Carbonic Anhydrase Activity in Mitochondria from Rat Liver*

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An 18O exchange method has been used to determine the location of carbonic anhydrase in mitochondria from rat liver and to examine the role of this enzyme in the kinetics of CO2 in resting and respiring mitochondria. Using digitonin subfractionation, we have determined that a substantial fraction, 40 to 60%, of the carbonic anhydrase activity in the mitochondrion from rat liver is located in the space between the inner and outer membranes; the remaining activity was found in the matrix with no detectable activity in the sedimented membranes. The total catalytic CO2 hydration activity measured in intact mitochondria from rat liver was about 1% of that found in an equal volume of rat erythrocytes. The apparent permeability constant representing the barrier for the diffusion of HCO3- from external solution to intramitochondrial carbonic anhydrase, 9 × 10^{-4} cm s^{-1}, is near in magnitude to the permeability constant for the diffusion of HCO3- across the rat erythrocyte membrane, 4 × 10^{-4} cm s^{-1}. Calcium-induced respiratory jumps were shown to cause changes in the rate of 18O exchange between CO2 and H2O that were consistent with a net uptake of CO2 by the mitochondria.

The presence of carbonic anhydrase in mitochondria from various tissues has been the subject of several investigations (1-9). Rat liver mitochondria have received the most attention (1-7) but the reports have been mixed. Some investigators have failed to detect any carbonic anhydrase activity in the mitochondrial fraction of rat liver homogenates (7). Others detected a small amount of activity but concluded that this was due to contamination from the soluble fraction and erythrocytes (1). Most investigators, however, agree that the small but significant amount of carbonic anhydrase activity (2-10%) of total activity of rat liver homogenates) recovered in the mitochondrial fractions is of mitochondrial origin (2, 5, 6). It is important to note that the carbonic anhydrase from the mitochondrial fraction of rat liver is more susceptible to inhibition by certain sulfonamides than is the cytosolic enzyme; these are most likely different isozymes (3). Carbonic anhydrase has also been detected in mitochondrial fractions from other tissues including dog liver (3, 4), guinea pig liver, and skeletal muscle (8, 9), rat kidney, and cerebral cortex (2). The mitochondria from guinea pig liver have been found to contain a considerable amount of activity, about \( \frac{1}{50} \) that of erythrocytes (8).

The exact location within the mitochondrion and the role of the mitochondrial carbonic anhydrase require further study. It has been postulated that this is a membrane-bound enzyme (2, 5, 6), whose physiological role is to provide a rapidly reacting buffer in the matrix space (5). Others suggested that it is an enzyme found in the matrix which could catalyze the hydration of CO2 and provide a counterion, bicarbonate, or carbonate, for the accumulation of calcium by respiring mitochondria (10). The activity of mitochondria from guinea pig liver was found to be soluble, not bound to sedimented membranes (9).

We have used an 18O exchange method to determine the location of carbonic anhydrase in mitochondria from rat liver and to examine the role of this enzyme in the kinetics of CO2 in both resting and respiring mitochondria. The main advantages of the 18O method in studying these properties are that it is a sensitive method for the detection of carbonic anhydrase activity and that it may be used to determine intracellular carbonic anhydrase activity and the membrane barriers to diffusion of bicarbonate (9, 11, 12). Using digitonin subfractionation, we have determined that a substantial fraction (40 to 60%) of the carbonic anhydrase activity in the mitochondrion of rat liver is located in the space between the inner and outer membranes; the remaining activity was found in the matrix with no detectable activity in the sedimented membranes. The total catalytic CO2 hydration activity measured in intact mitochondria from rat liver was about 1% of that found in an equal volume of rat erythrocytes. Moreover, the apparent permeability constant representing the barrier for diffusion of HCO3- into a mitochondrion is near in magnitude to the permeability constant for the diffusion of HCO3- across the red cell membrane.

**EXPERIMENTAL PROCEDURES**

**Solutions for Preparation of Mitochondria**—Solution 1 contained 220 mM d-mannitol, 70 mM sucrose, 2 mM Hepes, 0.5 mg/ml of bovine serum albumin (solution 1a was solution 1 without bovine serum albumin, solution 1b was solution 1 with 1% Triton X-100 (octyl phenoxy polyethoxethanol)). Solution 2 contained 180 mM d-mannitol, 70 mM sucrose, 25 mM Hepes, 0.5 mg/ml of bovine serum albumin (solution 2a was solution 2 with 25 mM KHCO3). Solution 3 contained 150 mM d-mannitol, 70 mM sucrose, 25 mM Hepes, 25 mM potassium succinate, 0.5 mg/ml of bovine serum albumin (solution 3a was solution 3 with 25 mM KHCO3). The pH of all solutions was 7.4.

**Preparation of Mitochondria**—Mitochondria were isolated from livers of male, albino Sprague-Dawley rats using the procedure of Schnaitman and Greenawalt (13). Mitochondria intended for digitonin subfractionation studies were isolated using solution 1a, as suggested by Kun et al. (14). All other mitochondria were prepared using solution 1. EDTA (1 mM) was included in the homogenization step when the mitochondria were intended for respiration studies. The mitochondrial pellet was washed three times, each time resuspending the pellet with a glass rod by gentle swirling. Mitochondrial preparations were occasionally checked for possible microsomal contamination by comparing their NADPH-cytochrome c reductase content (15) to that of microsomes prepared from the same livers. The mitochondrial preparations had a reductase activity (per mg of total protein)

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1. The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.
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protein) that was less than 10% of the reductase activity of preparations made with digitonin membranes.

Lysis of Mitochondria and Separation of Mitochondrial Membranes—Lysis was accomplished by resuspending the mitochondrial pellet in solution 1a at 80 to 100 mg of protein/ml and divided into several portions. These were treated with various amounts of digitonin (suspended by Schnaitman and Greenewalt (13) and centrifuged at 9000 x g for 15 min. The pellets were washed once and the supernatant fluids from both centrifugations (original and washing) were pooled together. The fluffy layer was included with the supernatant fraction. The pellets, resuspended in solution 1b, and supernatant fluids from each treatment were assayed for carbonic anhydrase, protein, malate dehydrogenase, and monomine oxidase content. On one occasion, the samples were also assayed for succinate-cytochrome c reductase content. The recovered amount of enzyme activities and total protein after each digitonin treatment was usually in the range of 90-110%. Because of this variability in total recovery, the enzyme was assayed in the pellet from each treatment was also assayed for the fraction of the activity recovered in the pellet and supernatant fluid, rather than the fraction of the original total activity. At the concentrations used (see Fig. 2), digitonin did not interfere with any of the assays. Most digitonin treatments were repeated with four different preparations.

Protein Determination—Mitochondrial protein was determined by the method of Lowry et al. (16) using bovine serum albumin as the standard. The samples were diluted to the appropriate concentration range with 0.5 M NaOH. A spectrophotometric difference method (17) was also used periodically, especially for quick determinations. The two methods gave similar results (within 10%). Determinations were made in triplicate.

Enzyme Assays—Monomine oxidase was assayed by following the oxidation of benzylamine spectrophotometrically at 250 nm at 37 °C as described by Schnaitman et al. (18). Each determination was repeated four times. Malate dehydrogenase was assayed by determining the rate of NADH formation at 340 nm at 30 °C as described by Ochoa (19). Each determination was repeated four times. Succinate-cytochrome c reductase was assayed as described by Sottocasa et al. (20). Each determination was repeated four times.

Carbonic anhydrase activity was measured by the 18O exchange technique described by Silverman et al. (11). Because of the hydration-dehydration cycle, oxygen 18 in CO2 and HCO3- is exchanged with oxygen 16 of water. This method involves the measurement of the isotopic content of 18O-labeled CO2 with a mass spectrometer equipped with a CO2 inlet. The CO2 inlet is described in a previous publication (10). Gas passing across the membrane enters a mass spectrometer, providing a continuous monitor of isotopic content of CO2. In order to determine the extent of lysis in the liver of guinea pigs (9), the large intracellular concentration of carbonic anhydrase was homogenous in solution. The concentrations of these preparations were chosen to give oxygen 18 exchange rates which were comparable to those obtained with intact mitochondria. After 30 min in solutions containing 33 mm Ca2+ or Sr2+, the suspensions of mitochondria showed no more lysis than suspensions maintained in the absence of Ca2+ and Sr2+. Inhibition of carbonic anhydrase was achieved using 10 mM benzamidethanol (Ki ~ 2 x 10^(-8) M).

The 18O content of CO2 was determined by periodically withdrawing small samples (0.1 ml) from the reaction medium during measurements of the catalyzed rate of 18O exchange. The samples were immediately acidified and the isotopic content of the CO2 generated was measured on the mass spectrometer. The 18O content of HCO3- was calculated from the 18O content of CO2 generated by acidification, the 18O content of CO2 alone as determined from the CO2 inlet, and the fraction of total CO2 that is bicarbonate calculated from the Henderson-Hasselbalch equation.

Volume of Mitochondria—The total volume of all mitochondria in suspension was correlated with protein content and estimated in the following manner. The weight of mitochondria was estimated from the protein content of the suspension by assuming that rat liver mitochondria contain 70% protein (20, 21) and that the density of mitochondria is 1.0 mg/ml. This gives a value of 1.4 µl/mg of protein. This can be compared with the value 1.0 µl/mg of protein which has been assumed for the mitochondrial matrix (22).

Kinetic Theory—The exchange of 18O between CO2 and water occurs in the absence of enzyme and is catalyzed by carbonic anhydrase.

\[
\text{C}^\text{18O}_2 + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{H}^+ \rightarrow \text{CO}_2 + \text{H}_2\text{O}^{18} \quad (1)
\]

We observed this exchange by measuring, with a mass spectrometer, atom fractions of 18O in CO2 in homogeneous solution or in the external solution in mitochondrial suspensions. The atom fractions of 18O in all CO2, 18, and in doubly labeled CO2, c are:

\[
\frac{1}{2}(\text{CO}_2\text{O}^{18}) + \frac{1}{2}(\text{CO}_2\text{O}^{16}) = \frac{1}{2}(44) + \frac{1}{2}(46) = 45 \quad (2)
\]

where (44), (46), and (48) are heights of the corresponding mass peaks. The decrease in the 18O, c and cO, follows first order kinetics for the uncatalyzed exchange and for the catalyzed exchange when carbonic anhydrase is homogeneous in solution. The uncatalyzed exchange is given by:

\[
18O = Ae^{-kt}, \quad c = c_0e^{-kt} = Re^{-kt} \quad (3)
\]

The first order rate constants k and b can be expressed as the sum of catalyzed and uncatalyzed components:

\[
\theta = \theta_{\text{catalyzed}} + \theta_{\text{uncatalyzed}} \quad \gamma = \frac{\theta_{\text{catalyzed}}}{\theta_{\text{total}}} \quad (4)
\]

The effect on the 18O exchange processes of Equation 1 and on the kinetic expressions of Equation 3 caused by the addition of cells or organelles containing carbonic anhydrase is presented elsewhere (9, 11, 12, 23). The various chemical and diffusion steps involved are shown in Fig. 1. Except for the case of respiring mitochondria, chemical and diffusion of the equilibration is assumed; that is, chemical equilibrium is assumed inside and outside the mitochondria and the flux into the mitochondrion of both CO2 and HCO3- is equal to the flux out. In the case of red cells (11, 12, 24) and mitochondria from the livers of guinea pigs (9), the large intracellular concentration of carbonic anhydrase and the rapid access of CO2 to that carbonic
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The velocity of the catalyzed dehydration at chemical equilibrium, which equals the velocity of the catalyzed hydration, is given by

\[ v = \frac{k_{\text{cat}}}{K_{\text{cat}}} \frac{[E][\text{HCO}_3^-]}{[\text{CO}_2]} \]

where \( k_{\text{cat}} \approx 1.6 \times 10^{6} \text{ s}^{-1} \) and \( K_{\text{cat}} \approx 0.25 \text{ M at pH 7.4} \) for human carbonic anhydrase II. Then \( k' \), for dehydration is given by the following at [HCO_3^-] = 0.024 M which is much less than \( K_{\text{cat}} \).

\[ k' = \frac{k_{\text{cat}}}{K_{\text{cat}}} \frac{[E]}{[\text{HCO}_3^-]} \]

To convert to \( k \), for hydration we multiply by the equilibrium ratio [HCO_3^-]/[CO_2].

curved surface area of a cylinder is \( \pi r^2 \), which we have used in these calculations. \( V_2 \) was estimated from the mitochondrial protein content as described under "Experimental Procedures." The ratio of volumes also relates \( k' \) to \( k' \) and \( k \) to \( k' \); \( k'/k = (V_1/V_2) k' \); (23) valid for nonrespiring mitochondria at diffusion equilibrium.

**RESULTS**

**Localization of Carbonic Anhydrase**—Fig. 2 shows the release of protein, carbonic anhydrase, and several enzymes used as markers for different mitochondrial fractions from the 9000 \( \times g \) pellet of rat liver mitochondria after treatment with various concentrations of digitonin, which causes the destruction of the outer membrane at lower concentrations (\( \approx 0.1 \) mg of protein) and the inner membrane at higher concentrations. Monosamine oxidase serves as a marker for outer membrane, succinate-cytochrome c reductase for internal membrane, malate dehydrogenase for the matrix, and protein for matrix and inner membrane (13).

No carbonic anhydrase activity as determined by \( ^{18}O \) exchange rates could be detected in suspensions of mitochondrial membranes (inner and outer) isolated from detergent-treated mitochondria.

**\( ^{18}O \) Exchange in Suspensions of Nonrespiring Mitochondria**—The rate constants \( \theta \) and \( \gamma \) for \( ^{18}O \) depletion of CO_2 in suspensions of nonrespiring mitochondria from different preparations are given in Table I. All values of \( \theta \) and \( \gamma \) reported for experiments in suspensions of mitochondria are total values, i.e., they contain values for the uncatalyzed and catalyzed \( ^{18}O \) exchange. The uncatalyzed values were (mean and standard deviation): \( \delta_{\text{uncat}} = (0.90 \pm 0.08) \times 10^{-5} \text{ s}^{-1} \); \( \gamma_{\text{uncat}} = (1.82 \pm 0.13) \times 10^{-4} \text{ s}^{-1} \). Measurements of \( ^{18}O \) content of CO_2 in the fluid external to the mitochondria were begun about 20 s after addition of mitochondria to the solution in the CO_2 inlet vessel. This allowed for sufficient mixing time. The observed rate of decrease of \( ^{18}O \) content of CO_2, \( \tau = \tau_{\text{m}} \) obeyed first order kinetics with a rate constant \( \delta \). Lysis of mitochondria by detergent (1.0% Triton X-100) caused an increase in \( \theta \) by 50–100%. The value of \( \theta / \gamma \) of a suspension of lysed mitochondria was 2.0. The catalyzed component of \( \theta \) in solutions obtained from lysed mitochondria was inhibited by 50% in the presence of 15 mM NaI. This is the concentration of iodide found to

**Fig. 1. Chemical and diffusion processes of CO_2 and HCO_3^-**. The rate constants \( k \), \( k' \), and \( k'' \), are the rate constants. \( k' \) and \( k'' \) are diffusion rate constants as described in the text. Medium 1 is external solution and Medium 2 is intramitochondrial solution containing carbonic anhydrase.

**Fig. 2.** The percentage of total recovered protein or enzymatic activity sedimented at 9000 \( \times g \) for 15 min plotted against digitonin concentration. The average and standard deviation (only half the error bars are shown for clarity) from 3 or 4 experiments with different mitochondrial preparations are given. Points without error bars are the values obtained from 1 or 2 experiments only. Monoamine oxidase, \( \delta \); carbonic anhydrase, \( \gamma \); protein, \( \Delta \); malate dehydrogenase, \( \beta \); and succinate-cytochrome c reductase, \( \beta \).
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Table I

| Mitochondrial preparation | Experimental dataa | Results of Newton-Raphson iteration
|---------------------------|-------------------|-----------------------------|
|                           | $\theta'$ | $\gamma'$ | $V_{i}/V_{o}$ | $k_{e}$ | $k_{v}$ | $P$ for $HCO_{3}^{-}$ |
|                           | $x 10^{-3}$ s$^{-1}$ | $s^{-1}$ | $s^{-1}$ | $x 10^{12}$ cm s$^{-1}$ |
| 1                         | 3.57 ± 0.06 | 1.68 ± 0.03 | 120 | 1.03 ± 0.03 | 8.8 ± 3.0 | 11.1 ± 3.8 |
| 2                         | 4.96 ± 0.25 | 1.68 ± 0.03 | 100 | 1.40 ± 0.03 | 6.7 ± 3.4 | 8.9 ± 5.7 |
| 3                         | 3.11 ± 0.01 | 1.68 ± 0.02 | 210 | 1.55 ± 0.17 | 8.2 ± 0.4 | 16.2 ± 0.4 |

a Mean and standard deviation from at least three experiments.

| Assumed conditions | Rate constants for the exchange of $^{18}$O from uniformly labeled $CO_{2}$ and $^{18}$O from doubly labeled $CO_{2}$ in suspensions of nonrespiring mitochondria at different values of $V_{i}/V_{o}$ |
|-------------------|-----------------------------|
| $r^{18}$ | $k_{e}$ | $k_{v}$ | $P$ for $HCO_{3}^{-}$ |
| cm$^{-1}$ | $x 10^{12}$ s$^{-1}$ | $s^{-1}$ | $s^{-1}$ | $x 10^{12}$ cm s$^{-1}$ |
| 2.5 | 60.0 | 6.11 | 7.60 | 1.39 | 42.8 | 3.88 | 4.85 |
| 2.6 | 60.0 | 6.11 | 7.60 | 1.39 | 42.9 | 3.87 | 4.84 |
| 2.5 | 60.0 | 6.11 | 7.60 | 1.39 | 43.1 | 3.75 | 4.69 |
| 2.5 | 60.0 | 6.09 | 7.60 | 1.39 | 44.8 | 3.88 | 4.85 |
| 2.5 | 60.0 | 6.15 | 7.60 | 1.39 | 39.2 | 3.84 | 4.80 |
| 2.5 | 60.0 | 6.11 | 7.40 | 1.40 | 29.9 | 4.42 | 5.53 |
| 2.5 | 60.0 | 6.11 | 8.00 | 1.36 | 105.6 | 3.34 | 4.18 |
| 2.5 | 60.0 | 6.11 | 8.40 | 1.35 | 262.4 | 3.18 | 3.98 |
| 5.0 | 3.0 | 6.11 | 7.60 | 1.39 | 42.9 | 3.85 | 9.63 |
| 10.0 | 1.5 | 6.11 | 7.60 | 1.39 | 42.9 | 3.80 | 19.10 |

a Newton-Raphson iteration as described by Tu et al. (12) but for two variables only, $k_{e}$ and $k_{v}$.

b Radius of mitochondrion as a cylinder.

c Rate constant for exit of $CO_{2}$ out of a mitochondrion.

d Equilibrium constant $K = \left[HCO_{3}^{-}\right]/\left[H^{+}\right]/\left(CO_{2}\right)$. 

e Intramitochondrial pH.

f $P$ was calculated from Equation 5 of the text.

Ca$^{2+}$ was added at the arrows. The addition of 11 mM Ca$^{2+}$ (about 2 to 4 $\mu$mol/mg of protein) to different mitochondrial suspensions caused a decrease by 25–90% in $\theta$, the rate constant for $^{18}$O depletion from $CO_{2}$. This decreased value of $\theta$ persisted for about 100 s, after which $\theta$ returned to its original value. At the higher calcium concentration of 33 mM, we observed an increase in the $^{18}$O content of $CO_{2}$. Similar effects, although smaller in magnitude, were obtained with respiratory jumps induced by adding SrCl$_2$.

Several control experiments were done to establish that the effect of Ca$^{2+}$ and Sr$^{2+}$ on $\theta$ was the result of respiration-dependent events characteristic of mitochondria. Ca$^{2+}$ was shown to have no effect on the uncatalyzed $^{18}$O exchange or the exchange catalyzed by soluble bovine carbonic anhydrase, intact rat erythrocytes, or detergent-treated mitochondria. The relatively high concentrations of Cl$^{-}$, about 20 mM, how-
was very little or no damage to the inner membrane. The reported for mitoplast preparations by other researchers, 0.63 known to pass across membranes much more rapidly than membrane it was only minimal and it could not account for sient rise in sions of mitochondria. This result was expected since CO\textsubscript{2} is C\textsubscript{1}- and thus Con has more rapid access to carbonic membrane marker. The profiles of these two enzymes taken to- C\textsubscript{1}-; and thus Con has more rapid access to carbonic membrance and matrix, the value of P for HCO\textsubscript{3}- is of close, therefore, that if there were any damage to the inner membrane it was only minimal and it could not account for all the release from the pellet of carbonic anhydrase activity seen here at low digitonin concentrations. At 0.69 mg of digitonin/mg of protein there was a release of carbonic anhydrase activity that was about 50% of the activity released upon lysis of mitochondria. Such release can only be explained by the presence of carbonic anhydrase in a compartment outside the mitoplasts. The mitochondrial outer and inner membranes were found to contain insignificant amounts of carbonic anhydrase activity. Hence, these data strongly suggest that carbonic anhydrase is present not only in the matrix, as it was generally assumed (9, 10) but also in the intermembrane space. That there is enzyme in the matrix is indicated by the residual activity in the pellets after treatment with digitonin concentrations higher than 0.1 mg/mg of protein.

\[ ^{18}\text{O} \text{Exchange in Suspensions of Nonrespiring Mitochondria—} \text{The} ^{18}\text{O} \text{exchange method provides information to de-} \text{cribe} \text{CO}_2 \text{kinetics in cells containing carbonic anhydrase,} \text{ particu-} \text{larly which of the steps shown in Fig. 1 is rate-deter-} \text{mining. Due to the large concentration of carbonic anhydrase} \text{in} \text{red cells, diffusion into the red cell is rate-limiting for} \text{^{18}O} \text{loss from both CO}_2 \text{and HCO}_3^- (11, 12, 27). The data obtained from mitochondria of rat liver indicate that} \text{^{18}O} \text{depletion from HCO}_3^- \text{in suspensions of mitochondria is in part controlled by diffusion of HCO}_3^- \text{into the mitochondria, and in part con-} \text{trolled by the rate of the catalyzed dehydration in the mito-} \text{chondria. Evidence is the value of} \gamma/\theta = 1.7 \text{(Table I); an} \text{^{18}O} \text{depletion which is completely chemically controlled must have} \gamma/\theta = 2.0 \text{(27). Further evidence in Table I is that the} \text{rate constant for dehydration of HCO}_3^- \text{inside the mitochondria} \text{k}^- \text{, is of magnitude comparable to the rate constant for diffusion of HCO}_3^- \text{out of the mitochondria} \text{k}^+. \text{Although it can be inter-} \text{preted in several ways, the small increase in} \text{^{18}O} \text{exchange (50–100%) observed upon lysis of mitochondria is consistent with a permeability barrier to diffusion of HCO}_3^- \text{into mitochondria. It is apparent from the results that diffusion of CO}_2 \text{into mitochondria is not a rate-limiting event. This can be concluded from Table II which shows that values of} \text{k}_n \text{, the rate constant for the diffusion of CO}_2 \text{out of a mitochondrion, differing by a factor of} 100 \text{do not affect the solution of the kinetic equations. The range of values of} \text{k} \text{chosen in Table II can be compared with the value of} \text{k}_n \text{found for red cells of about} 200 \text{s}^{-1} \text{(12). This conclusion is easily reconciled with the low activity of carbonic anhydrase in a mitochondrion from rat liver, which is about} \text{1% of that of rat erythrocytes.}}

\[ \text{The values of} \text{k}^+ \text{, for diffusion of HCO}_3^- \text{out of mitochondria given in Table I are roughly equal to values of} \text{k}^+_n \text{,} \text{10 s}^{-1} \text{found for rat erythrocytes. However, when differences in size} \text{between erythrocytes and mitochondria are taken into consid-} \text{eration using Equation 5 it is seen that: P for HCO}_3^- \text{, the} \text{apparent permeability constant of mitochondria to HCO}_3^- \text{, at} 9 \times 10^{-5} \text{cm s}^{-1} \text{is somewhat smaller than the value of about} 4 \times 10^{-4} \text{cm s}^{-1} \text{calculated for red cells (12). Dodgson et al. (9) estimated P for HCO}_3^- \text{in mitochondria from guinea pig liver to be in the range of} 10^{-6} \text{to} 10^{-4} \text{cm s}^{-1}. \text{This permeability constant for HCO}_3^- \text{which we have esti-} \text{mated is a composite of the permeability barriers between external solution and intramitochondrial carbonic anhydrase. Since we have detected carbonic anhydrase in both the inter-} \text{membrane space and matrix, the value of P for HCO}_3^- \text{is of limited significance since it may reflect the permeability bar-} \text{rier of two membranes. Moreover, we have not included in the calculations the effect of our experimental conditions on the shape of the mitochondria, which frequently swell when isolated (28). As shown in Table II, our calculations were sensitive to the radius of the mitochondrion which we have.
assumed to be cylindrical in shape. It is also shown in Table II that the value of intramitochondrial pH assumed in the calculations had a large effect on \( k_t \); however, changes in the assumed value of pK for the CO₂-HCO₃⁻ equilibrium did not affect the calculations significantly.

\(^{18}O\) Exchange in Suspensions of Respiring Mitochondria—
Elder and Lehninger (10) showed that Ca²⁺ accumulates in respiring mitochondria in a 1:1 ratio with CO₂ and suggested that a role of mitochondrial carbonic anhydrase is to hydrate CO₂ and provide a counter ion for calcium uptake. This was confirmed when they found that calcium uptake in respiring mitochondria is decreased by 68% in the presence of 10⁻⁵ M acetazolamide. Elder and Lehninger suggested the accumulation in the mitochondria of calcium carbonate. Granules of calcium phosphate have been shown to deposit along the inner membrane and matrix of rat liver and heart mitochondria (29). Presumably, a similar precipitation of Ca²⁺ may occur when phosphate is replaced by carbonate. Precipitation of CaCO₃ containing \(^{18}O\) would sequester the labeled oxygen in a form which is not susceptible to exchange with water. This explanation is consistent with the observed decrease in the rate constant \( \theta \) for exchange of \(^{18}O\) between CO₂ and water observed upon stimulating respiration by addition of Ca²⁺ (Fig. 3). The value of \( \theta \) observed in suspensions of nonrespiring mitochondria results from \(^{18}O\) labeled CO₂ passing into the mitochondria being replaced by CO₂ of lower \(^{18}O\) content passing out of the mitochondria. A decrease in \( \theta \) upon stimulating respiration is consistent with the unidirectional uptake of labeled CO₂ the mere disappearance of CO₂ from the external fluid without its replacement by CO₂ of lower \(^{18}O\) enrichment will not result in \(^{18}O\) depletion of CO₂ outside the mitochondria. About 100 s after the respiratory jump the CO₂ kinetics of the mitochondria return to rates of \(^{18}O\) exchange very similar to those measured before the jump. The transient increase in \( \tau \) and \( \psi \) that was observed with the higher Ca²⁺ concentrations (33 mm in Fig. 3) arises from the difference in the enrichment of the CO₂ and HCO₃⁻ species in the external solution. Due to its slow diffusion into and out of the mitochondria, bicarbonate has a larger \(^{18}O\) content than CO₂ in suspensions of intact mitochondria. When the system is disturbed, as when the external reaction rate is increased or the intramitochondrial rate decreased, there is a tendency to equalize the enrichment of the two species (24).

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