CO\(_3\) \(-\) incorporation. Inhibition by Ca\(^{2+}\) is most pronounced at low levels (1 mM) of pyruvate. No marked decrease in the intramitochondrial concentrations of total ATP and acetyl-CoA or increase in the concentration of total ADP, accompany the inhibition. These observations indicate that the effect of Ca\(^{2+}\) is not mediated by a change in the intramitochondrial concentrations of these metabolites. In contrast, a decrease of about 20% in the concentration of intramitochondrial pyruvate and about 37% in the initial rate of pyruvate transport was observed in the presence of Ca\(^{2+}\). Therefore, it is likely that a decrease in the concentration of intramitochondrial pyruvate contributes to the inhibition by Ca\(^{2+}\) of H\(^{14}\)CO\(_3\) \(-\) incorporation, especially at low concentrations of exogenous pyruvate. However, changes in the concentration of this substrate do not appear to be able to account for the 90% inhibition of H\(^{14}\)CO\(_3\) \(-\) incorporation observed at 1 mM pyruvate. It is considered unlikely that inhibition by Ca\(^{2+}\) of mitochondrial HCO\(_3\) \(-\) transport contributes to the inhibition of H\(^{14}\)CO\(_3\) \(-\) incorporation since Elder and Lehninger (37) have shown that Ca\(^{2+}\) stimulates the accumulation of HCO\(_3\) \(-\) by isolated mitochondria.

The results of the ATP transport studies are consistent with the formation of a pool of CaATP\(^2-\) in the mitochondrial matrix. The inhibition of pyruvate carboxylase by CaATP\(^2-\) and Ca\(^{2+}\), in competition with MgATP\(^2-\) and Mg\(^{2+}\), respectively (8–10), may account for a significant part of the inhibition of H\(^{14}\)CO\(_3\) \(-\) incorporation. The proposed direct action of CaATP\(^2-\) or Ca\(^{2+}\) on pyruvate carboxylase is supported by the rapid nature of the inhibitory effect, its dependence upon the entry of Ca\(^{2+}\) into the mitochondrial matrix, the establishment of a steady state inhibited rate, and the ability of Mn\(^{2+}\), an antagonist of the interaction of Ca\(^{2+}\) with isolated pyruvate carboxylase (30), to reverse the inhibition by Ca\(^{2+}\). The rapid nature and kinetic properties of the inhibition are consistent with direct effects of CaATP\(^2-\) and Ca\(^{2+}\) on the reaction catalyzed by the pyruvate carboxylase enzyme, rather than an effect mediated by a slower process such as a possible phosphorylation or dephosphorylation of the enzyme (23).

The conclusions reached here differ from those of Walter et al. (12, 38) who reported little inhibition by Ca\(^{2+}\) of pyruvate carboxylation in isolated liver mitochondria in a mannitol/sucrose medium (12). In contrast, these authors observed that at high concentrations of sucrose, the rate of pyruvate carboxylation is markedly decreased, and the inhibitory effect of Ca\(^{2+}\) is more pronounced. In the present series of experiments, the inhibitory effect of Ca\(^{2+}\), tested in a medium of the same composition as the mannitol/sucrose medium used by Walter et al. (12, 38), was greater than that reported by these authors, although less than that observed in the KCl medium employed here. The apparent difference in the results obtained in the two laboratories may lie partly in the nature of the incubation media employed and in differences in the techniques used for the assay of pyruvate carboxylation (cf. Fig. 1).

Physiological Implications of Inhibition of Pyruvate Carboxylase by Ca\(^{2+}\) — The concentrations of endogenous intramitochondrial Ca\(^{2+}\) in isolated mitochondria were found to be generally no less than 20 nmol/mg of protein. The use of uncoupling agents or ionophores, such as A23187, to lower the concentration of endogenous Ca\(^{2+}\) was avoided because it is known that these compounds may alter a number of other metabolic parameters (39) which could in turn influence the activity of pyruvate carboxylase. Nevertheless, some insight into the behavior of the rate of pyruvate carboxylation at lower intramitochondrial Ca\(^{2+}\) concentrations can be gained by extrapolation of the curve of the rate of H\(^{14}\)CO\(_3\) \(-\) incorporation as a function of intramitochondrial Ca\(^{2+}\) concentration to lower Ca\(^{2+}\) concentrations (broken line, Fig. 3). Examination of the resulting curve (Fig. 3), as well as the data of Table I, indicates that changes in intramitochondrial Ca\(^{2+}\) concentration which lie within the estimated physiological range of 3 to 20 nmol/mg of protein (2, 3, 12) would have significant effects on the activity of pyruvate carboxylase. Moreover, the effects of Ca\(^{2+}\) were found to be more pronounced at concentrations of pyruvate (1 mM) which are close to the estimated physiological range of about 0.01 to 0.4 mM (0.01 to 0.4 μmol/g wet weight, summarized in Ref. 40).

Further evidence which is consistent with the proposal that pyruvate carboxylase can respond to changes in the intramitochondrial Ca\(^{2+}\) concentration likely to occur in vivo is provided by the observation that the lower range of intramitochondrial Ca\(^{2+}\) concentrations (20 to 40 nmol/mg) in which pyruvate carboxylation is inhibited is also that in which pyruvate dehydrogenase activity is increased. For the latter enzyme, there is some evidence that Ca\(^{2+}\) does play a role in the regulation of the reaction rate under physiological conditions (39, 41).

The results obtained with coupled isolated mitochondria indicate that Ca\(^{2+}\) can contribute to the regulation of the activity of pyruvate carboxylase in vivo. However, it will be necessary to assess how the regulation of this reaction by Ca\(^{2+}\)
is linked with regulation by other parameters, including the intramitochondrial concentrations of adenine nucleotides, acetyl-CoA, and pyruvate, and the oxidation-reduction potential.

Acknowledgment — We are grateful to Miss Maria Glistak for skilled technical assistance.

REFERENCES