Respiration-Dependent Transport of Carbon Dioxide into Rat Liver Mitochondria†

Joe Allen Elder and Albert Lester Lehninger*

ABSTRACT: Rat liver mitochondria respiring in a phosphate-free bicarbonate buffer accumulate substantial amounts of Ca^{2+}, to levels suggesting that the bicarbonate buffer is furnishing the required counteranion for Ca^{2+}. When the HCO_3^-–CO_2 buffer was labeled with ^{14}C, the isotope was accumulated in large amounts. The $^{45}Ca^{2+}$:^{14}C uptake ratio of 1.0 and the finding that the accumulated ^{14}C was completely released as a gaseous product, presumably CO_2, by exposure of the loaded mitochondria to dilute acid, indicated that $CaCO_3$ accumulated in the matrix. Up to about 300 nmol of Ca^{2+}/mg of protein, the accumulation of carbonate was as rapid as accumulation of phosphate from a phosphate-buffered medium. Carbonate accumulation also occurs when Ca^{2+} is replaced by Sr^{2+} or Mn^{2+}, but not when the divalent cation is Mg^{2+}. Entry of both Ca^{2+} and CO_3^{2-} is blocked by respiratory inhibitors, uncoupling agents, and by La^{3+} and ruthenium red, which are inhibitors of energy-linked Ca^{2+} uptake by mitochondria. Mersalyl and atractyloside do not inhibit accumulation of carbonate coupled to Ca^{2+} uptake. When phosphate is present, it competes with the accumulation of carbonate as the counteranion for Ca^{2+}. Kinetic analysis of the stimulation of oxygen uptake by anions in the presence of excess Ca^{2+} has revealed that HCO_3^- does not stimulate respiration whereas dissolved CO_2 does, thus identifying CO_2 as the permeant entering species of the bicarbonate buffer, the precursor of carbonate in the matrix. Diamox, a specific inhibitor of carbonic anhydrase, blocks the respiration-dependent accumulation of both Ca^{2+} and carbonate. It is concluded that carbonate accumulating with Ca^{2+} in the matrix of respiring mitochondria is formed from entering CO_2 by the action of carbonic anhydrase located in the mitochondrial inner membrane or matrix.

Bicarbonate is a major intracellular anion, which largely derives from the decarboxylation reactions of the tricarboxylic acid cycle in the mitochondrial matrix. However, relatively little information is available regarding the movement of bicarbonate or CO_2 through the mitochondrial membrane and its relationship to the transmembrane movements of other anions and cations.

In this paper we report the results of one approach to this problem. It is based on the fact that respiration-dependent accumulation of Ca^{2+} in the mitochondrial matrix takes place only if it is accompanied by entry of an electrically equivalent counteranion such as phosphate (Lehninger *et al.*, 1967; Gear *et al.*, 1967; Lehninger, 1972). We have found that a bicarbonate buffer system can replace phosphate as a source of counteranion for the accumulation of Ca^{2+} by respiring rat liver mitochondria. The entering species has been shown to be dissolved CO_2, which accumulates in a form tentatively identified as carbonate ion. The accumulation of both carbonate and Ca^{2+} by the respiring mitochondria is inhibited by Diamox (acetazolamide), an inhibitor of carbonic anhydrase. An abstract of this work has been published (Elder, 1972).

Experimental Procedure

Preparation of Mitochondria. Mitochondria were isolated from livers of male Sprague–Dawley albino rats in 220 mM mannitol, 70 mM sucrose, 0.5 mg/ml of bovine serum albumin, and 2 mM potassium Hepes[1] buffer (pH 7.4), or in 0.25 M sucrose by the procedure of Schnaitman and Greenawalt (1968), except that the final two washes were at 9750 g. The mitochondrial pellet was resuspended in isolation medium to 50 mg of protein/ml. Protein was determined by the method of Murphy and Kies (1960).

Incubation Medium. The incubation medium consisted of 120 mM KCl, 5 mM potassium Hepes, 10 mM potassium succinate, and 2 mM bicarbonate buffer (pH 7.4). To prepare this medium, aliquots of stock solutions of KCl, Hepes, and succinate were diluted to 0.9 of the final desired volume and the pH was adjusted to 6.55. The addition of 0.1 volume of 20 mM K_2CO_3 (pH 11.0) gave the above final concentrations and a final pH of 7.4, following equilibration at 25° for 15 min. This procedure, rather than gassing of a bicarbonate solution with CO_2, was chosen to allow preparation of ^{14}C-labeled bicarbonate buffers from aliquots of a ^{14}C-labeled K_2CO_3 stock solution without loss of radioactivity. At pH 7.4 and 25° the ratio of the HCO_3^- concentration to the CO_2 concentration is 10.5:1; the medium thus contains 1.83 mM HCO_3^- and 0.17 mM CO_2.

The incubations were carried out in 14-ml centrifuge tubes which were covered with two layers of Parafilm after addition of K_2CO_3. Additions made subsequent to the K_2CO_3 were injected through the Parafilm cover, which was quickly resealed.

Uptake of ^{14}C-Labeled HCO_3^-–CO_2. In the standard test system, rat liver mitochondria (3.75 mg of protein) were incubated at room temperature for 2 min in 10 ml of the medium containing ^{14}C-labeled HCO_3^-–CO_2 mixture. The tubes were then spun in the Sorvall RC2-B centrifuge at 20,000 rpm for 30 sec at 0–4° in the SE-12 rotor; the total time of the entire centrifugation was 2.5 min. The supernatant medium was decanted after removal of a 0.2-ml sample. The inner surface of the tube was wiped dry without disturbing the pellet. The pellet was dissolved in 0.5 ml of 10 mM NaOH and trans-

† From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received November 20, 1972.* Supported by Grant GM-05919 from the U. S. Public Health Service and by Grant GB-20519 from the National Science Foundation.

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; N_2ph, 2,4-dinitrophenyl; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone.

ferred with another 0.5 ml of NaOH to a vial containing 10 ml of scintillation fluid and 1 ml of NaOH. Both the supernatant sample and the solubilized pellet were counted with an equal amount of base present to prevent loss of CO_2 during counting. The dioxane-based scintillation fluid contained 100 g of naphthalene and 5 g of diphenyloxazole per l. The samples were counted in a Beckman LS-100 liquid scintillation system.

Uptake of $^{45}Ca^{2+}$. Samples from incubations with $^{45}Ca^{2+}$ were treated exactly as above, except that water was used instead of NaOH.

Oxygen Uptake and pH. Oxygen uptake was measured with a Clark electrode (see Lessler and Brierley, 1969); simultaneously the pH of the medium was recorded from a glass electrode and potentiometer.

Reagents. Carbonic anhydrase and mersalyl were obtained from Sigma Chemical Co., St. Louis, Mo., $^{45}CaCl_2$ from International Chemical and Nuclear Corp., Irvine, Calif., sodium [^{14}C]bicarbonate from New England Nuclear, Boston, Mass., and Diamox (sodium acetazolamide) from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

TABLE I: Effect of HCO_3^- + CO_2 on Ca^{2+} Uptake.[a]

Ca^{2+} Added (nmol/mg of Protein)	Ca^{2+} Uptake (nmol/mg of Protein)		
	Control (Cl as Anion)	P_i as Anion	HCO_3^- + CO_2 as Anion Source
80	76.3	79.3	79.5
160	97.0	158	160
240	93.8	236	239
320	88.3	9.3	270
400	85.6	14.4	224

[a] The control system contained 120 mM KCl, 10 mM potassium succinate, 5 mM potassium Hepes (pH 7.4), $^{45}Ca^{2+}$ as shown, and rat liver mitochondria (3.75 mg of protein) in a volume of 10 ml. The concentration of P_i and HCO_3^- + CO_2 was 2 mM. After a 2-min incubation at room temperature, the mitochondria were centrifuged and the $^{45}Ca^{2+}$ remaining in the supernatant was determined.

Results

Effect of HCO_3^- + CO_2 *on* Ca^{2+} *Uptake by Respiring Rat Liver Mitochondria.* In the first series of experiments (Table I) it is shown that a mixture of bicarbonate and CO_2 is capable of supporting respiration-linked accumulation of Ca^{2+} under conditions in which a permeant anion is required. Earlier work has shown (Lehninger *et al.*, 1967; Gear *et al.*, 1967; Lehninger, 1972) that in the absence of phosphate or other permeant anions, the uptake of Ca^{2+} supported by oxidation of succinate is limited to about 80–100 nmol of Ca^{2+}/mg of mitochondrial protein, regardless of the amount of Ca^{2+} available in the medium. This type of Ca^{2+} uptake is called "membrane loading" since the Ca^{2+} so accumulated does not pass into the matrix but is bound to specific sites on the mitochondrial membrane which require electron transport for stoichiometric activation (Gear *et al.*, 1967; Lehninger, 1972). Accumulation of Ca^{2+} by rat liver mitochondria in a KCl medium, but in the absence of permeant anions, is shown in Table I; a maximum of about 100 nmol of Ca^{2+} bound per mg of protein was observed. When the permeant anion phosphate was present, up to 236 nmol of Ca^{2+}/mg of protein was taken up; under these conditions phosphate was also accumulated (Chappell and Crofts, 1965); this process is called "matrix loading" (Lehninger, 1972). When the amount of Ca^{2+} in the medium exceeded about 240 nmol/mg of mitochondrial protein, the rapid respiration-dependent uptake of Ca^{2+} and phosphate is followed by a rapid discharge of both Ca^{2+} and phosphate from the mitochondria (Table I), in confirmation of earlier observations (Lehninger *et al.*, 1967; Chappell and Crofts, 1965; Rossi and Lehninger, 1964). Such release of Ca^{2+} and phosphate is prevented when ADP is also present in the medium, in which case Ca^{2+} loads up to 2500 nmol/mg may be retained quantitatively in the matrix (Vasington and Murphy, 1962; Carafoli *et al.*, 1965a,b). The role of ADP is not entirely clear but its presence apparently prevents osmotic swelling of the mitochondria by inducing precipitation of insoluble granules of calcium phosphate (*cf.* Lehninger *et al.*, 1967; Rossi and Lehninger, 1964; Carafoli *et al.*, 1965a,b; Greenawalt *et al.*, 1964).

As is shown in Table I, replacement of phosphate by a bicarbonate–CO_2 buffer at pH 7.4 resulted in accumulation and retention of Ca^{2+} by the respiring mitochondria in the absence of ADP, even when the amount of Ca^{2+} initially present was as high as 400 nmol/mg of protein (Table I). This type of experiment strongly suggested that the bicarbonate buffer system was yielding a counteranion, either HCO_3^- or CO_3^{2-}, for the Ca^{2+} entering the respiring mitochondria.

Respiration-Dependent Uptake of ^{14}C*-Labeled* HCO_3^- + CO_2 *during* Ca^{2+} *Uptake.* That one or more of the molecular or ionic species present in a bicarbonate buffer system at pH 7.4 actually entered the respiring mitochondria with Ca^{2+} was demonstrated in experiments in which the Ca^{2+} and the HCO_3^-–CO_2 buffer were labeled with ^{45}Ca and ^{14}C, respectively, in matched pairs of otherwise identical reaction systems. The experiments in Table II show the uptake of $^{45}Ca^{2+}$ and of labeled HCO_3^- + CO_2 buffer by respiring rat liver mitochondria exposed to increasing concentrations of Ca^{2+}, with the total bicarbonate buffer concentration held at 2.0 mM. Phosphate was not added to these systems. It is seen that both $^{45}Ca^{2+}$ and ^{14}C label were taken up by the respiring mitochondria. Moreover, the net amounts of labeled carbon accumulated were nearly equivalent to the amount of Ca^{2+} taken up over the entire range of Ca^{2+} concentrations added; the ^{45}Ca:^{14}C uptake ratio was found to be about 0.95 at Ca^{2+} concentrations up to 320 nmol and somewhat less at 400 nmol of Ca^{2+}/mg of protein. The ^{14}C label accumulated by rat liver mitochondria under identical conditions in the absence of Ca^{2+} averaged 60 nmol/mg of protein for 19 determinations. This value has been subtracted from the total amount of ^{14}C label accumulated in the presence of Ca^{2+} to give the net uptake recorded in Table II.

In view of the approximately 1:1 stoichiometry of $^{45}Ca^{2+}$ and net ^{14}C uptake it appears most likely that the anionic species accumulating in the mitochondria is the carbonate (CO_3^{2-}) anion, particularly in view of earlier observations which indicate that mitochondria become alkaline during respiration (Gear *et al.*, 1967; Chance and Mela, 1966; Addanki *et al.*, 1967), a condition which would favor accumulation of CO_3^{2-} in the matrix rather than HCO_3^-. Accumulation of organic carbon was excluded by the following type of experiment. Mitochondrial pellets in which Ca^{2+}-dependent uptake of ^{14}C-labeled HCO_3^- + CO_2 had taken

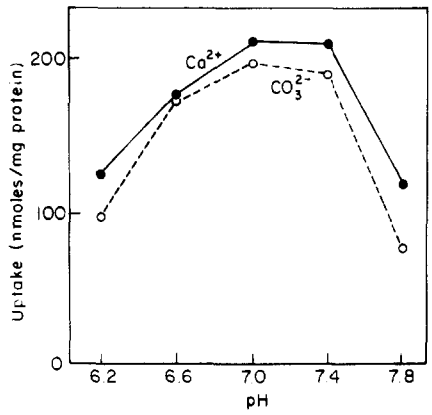

FIGURE 1: Effect of pH on Ca^{2+} and CO_3^{2-} uptake. Mitochondria were incubated as described in Table II except Ca^{2+} concentration was 240 nmol/mg of protein and the pH was adjusted from 6.2–7.8.

place were acidified and flushed with nitrogen for 15 min prior to counting. Less than 2% of the counts remained in the acidified aqueous phase, indicating that about 98% of the accumulated radioactivity was in a form volatile in acid, presumably as CO_3^{2-}. Evidently there was little or no incorporation of ^{14}C into nonvolatile intermediates of the tricarboxylic acid cycle. In the remainder of this paper it will be assumed that CO_3^{2-} is the form which accumulates with Ca^{2+}.

Data in Table II also show that Sr^{2+} and Mn^{2+} supported uptake of carbonate, although less actively than Ca^{2+}, but Mg^{2+} did not. This finding is consistent with much evidence that Ca^{2+}, Sr^{2+}, and Mn^{2+} are transported into mitochondria by the same mechanism, whereas Mg^{2+} is not accumulated by rat liver mitochondria (Lehninger *et al.*, 1967; Chappell *et al.*, 1963; Carafoli *et al.*, 1964, 1965a,b).

TABLE II: Uptake of $^{45}Ca^{2+}$ and ^{14}C-Labeled HCO_3^-–CO_2 by Respiring Mitochondria.[a]

Cation Added (nmol)/mg of Protein	Addns	Net Uptake (nmol/mg of Protein) $^{45}Ca^{2+}$	Net Uptake (nmol/mg of Protein) ^{14}C	Uptake Ratio $^{45}Ca^{2+}$:^{14}C
80	Ca^{2+}	65.9	66.4	0.99
160	Ca^{2+}	130	146	0.89
240	Ca^{2+}	197	223	0.88
320	Ca^{2+}	257	256	1.00
400	Ca^{2+}	197	238	0.82
240	Sr^{2+}		197	
240	Mn^{2+}		82	
240	Mg^{2+}		0	

[a] Rat liver mitochondria (3.75 mg of protein) were incubated in a closed tube for 2 min at room temperature in 120 mM KCl, 10 mM potassium succinate, 5 mM potassium Hepes, and 2 mM HCO_3^-–CO_2 (pH 7.4), with $^{45}Ca^{2+}$, Sr^{2+}, Mn^{2+}, or Mg^{2+} as shown. A duplicate set of tubes contained ^{14}C-labeled HCO_3^-–CO_2. The mitochondria were centrifuged and the radioactivity in the pellets was determined. The pellets containing ^{14}C label were dissolved in NaOH. The ^{14}C-uptake data were corrected by subtracting the amount of ^{14}C label accumulated by mitochondria under identical conditions in the absence of added Ca^{2+}.

TABLE III: Effect of Inhibitors and Uncouplers on Uptake of Ca^{2+} and CO_3^{2-}.[a]

		Uptake (nmol/mg of Protein) Ca^{2+}	Uptake (nmol/mg of Protein) CO_3^{2-}
Control		217	210
Cyanide	100 μM	58.8	20.9
Antimycin A	0.45 μM	4.0	0
Rotenone	0.25 μM	211	207
Rotenone (succinate omitted)		23.9	0
N_2phOH	50 μM	6.3	0
FCCP	0.50 μM	1.4	0
Oligomycin	6 μg/mg	211	198
La^{3+}	2 nmol/mg	5.2	0
Ruthenium red	1 nmol/mg	3.9	0
Mersalyl	10 μM	177	168
Atractyloside	1 μM	203	157

[a] Conditions were exactly as described in Table II except the Ca^{2+} concentration was 240 nmol/mg of protein.

Effect of pH. Figure 1 shows that the bicarbonate buffer system can support Ca^{2+} uptake by rat liver mitochondria over a wide range of pH, between pH 6.2 and 7.8, with an optimum at pH 7.0–7.4. Approximately equal net amounts of $^{45}Ca^{2+}$ and ^{14}C accumulated in the range pH 6.2–7.4.

Effect of Inhibitors of Respiration and Phosphorylation on Carbonate Accumulation. As is shown in Table III, the accumulation of both labeled carbonate and Ca^{2+} in the presence of succinate was inhibited by cyanide and by antimycin A, indicating dependence of their uptake on respiration. Rotenone, an inhibitor of electron transport at site I, did not inhibit succinate-supported Ca^{2+} and CO_3^{2-} uptake, as expected. Omission of succinate, particularly in the presence of rotenone, which blocks endogenous NAD^+-linked respiration, also caused failure of both Ca^{2+} and CO_3^{2-} to be accumulated. The uncoupling agents N_2phOH and FCCP blocked uptake of both ions, showing that energy-coupling mechanisms are involved. Oligomycin, however, failed to block uptake of either ion, as expected (Lehninger *et al.*, 1967).

Effect of Inhibitors of Mitochondrial Transport Systems. Experiments in Table III also show that the uptake of both Ca^{2+} and CO_3^{2-} is inhibited by La^{3+} (Mela, 1968; Lehninger and Carafoli, 1971) and ruthenium red (Moore, 1971), specific inhibitors of Ca^{2+} transport into respiring mitochondria. Failure of Ca^{2+} to be taken up thus results in failure of CO_3^{2-} to accumulate, presumably because the latter cannot accumulate without a countercation. On the other hand, mersalyl, an inhibitor of the phosphate–hydroxyl carrier of rat liver mitochondria (Tyler, 1968), and atractyloside, an inhibitor of the ADP–ATP carrier (Pfaff *et al.*, 1965), failed to inhibit either Ca^{2+} uptake or CO_3^{2-} uptake, showing the independence of both Ca^{2+} and CO_3^{2-} entry from movements of either phosphate or ADP under these conditions.

Effect of Phosphate on Accumulation of Carbonate. Addition of phosphate to the incubation medium caused inhibition of the respiration-coupled uptake of carbonate, without reducing

TABLE IV: Effect of Phosphate on Uptake of Ca^{2+} and CO_3^{2-}.[a]

Phosphate (nmol/mg of Protein)	Uptake (nmol/mg of Protein)	
	Ca^{2+}	CO_3^{2-}
0	210	213
120	206	108
240	209	60.0
360	42.4	6.4
480	12.4	4.4
600	10.5	
600 + mersalyl	211	211

[a] Mitochondria were incubated as described in Table II, except that the Ca^{2+} concentration was 240 nmol/mg of protein and phosphate was added as shown. The concentration of mersalyl was 10 μM.

TABLE V: Effect of ADP and Mg^{2+} on Uptake of Ca^{2+} and CO_3^{2-}.[a]

Additions	Ion Uptake (nmol/mg of Protein)	
	Ca^{2+}	CO_3^{2-}
Control	246	236
1 mM ADP	218	29.4
6 mM $MgCl_2$	266	260
ADP + $MgCl_2$	336	48.1

[a] The mitochondria were incubated as described in Table II; the Ca^{2+} concentration was 400 nmol/mg of protein. ADP and Mg^{2+} were added at the concentrations shown.

the uptake of Ca^{2+}, at phosphate concentrations up to 240 nmoles per mg of protein (Table IV). The degree of inhibition increased with the concentration of phosphate added. Phosphate is evidently accumulated preferentially by the mitochondria under these test conditions, in which the HCO_3^-–CO_2 concentration in the medium was held constant at 2.0 mM. When phosphate concentration was increased above 240 nmol/mg of protein, little or no accumulation of Ca^{2+} or CO_3^{3-} occurred, presumably because the entry of large amounts of Ca^{2+} and phosphate is followed by swelling and their release, since the reaction system did not contain ADP (*cf.* eq 1).

That phosphate was actually entering the mitochondria *via* the phosphate–hydroxyl transport system was shown by the effect of mersalyl (Tyler, 1968), an inhibitor of this system, which completely prevented the uptake of phosphate and allowed full accumulation of both Ca^{2+} and CO_3^{2-} (Table IV).

Effect of ADP and Mg^{2+} on Accumulation of Ca^{2+} and CO_3^{2-}. Table V shows the effect of ADP and Mg^{2+} on uptake of Ca^{2+} and CO_3^{2-}. In the presence of 1 mM ADP, Ca^{2+} uptake was normal in the presence of $HCO_3^- + CO_2$, but CO_3^{2-} accumulation was greatly reduced to about 15 per cent of its control value. As already seen in Table II, CO_3^{2-} does not accumulate with Mg^{2+} in the absence of Ca^{2+}; however, Mg^{2+} at 6 mM increased both Ca^{2+} and CO_3^{2-} uptake. The CO_3^{2-} uptake in the presence of ADP and Mg^{2+} was less than that with Mg^{2+} alone. In the presence of ADP most of the accumulated Ca^{2+} is accompanied by an anion other than CO_3^{2-}, presumably phosphate formed by the sequential action of adenylate kinase and mitochondrial ATPase on ADP. The phosphate so formed can effectively compete with CO_3^{2-}, as already seen above; calcium phosphate would thus be accumulated within the matrix.

Diamox Inhibition of Ca^{2+} and CO_3^{2-} Uptake. The uptake of both Ca^{2+} and CO_3^{2-} was greatly reduced by very low concentrations of Diamox, a specific inhibitor of carbonic anhydrase (*cf.* Maren, 1967) (Table VI). However, Diamox did not inhibit uptake of Ca^{2+} when phosphate replaced the HCO_3^-–CO_2 system as source of anions. These experiments therefore indicate that carbonic anhydrase or some other Diamox-sensitive factor is required for accumulation of CO_3^{2-}.

Identification of Dissolved CO_2 as the Entering Species. In a bicarbonate buffer system at pH 7.4 there are four species potentially capable of entering the matrix and furnishing the CO_3^{2-} anion which is tentatively concluded to be the form accumulating in the mitochondria with Ca^{2+} during respiration. They are (1) dissolved CO_2, (2) undissociated carbonic acid (H_2CO_3), (3) the bicarbonate anion (HCO_3^-), and (4) the carbonate anion (CO_3^{2-}). In order to determine which of these is actually the species passing through the mitochondrial membrane, and thus the precursor of the carbonate ion which presumably accumulated with Ca^{2+}, a series of kinetic tests was carried out. These tests utilized the well-known fact that the rate of the reversible hydration of dissolved carbon dioxide at pH 7.4 is relatively slow in the absence of carbonic anhydrase. For the uncatalyzed hydration of CO_2 the first-order rate constant is rather low, in the range 0.0257–0.044 sec^{-1} at pH 7.4 and 25° (Garg and Maren, 1972). The rate of hydration of CO_2, particularly at the low CO_2 concentrations used in our experiments, is low enough to be rate limiting for the respiration-coupled entry of Ca^{2+} into mitochondria, if CO_2 is the only species capable of furnishing its counteranion in the matrix and if its hydration is uncatalyzed.

As is shown in Figure 2 rat liver mitochondria in a bicarbonate buffer system yield, on successive additions of 80 nmol of Ca^{2+}/mg of protein, a series of jumps each followed by return to a constant state 4 respiratory rate. Each of these jumps is complete in about 40 sec. The Ca^{2+}: O stoichiometry for these respiratory jumps, with succinate as substrate, is about 3.95, equivalent to a Ca^{2+}: site ratio of 1.98, which is in close agreement with previously recorded values for the

TABLE VI: Effect of Diamox on Uptake of Ca^{2+} and CO_3^{2-}.[a]

Diamox (μM)	Uptake (nmol/mg of Protein)	
	Ca^{2+}	CO_3^{2-}
0	210	214
1	184	171
10	66.2	47.0
100	41.3	30.5
0	207	214
10	35.7	15.3
100	22.2	4.7

[a] Mitochondria were incubated as described in Table II with 240 nmol of Ca^{2+}/mg of protein and Diamox as shown.

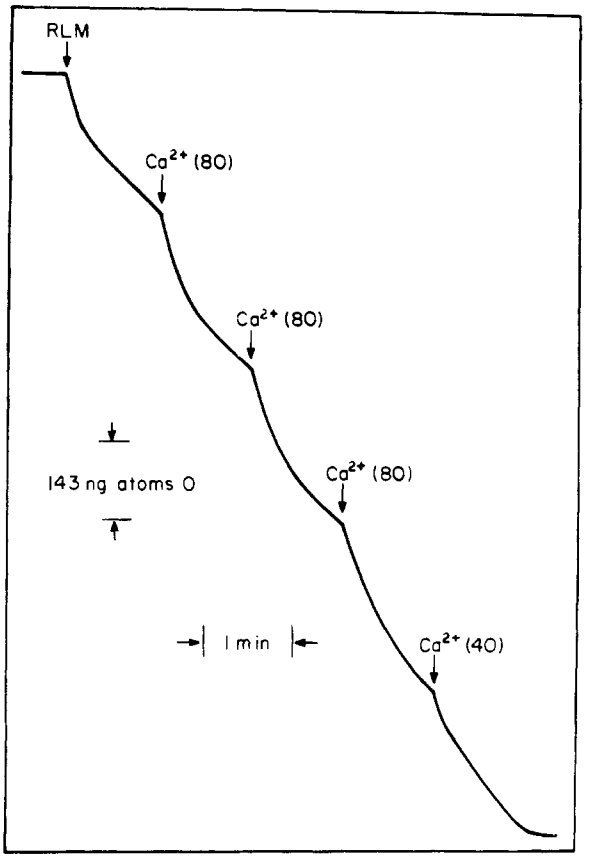

FIGURE 2: Ca^{2+} stimulated respiration in a HCO_3^- + CO_2 medium. The system contained 120 mM KCl, 5 mM potassium Hepes, 10 mM potassium succinate, and 2 mM HCO_3^- + CO_2 (pH 7.4) in a volume of 3 ml at 25°. Rat liver mitochondria (RLM, 7.5 mg of protein) and $CaCl_2$ (nmol/mg of protein) were added as indicated.

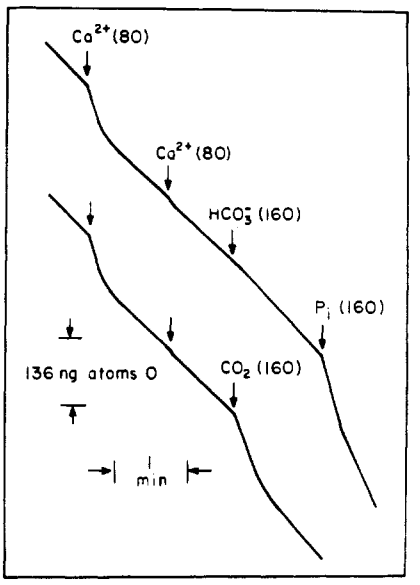

FIGURE 3: Test of CO_2 *vs.* HCO_3^- as permeant species. Mitochondria (7.5 mg of protein) were added to a medium containing 120 mM KCl, 10 mM potassium succinate, and 5 mM potassium Hepes (pH 7.4) in a volume of 2.9 ml at 25°. Ca^{2+}, P_i, CO_2, and HCO_3^- were added as indicated; amounts are given in nmoles per mg of protein. HCO_3^- was added as freshly prepared $KHCO_3$ solution, pH 8.2 (<1% CO_2); CO_2 was added as a freshly prepared 100% CO_2-saturated water solution (<1% HCO_3^-). The change in pH of the medium upon addition of HCO_3^- and CO_2 was $<+0.01$ and <-0.20 unit, respectively. The pH of the medium and the oxygen concentration were recorded simultaneously employing a dual-channel recorder.

stoichiometry between Ca^{2+} accumulation and electron transport (Rossi and Lehninger, 1964). This experiment was carried out with a previously equilibrated 2 mM HCO_3^-–CO_2 buffer system.

Tests were then made of the respiratory response of rat liver mitochondria to pulses of Ca^{2+} in a medium strongly buffered at pH 7.4 with 5 mM Hepes buffer, to which freshly prepared aqueous solutions of $KHCO_3$ or of CO_2 were added as source of the counteranion for the entering Ca^{2+}. The oxygen electrode traces in Figure 3 show the results of such tests. A first addition of 80 nmol of Ca^{2+}/mg of protein, with only the impermeant chloride anion present, was made to saturate the anionic sites on the membrane capable of loading Ca^{2+}; the usual respiratory jump ensued. However, addition of a second pulse of 80 nmol of Ca^{2+}/mg of protein evolved no stimulation of oxygen uptake, as expected when no permeant or potentially permeant anion is present; most of the second pulse of Ca^{2+} remains free in the medium (*cf.* Table I). When the permeant anion phosphate is now added, an immediate jump in oxygen uptake occurs, corresponding to entry of both Ca^{2+} and phosphate counteranion into the matrix (Chance, 1965). However, when an aliquot of a freshly prepared solution of bicarbonate, containing little or no dissolved CO_2, was added after the second pulse of Ca^{2+}, no jump in the oxygen uptake rate occurred, indicating that the addition of HCO_3^- in the absence of CO_2 supplied no permeant anion at a high enough rate to support entry of Ca^{2+} into the matrix. That the mitochondria in this test were still fully functional and responsive to a permeant anion was shown by a subsequent addition of phosphate, which produced an immediate increase in rate of oxygen uptake, corresponding to stoichiometric uptake of Ca^{2+} and phosphate. On the other hand, when a freshly prepared solution of CO_2 in water, presumably containing little or no HCO_3^- or H_2CO_3, was added to mitochondria after the second addition of Ca^{2+}, an immediate stimulation of oxygen uptake took place.

These experiments indicate that dissolved CO_2 can readily enter rat liver mitochondria and support maximum respiration-dependent uptake of Ca^{2+}, whereas the HCO_3^- anion cannot. This conclusion is consistent with the fact that the electrically neutral CO_2 molecule readily passes through membranes (Caldwell, 1958; Forster, 1969). The bicarbonate anion cannot pass the membrane readily, presumably because it is electrically charged and no specific carrier or transport system for HCO_3^- is apparently present in the mitochondrial membrane.

The second major conclusion from the experiments in Figure 3 is that if dissolved CO_2 is the entering species it must be very quickly converted into the CO_3^{2-} anion within the mitochondria in order to provide the counteranion for the rapidly entering Ca^{2+}. Presumably the conversion of dissolved CO_2 into the carbonate anion in the somewhat alkaline mitochondrial matrix occurs by the following steps (reactions 1 and 2). The rate-limiting step of this sequence would be expected to be the hydroxylation of CO_2 to bicarbonate (re-

$$CO_2\,(\text{diss}) + OH^- \rightleftharpoons HCO_3^- \qquad (1)$$

$$HCO_3^- + OH^- \rightleftharpoons CO_3^{2-} + HOH \qquad (2)$$

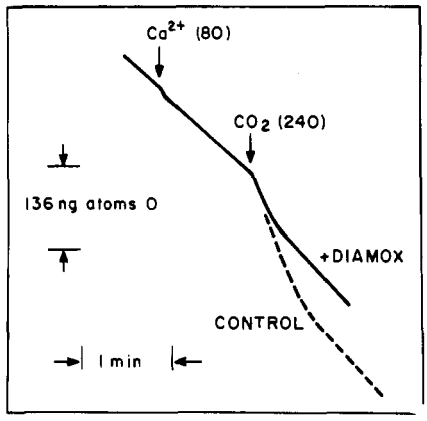

FIGURE 4: Effect of Diamox on CO_2-stimulated respiration. The system was as described in Figure 2. The Ca^{2+} addition represents the second Ca^{2+} pulse. Diamox (10 μM) was added at time zero. CO_2 was added as a fresh 100% CO_2-saturated water solution.

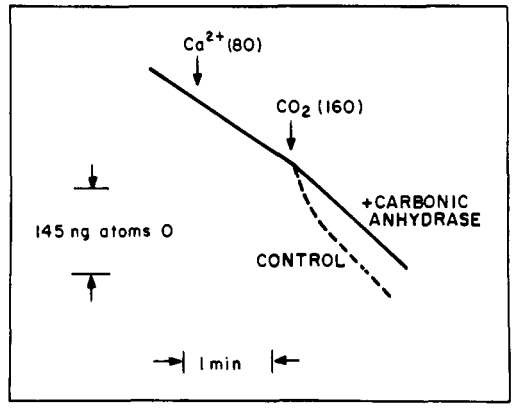

FIGURE 5: Effect of carbonic anhydrase on CO_2-stimulated respiration. The system was as described in Figure 2. The Ca^{2+} addition respresents the second Ca^{2+} pulse. Carbonic anhydrase (20 μg) was added at time zero. CO_2 was added as a freshly prepared 100% CO_2-saturated water solution.

action 1). In order for this reaction to proceed rapidly enough to provide counteranion for the entering Ca^{2+}, it must in all likelihood be catalyzed, presumably by carbonic anhydrase present in the mitochondrial matrix. That this explanation is probably correct is shown by the oxygen electrode trace in Figure 4. Addition of freshly prepared dissolved CO_2 after a second pulse of 80 nmol of Ca^{2+}/mg of protein evoked an immediate increase in respiratory rate, as already shown in Figure 3. However, when 10 μM Diamox was present in the system from the beginning, addition of CO_2 after the Ca^{2+} pulse yielded a much smaller increase in oxygen uptake. This result indicates that the mitochondria contain carbonic anhydrase and that it is required to convert the dissolved CO_2 entering the mitochondria into an anionic species, presumably carbonate.

Diamox at 10 and 100 μM has no effect on the oxidation rate of succinate, α-ketoglutarate, β-hydroxybutyrate, or isocitrate, or on ADP acceptor control of respiration (see Discussion).

These general conclusions were supported by another type of experiment. The oxygen electrode traces in Figure 5 show that a respiratory jump induced by Ca^{2+} in a system containing dissolved CO_2 is almost completely inhibited if 20 μg of purified bovine erythrocyte carbonic anhydrase is added to the suspending medium. The presence of the latter would cause rapid conversion of 90% of the 160 nmol of CO_2/mg of protein initially added to the system into bicarbonate; the resulting mixture would contain 16 nmol of CO_2 and 144 nmol of HCO_3^- per mg of protein at pH 7.4 and 25°. The great reduction in the concentration of dissolved CO_2 in the medium caused by the addition of carbonic anhydrase could be expected to cause a proportional decrease in the rate of entry of dissolved CO_2, since the rate of physical diffusion of CO_2 is proportional to its concentration.

Discussion

The data in this paper demonstrate that CO_2 readily passes through the mitochondrial membrane into the matrix whereas the bicarbonate anion does not, under conditions in which an anion is required in the matrix to accompany respiration-dependent entry of Ca^{2+}. This conclusion is consistent with the osmotic swelling experiments of Chappell and Crofts (1966) who concluded that the bicarbonate anion does not penetrate the mitochondrial membrane. The evidence presented strongly suggests that the carbonate anion is the species accumulating with Ca^{2+} under the condition we have employed. The nearly exact 1:1 stoichiometry in the uptake of ^{45}Ca and ^{14}C and the volatility of the accumulated ^{14}C on acidification support this view. Since calcium carbonate would be expected to be insoluble in the somewhat alkaline mitochondrial matrix, it is possible that calcium carbonate actually precipitates in the matrix, particularly when larger amounts are accumulated. Electron microscopic examination of this question is under way. Presumably the carbonate is formed in the alkaline matrix from the bicarbonate which results from the reaction of the CO_2 with hydroxyl ions generated by electron transport: $OH^- + CO_2 \rightleftharpoons HCO_3^-$.

The hydroxylation of carbon dioxide is a relatively slow process and is promoted biologically by carbonic anhydrase. Our data are consistent with the view that carbonic anhydrase is involved in these reactions in rat liver mitochondria, since the respiration-dependent accumulation of carbonate (as well as the accumulation of Ca^{2+}) is blocked by Diamox, a specific inhibitor of carbonic anhydrase (Maren, 1967). Moreover, our findings suggest that the carbonic anhydrase is present within the mitochondria, in either the inner membrane or matrix, more likely the latter.

There is some uncertainty in the literature as to the presence of carbonic anhydrase in mitochondria. Carbonic anhydrase is largely localized in the cytosol of animal tissues (Maren, 1967). Although some studies have claimed that the enzyme is absent from mitochondria of various animal tissues, including rat liver (Bernstein *et al.*, 1968; Datta and Shepard, 1959; Deprez and Francois, 1972), other investigators have reported the presence of small, but distinctly measurable, amounts of carbonic anhydrase in a number of different types of mitochondrial preparations, including rat liver mitochondria (Karler and Woodbury, 1960; Maren *et al.*, 1966; Maren and Ellison, 1967; Holton, 1969; Rossi, 1969).

Rossi (1969) has reported in a short communication that about 4% of the total carbonic anhydrase activity of rat liver is present in the mitochondria; this activity was found to be membrane bound and Diamox sensitive. Maren *et al.* (1966) found that rat liver contains at least two species of carbonic anhydrase, one in the cytosol and the other in the mitochondria. The activity found in the cytosol is not inhibited by Diamox whereas that in the mitochondria is sensitive to

carbonic anhydrase inhibitors. Presumably it is the latter type of activity that is involved in the Diamox-sensitive process of carbonate accumulation occurring in the well-washed rat liver mitochondria employed in this study. The information available on carbonic anhydrase activity in mitochondria is thus rather meager, but the simplest working hypothesis is that rat liver mitochondria contain carbonic anhydrase activity in the matrix, which catalyzes hydroxylation of dissolved carbon dioxide entering through the inner membrane to yield successively bicarbonate and carbonate.

The data reported in this paper also suggest the possibility that mitochondria may participate in the energy-dependent deposition of calcium carbonate under biological circumstances. For example, calcium carbonate is deposited in the shells of mollusks, crustaceans, and other invertebrates, and in egg shells of birds. Mitochondria of the calciferous gland of the earthworm, which actively secretes $CaCO_3$, have been reported to contain crystals of calcite (Crang *et al.*, 1968). It appears possible that mitochondria may participate in these processes, as they appear to do in the deposition of calcium phosphate under both normal and pathological conditions (Lehninger, 1970). In tissues not specialized for the production of $CaCO_3$, the accumulation of this salt within mitochondria would be consistent with the idea that mitochondria can buffer the intracellular concentration of ionic calcium by the respiration-dependent segregation of calcium salts in the matrix (Lehninger, 1965; Borle, 1972).

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