Role of Calcium as an Inhibitor of Rat Liver Carbamylphosphate Synthetase I

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The mechanism of Ca\(^{2+}\) inhibition of carbamylphosphate synthetase has been investigated using purified enzyme obtained from livers of rats fed a high protein diet. Binding of Mn\(^{2+}\) to the enzyme was measured by EPR techniques at pH 7.8, and Scatchard plots of the data indicated one Mn\(^{2+}\)-binding site with a K\(_d\) of 13 \(\mu M\). From competition studies between Mn\(^{2+}\) and Ca\(^{2+}\) or Mg\(^{2+}\) binding, values of 180 \(\mu M\) were obtained for K\(_d\) (Mg) and 193 \(\mu M\) for K\(_d\) (Ca). A nonlinear least squares curve fitting program was used to calculate the K\(_d\)'s for MgATP\(^{2-}\) at the metal-nucleotide binding sites using a simplified rate equation of the enzyme reaction mechanism. Values of 140 and 2420 \(\mu M\) were obtained for K\(_d\) (MgATP) at the first and second sites, respectively, at pH 7.8, with a free Mg\(^{2+}\) of 1 mM and other substrates and activators present at saturating concentrations. Variations of the bicarbonate, N-acetylglutamate, and ammonia concentrations in the absence and presence of different amounts of total calcium, from which free Ca\(^{2+}\), free Mg\(^{2+}\), MgATP\(^{2-}\), and CaATP\(^{2-}\) concentrations were calculated, permitted values for K\(_d\) (CaATP) to be obtained by graphic procedures. Mean values of 375 and 120 \(\mu M\) were obtained for K\(_d\) (CaATP) at the first and second sites, respectively. Using the above kinetic constants, a computer model of the enzyme reaction was constructed and tested using two further sets of kinetic data obtained by varying the concentrations of Mg\(^{2+}\), Ca\(^{2+}\), MgATP\(^{2-}\), and CaATP\(^{2-}\). Poor fits were obtained unless the formation of a mixed complex involving CaATP\(^{2-}\) competition with MgATP\(^{2-}\) at the second metal-nucleotide-binding site was incorporated into the rate equation. Nonlinear least squares curve fitting of both sets of experimental data gave a well determined value of 124 \(\mu M\) for this final CaATP\(^{2-}\)-inhibitory constant. Sensitivity tests for variation of the primary kinetic constants with the computer model showed that the inhibitory effect of free Ca\(^{2+}\) was weak and that the observed calcium inhibition of carbamylphosphate synthetase can be accounted for primarily by competitive interaction of CaATP\(^{2-}\) at the second MgATP\(^{2-}\)-binding site. With 1 mM free Mg\(^{2+}\) and 5 mM MgATP\(^{2-}\), half-maximal inhibition of enzyme activity was obtained with 0.2 mM CaATP\(^{2-}\).

Carbamylphosphate synthetase I catalyzes the first step of urea biosynthesis and involves the formation of carbamylphosphate from NH\(_3\), HCO\(_3\)\(^{-}\) and two molecules of MgATP\(^{2-}\) according to Reaction 1:

\[
\text{NH}_3 + \text{HCO}_3^- + 2\text{MgATP}^{2-} \rightarrow \text{N-acetylglutamate} + 2\text{MgADP} + 2\text{HPO}_4^{2-} + 2\text{Mg}^{2+} + \text{H}^+ + \text{K}^+
\]  

(1)

It accounts for 20–30% of the protein of rat liver mitochondria (1) and enzyme activity is strictly dependent on the presence of Mg\(^{2+}\) and N-acetylglutamate as obligatory activators (2, 3). An understanding of its regulation is important since the formation of carbamylphosphate is generally considered to be the primary site for regulation of urea synthesis (4, 5).

In a previous study, it was shown that addition of Ca\(^{2+}\) to either lysed or intact mitochondria caused an inhibition of carbamylphosphate synthetase activity, which could be reversed by the addition of Mg\(^{2+}\) (6). Although it was concluded that Ca\(^{2+}\) interfered with the activation of carbamylphosphate synthetase by Mg\(^{2+}\), it could not be ascertained whether this effect was exerted by competition of free Ca\(^{2+}\) at the Mg\(^{2+}\)-binding site or by CaATP\(^{2-}\) at either or both of the MgATP\(^{2-}\)-binding sites. The present work was initiated to obtain more information on the mechanism of Ca\(^{2+}\) inhibition, using purified rat liver carbamylphosphate synthetase, with the view of assessing the possible physiological significance of the effect. This involved an examination of metal-binding constants at the free Mg\(^{2+}\)-binding site and the kinetic constants and metal-nucleotide specificity for the two MgATP\(^{2-}\)-binding sites.

Direct binding studies with the purified enzyme showed that although Ca\(^{2+}\) binding was competitive with Mg\(^{2+}\) or Ma\(^{2+}\) at the free metal-binding site, the binding constant for Ca\(^{2+}\) was approximately 15-fold higher than the value of 10 \(\mu M\) estimated from previous studies for the free Ca\(^{2+}\) concentration in the matrix space of rat liver mitochondria (7). In contrast, the major inhibitory effect of calcium on the isolated enzyme could be accounted for by CaATP\(^{2-}\) competition for MgATP\(^{2-}\) at the metal-nucleotide-binding sites. Analysis of the nonlinear kinetic plots produced as a consequence of binding of MgATP\(^{2-}\) at the two metal-nucleotide binding sites, as well as competitive interaction of CaATP\(^{2-}\) at both sites, was greatly aided by the development of a kinetic model, based in part on previous studies of the enzyme mechanism (8–11). This model incorporated values of the kinetic constants for Ca\(^{2+}\) and Mg\(^{2+}\) interaction at the metal-binding site and the apparent K\(_d\) values for MgATP\(^{2-}\) at each of the two metal-nucleotide-binding sites as determined in the present...
study, and was used to evaluate inhibition of enzyme activity attributable to Ca\(^{2+}\) and CaATP\(^{2-}\) in the presence of saturating concentrations of bicarbonate, ammonia, and N-acetylglutamate. A preliminary account of this work has been published in abstract form (12).

**EXPERIMENTAL PROCEDURES**

**Purification of Carbamylphosphate Synthetase from Rat Liver**

Carbamylphosphate synthetase I used for the metal-binding studies was purified to homogeneity from livers of rats fed a high protein diet as described elsewhere (13). For the kinetic studies, the purification scheme was abbreviated as described by Powers (14). The enzyme was stored at -70°C in buffer containing 50 mM HEPES, pH 8.0, 2 mM ammonium sulfate, 0.1 M sodium acetate, 10 mM Mg acetate and 2 mM dithioerythritol in 20% glycerol. When assayed in buffer containing 50 mM HEPES, pH 7.8, 50 mM KCl, 20 mM ammonium acetate, 20 mM Mg acetate, 10 mM ATP, 15 mM N-acetylglutamate, 2 mM dithioerythritol, and 10 μM EGTA, the enzyme had a specific activity of 2-3 pmol of carbamylphosphate/mg of protein-min as previously reported (13). The enzyme preparation was desalted using the technique described by Penefsky (15).

Prior to any binding studies, 1 ml of the enzyme preparation (6 mg/ml) was dialyzed for 2 h against 100 ml of buffer containing 50 mM HEPES, pH 7.8, 0.1 M NaCl, 2 mM dithioerythritol, and 0.5 mM EDTA, followed by three changes with 250 ml of a similar buffer but with 5 mM EDTA. The protein solution was then passed through a Sephadex G-25 column (0.5 x 20 cm) pre-equilibrated with the same EDTA-free buffer to remove any remaining EDTA contamination and concentrated using Amicon filters. The dialyzed enzyme preparation retained its original specific activity and contained no detectable Mn\(^{2+}\), Mg\(^{2+}\), or Ca\(^{2+}\) when assayed by atomic absorption spectroscopy.

**Determination of Carbamylphosphate Synthetase Activity**—Enzyme activity was determined by radiometric or colorimetric techniques. The colorimetric method of Ceriotti and Gazzaniga (16) was used as described by Lusty (13) for the experiments presented in Table I. The radiometric assay was used for the kinetic studies and involved measurement of [\(^{14}C\)carbamide] incorporation into citrulline in the presence of excess ornithine and ornithine transcarbamylase. The assay buffer (final volume, 0.5 ml) consisted of 50 mM HEPES, pH 7.8, 50 mM Na acetate, 50 mM KCl, Na\(^{4+}\)CO\(_3\) (1-2 μCi/mill), 10 mM ammonium acetate, 5 mM ATP, 6 mM Mg acetate, 10 mM N-acetylglutamate, 2 mM dithioerythritol, 10 μM EGTA, 10 mM ornithine, 10 units of ornithine transcarbamylase, and 10-30 μg of carbamylphosphate synthetase. The reaction was started by the addition of enzyme and 1-ml aliquots were removed after 5, 10, and 15 min at 37°C. The protein solution was then passed through a Sephadex G-25 column (0.5 x 20 cm) to remove any remaining EDTA contamination and concentrated using Amicon filters. The dialyzed enzyme preparation retained its original specific activity and contained no detectable Mn\(^{2+}\), Mg\(^{2+}\), or Ca\(^{2+}\) when assayed by atomic absorption spectroscopy.

**Metal-binding Studies**—Binding of Mn\(^{2+}\) to rat liver carbamylphosphate synthetase I was determined as described by Cohn and Townsend (17) using a Varian EPR-E12 spectrometer operating at 9 GHz. Binding titrations were performed at 37°C in 0.05 ml final volume of buffer containing 50 mM HEPES, pH 7.8, 100 mM NaCl, and 2 mM dithioerythritol at a fixed enzyme concentration in the range from 30 to 60 μM with the Mn\(^{2+}\) concentration varied to provide from 20 to 80% saturation of the enzyme. The protein concentration was calculated from its absorbance at 290 nm (13). The number and Kd value of the Mn\(^{2+}\)-binding sites were determined from linear regression analysis of Scatchard plots. The dissociation constants for Mn\(^{2+}\) and Mg\(^{2+}\) were determined under the same experimental conditions by measuring the increase in the Kd for Mn\(^{2+}\) with the Mn\(^{2+}\) titration being conducted in the presence of 0.5 mM Ca\(^{2+}\) or Mg\(^{2+}\) and applying the equation for simple competitive binding of two metals for a common site (18).

**Preparation of ATP/PS Diastereoisomers**—The two diastereoisomers of ATP/PS were prepared enzymatically from ADP/PS essentially as described by Ekstein and Goody (19) with the modifications as outlined by Jaffe and Cohn (20). Both isomer stock solutions were extensively extracted with 8-hydroxyquinoline in chloroform to prevent metal contamination.

**Calculation of Free and ATP-ligated Metal Concentrations**—Since the kinetic experiments were based on the addition of different mixtures of Mg\(^{2+}\), Mn\(^{2+}\), or Ca\(^{2+}\) and ATP, it was necessary to calculate the concentrations of free metal and metal-ATP complexes present in the incubation medium. This problem was considerably simplified since experiments were carried out at pH 7.8 and the contributions of the protonated forms of ATP\(^{2-}\) both as significant metal-binding contributors and as kinetically competent species can safely be neglected (21). Calculations were performed using an iterative program to solve equations for the binding of two metals with two ligands (22). Contributions to metal binding from other components of the reaction mixture were negligible. The dissociation constants used (61 and 31 μM for MgATP\(^{2-}\) and MnATP\(^{2-}\) complexes, respectively) are those reported by Morrison (21), after correction for the effects of pH, ionic strength, and presence of monovalent cations. A value of 190 μM was determined for the apparent dissociation constant for CaATP\(^{2-}\) by competition with the calcium indicator arsenazo II III (23) in the presence of 50 mM HEPES, pH 7.8, 100 mM sodium acetate, and 2 mM dithioerythritol. The amounts of total Mg\(^{2+}\) and total ATP necessary to generate a particular range of MgATP\(^{2-}\) concentrations at constant free Mg\(^{2+}\) were calculated according to O'Sullivan and Smithers (24).

**Kinetic Model of Calcium Inhibition of Carbamylphosphate Synthetase Activity**—The model was based on the ordered reaction sequence for metal and metal-nucleotide binding to carbamylphosphate synthetase from previous kinetic studies of Elliott and Tipton (8, 9), the pulse-chase experiments of Rubio et al. (10) and Britton et al. (11), and the equilibrium and kinetic studies of Fahien and Cohen (25) and Fahien et al. (26). The model accounts for the presence of one metal site and two separate metal-nucleotide sites and assumes, on the basis of the above studies, that binding of the metal and metal-nucleotides occurs in rapid equilibrium so that their apparent Kd values are numerically equal to their respective dissociation constants. The multiple equilibria that describe the reaction sequence in the presence of Ca\(^{2+}\) are depicted in Fig. 1.

The model describes the situation in which free Ca\(^{2+}\) competes with Mg\(^{2+}\) for the metal binding site and CaATP\(^{2-}\) competes with MgATP\(^{2-}\) for its binding at either or both metal-nucleotide sites. It is assumed that when Mg\(^{2+}\) binds to the metal site it induces an abortive E.Ca complex which precludes any further binding of substrates and activators. Additionally, CaATP\(^{2-}\) can bind to one or both metal-nucleotide sites to form the respective dead-end complexes. Kd and Kc represent the dissociation constants for Mg\(^{2+}\) and Ca\(^{2+}\), respectively. In the absence of Ca\(^{2+}\), and with saturating concentrations of bicarbonate, ammonia, and N-acetylglutamate, the first molecule of MgATP\(^{2-}\) binds to the E-Mg complex with a dissociation constant termed Kd, while the second molecule of MgATP\(^{2-}\) binds at the second metal-nucleotide site with a dissociation constant of Kc, so that a represents the ratio of dissociation constants for MgATP\(^{2-}\) and CaATP\(^{2-}\) at these sites.

When calcium is added, CaATP\(^{2-}\) can bind to either or both metal-nucleotide sites to produce in each case a dead-end complex. Kd refers to the dissociation constant for binding of CaATP\(^{2-}\) at site 1, and dKd is the dissociation constant for CaATP\(^{2-}\) binding at site 2. Thus, Kc represents the ratio of the dissociation constant for CaATP\(^{2-}\) at site 2 relative to that at site 1, when both are occupied by CaATP\(^{2-}\). In addition to the homologous complexes which contain the same metal-nucleotide species at both sites, the possibility of formation of heterologous complexes containing both MgATP\(^{2-}\) and CaATP\(^{2-}\) at either or both of the metal-nucleotide sites must be considered. Ka represents the ratio of dissociation constants for CaATP\(^{2-}\) and CaATP\(^{2-}\) at these sites.
Calcium Inhibition of Carbamylphosphate Synthetase

The rate equation that describes this multiple equilibrium, \(v\), derived as described by Segel (27), is:

\[
\begin{align*}
\frac{v}{V_{\text{max}}}[Mg]MgATP] = & \frac{[Mg][MgATP] / \left[ K_a [K] + [Mg][\alpha K] \right]}{\left[ \gamma + [Mg][\alpha K] + [Mg][\alpha K] \right]} \\
& + \left( \frac{[Mg][\alpha K] + [Mg][\alpha K]}{\beta K} \right) + \left( \frac{[Mg][\alpha K] + [Mg][\alpha K]}{\beta K} \right)
\end{align*}
\]  

In the absence of \(Ca^2+\), Equation 2 reduces to:

\[
\frac{v}{V_{\text{max}}}[Mg][MgATP] = \frac{[Mg][MgATP] / \left[ K_a [K] + [Mg][\alpha K] \right]}{\left[ \gamma + [Mg][\alpha K] + [Mg][\alpha K] \right]} + \left( \frac{[Mg][\alpha K] + [Mg][\alpha K]}{\beta K} \right)
\]

Computer Simulations and Statistical Fittings—A Fortran program was developed which allowed the simulation of titration curves according to Equation 2 using a PDP-11/23 computer interfaced with a matrix printer and a Tektronics graphics plotter. The program calculated the distribution of free and nucleotide-bound metal species from specified totals and solved for Equation 2 or its reciprocal using a predetermined set of kinetic parameters. The simulation was performed by mathematically incrementing total calcium concentrations at constant total ATP and \(Mg^{2+}\) concentrations, and the results were expressed in the form of either Dixon or double reciprocal plots. A nonlinear least squares regression analysis of Equation 3 was carried out using the BMDP routine (a derivative-free nonlinear least squares regression program) from the BMDP statistical package of the University of California as modified for the DEC-10 computer.

**RESULTS**

Calcium Inhibition of Carbamylphosphate Synthetase—When carbamylphosphate synthetase is assayed under standard conditions with saturating concentrations of bicarbonate, ammonia, and \(N\)-acetylglutamate in buffer containing 6 mM \(Mg^{2+}\) and 5 mM ATP the activity of the enzyme is inhibited by the addition of \(Ca^{2+}\). Fig. 2A (solid line) shows that the enzyme is inhibited by about 90% when the total calcium concentration reaches 5 mM. From the Dixon plot of these data (Fig. 2A, inset) an apparent \(K_d\) for total calcium of 0.7 mM is obtained. This value was relatively independent of ionic strength but was highly dependent on the concentrations of total \(Mg^{2+}\) and ATP (data not shown). Calcium competes with \(Mg^{2+}\) for chelation with ATP, and Fig. 2B shows the changes in the concentrations of free \(Mg^{2+}\), free \(Ca^{2+}\), \(MgATP^2-\), and \(CaATP^2-\) in the assay buffer with different additions of total calcium, calculated using values of 61 and 190 \(\mu\)M for the dissociation constants of \(MgATP^2-\) and \(CaATP^2-\), respectively. With total \(Mg^{2+}\) and ATP concentrations of 6 and 5 mM, respectively, addition of 5 mM total \(Ca^{2+}\) produced a free \(Ca^{2+}\) concentration of 3.5 mM and a \(CaATP^2-\) concentration of 1.5 mM, while the \(MgATP^2-\) concentration decreased from 4.75 to 3.5 mM and free \(Mg^{2+}\) increased from 1.2 to 2.6 mM. Theoretically, therefore, the observed inhibition of enzyme activity upon addition of \(Ca^{2+}\) could be caused by a fall of the \(MgATP^2-\) concentration or by an increase of free \(Ca^{2+}\) or \(CaATP^2-\). Similar results were obtained with \(Mn^{2+}\) rather than \(Mg^{2+}\) as the activator metal except that the total \(Ca^{2+}\) required to achieve half-maximal inhibition was 4-fold higher (data not shown). This difference of sensitivity of the enzyme to \(Ca^{2+}\) inhibition with \(Mn^{2+}\) or \(Mg^{2+}\) present can be ascribed to several factors, namely the smaller \(K_d\) for complexation of \(Mn^{2+}\) than \(Mg^{2+}\) with ATP (31 versus 61 \(\mu\)M) and the higher affinity of both the free metal site and the two metal-nucleotide-binding sites for \(Mn^{2+}\) compared with \(Mg^{2+}\) as the activator metal (see subsequent sections). Clearly, an
evaluation of the mechanism by which Ca$^{2+}$ inhibits carbamylphosphate synthetase activity requires a knowledge of the kinetic constants for interactions at both the free metal-binding site and the metal-nucleotide-binding sites.

**Competition of Ca$^{2+}$ at the Metal-Binding Site—Equilibrium binding studies of Mn$^{2+}$ to enzymes, as monitored by EPR techniques, have been widely used to determine the number and affinity of divalent metal-binding sites to a variety of enzymes, including *Escherichia coli* carbamylphosphate synthetase (17, 28–30). Fig. 3 (curve A) shows a Scatchard plot of Mn$^{2+}$ binding to highly purified rat liver carbamylphosphate synthetase. Extrapolation of the line to the abscissa confirms that there is only one free metal-binding site, while the reciprocal of the slope provides a value of 13 μM for the apparent $K_d$ for Mn$^{2+}$ binding. When the Mn$^{2+}$-binding titration was performed in the presence of 0.5 mM Ca$^{2+}$, the Scatchard plot deviated from linearity at low ratios of bound Mn$^{2+}$ (Fig. 3, curve B). However, the linear portion of the plot extrapolated to a value of unity on the abscissa, while the apparent $K_d$ for Mn$^{2+}$ binding was increased to 44 μM. This increase of the Mn$^{2+}$ dissociation constant in the presence of Ca$^{2+}$ indicates competition between the two metal ions for the same site. Calculations based on seven separate determinations provided a mean value of 193 ± 6 μM for the $K_d$ for Mn$^{2+}$ binding. Further experiments showed, as expected, that Mg$^{2+}$ was competitive with Mn$^{2+}$ for binding to the enzyme, and gave a value of 180 μM for the $K_d$ for Mn$^{2+}$. The experimentally determined dissociation constants for free Mn$^{2+}$ and Mg$^{2+}$ binding to carbamylphosphate synthetase determined by EPR techniques (13 and 180 μM, respectively) are very similar to the kinetically determined $K_a$ values of 14.7 and 170 μM (13). This finding strongly suggests that both the kinetic and equilibrium binding measurements refer to the same site and that the free metal is in rapid equilibrium with the metal-binding site. Therefore, the $K_d$ for free Ca$^{2+}$ at this site is expected to be equal to the $K_a$ for Ca$^{2+}$ binding as determined above, namely about 190 μM.

It is evident from the above considerations that, for the buffer system used for the standard assay conditions, the $K_a$ for free Mg$^{2+}$ is approximately equal to the $K'$ for free Ca$^{2+}$, both being in the range 170–200 μM. Using values for these parameters calculated from the metal-binding studies, and assuming saturating concentrations of all other reactants and activators, the reaction velocity can be calculated on the basis of a single site competition between Ca$^{2+}$ and Mg$^{2+}$ using the kinetic equation for simple competitive inhibition. Values for free Ca$^{2+}$ and free Mg$^{2+}$ were taken from the curves presented in Fig. 2B, and the calculated enzyme velocity is shown by the dotted line in Fig. 2A. The inhibition attributed to Ca$^{2+}$ inhibition calculated on this basis was considerably less than the observed inhibition of enzyme activity. A series of experiments using Mn$^{2+}$ as the activator metal were also performed with similar results. It is clear from these results that the overall inhibition of carbamylphosphate synthetase by calcium cannot be accounted for solely in terms of free Ca$^{2+}$ competition with the metal ion activator at the metal-binding site of the enzyme.

**Specificity and Affinity of the Metal-Nucleotide-binding Sites**—In order to investigate the specificity of rat liver carbamylphosphate synthetase for different metal nucleotides, titrations of enzyme activity were performed at pH 7.4 using saturating concentrations of bicarbonate, ammonia, and N-acetylimidazole, with varied metal-nucleotide concentrations in the range from 0.1 to 10 mM using either Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, or Ca$^{2+}$ as the metal ion. The free metal concentration was kept constant during the titration at 10 mM for Mg$^{2+}$, 5 mM for Co$^{2+}$, 0.2 mM for Mn$^{2+}$, and 0.1 mM for Zn$^{2+}$ and Cd$^{2+}$. Concentrations of free Mn$^{2+}$ higher than about 0.2 mM were found to be inhibitory. The two diastereoisomers of ATP$\beta$S (19, 20, 31) have also been used in an attempt to probe the stereospecificity of the metal nucleotide complex. Table I summarizes the results of these experiments using Mg$^{2+}$, Mn$^{2+}$, or Co$^{2+}$ as the metal ion. No detectable enzyme activity could be obtained with Ca$^{2+}$, Zn$^{2+}$, or Cd$^{2+}$ as the only metal ion present. These results are in agreement with a previous report on the inhibitory effects of certain heavy metal ions on rat liver carbamylphosphate synthetase I activity (14). Table I shows that the $V_{max}$ values for MgATP$\beta$S and MnATP$\beta$S were similar and about 10-fold higher than that for CaATP$\beta$S. The enzyme is stereospecific for the A diastereoisomer of ATP$\beta$S when complexed with Mg$^{2+}$, but activity is also observed with the B diastereoisomer with Mn$^{2+}$ or Co$^{2+}$ as the metal ligand ($V_{max}$ A/B = 2.7 and 2.3, respectively). Further experiments, in which ATP$\beta$S (A) and ATP$\beta$S (B) were both present at 1 mM together with 3 mM Mg$^{2+}$, showed

![Image](http://www.jbc.org/)

**Fig. 2.** Inhibition of rat liver carbamylphosphate synthetase by calcium. Initial rates of citrulline formation were measured in an assay mixture (see "Experimental Procedures") containing 6 mM total Mg and 5 mM total ATP. A (solid line) shows the measured mean inhibition of reaction rate after addition of calcium to several experiments, while the broken line shows a calculated rate assuming only competitive inhibition of Ca$^{2+}$ at the Mg$^{2+}$-binding site from the formula $v = V_{max} [Mg]/[Mg] + K_{Mg}$, where $K_{Mg}$ = 180 μM and $K_d$ = 193 μM. B shows the calculated concentrations of free Ca$^{2+}$, free Mn$^{2+}$, CaATP$\beta$S, and MgATP$\beta$S in the reaction mixture as a function of total calcium addition using values of 61 and 190 μM for the dissociation constants for chelation of Mg$^{2+}$ and Ca$^{2+}$, respectively, to ATP.

![Image](http://www.jbc.org/)

**Fig. 3.** Scatchard plots of Mn$^{2+}$ binding to rat liver carbamylphosphate synthetase in the absence of (A) and presence (B) of 0.5 mM Ca$^{2+}$. The amount of enzyme used was 30 μM in A and 40 μM in B.

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S. Cerdan and C. J. Lusty, unpublished observations.
that the enzyme activity was the same as that obtained with 1 mM ATP6S (A) as the only nucleotide present, indicating that MgATP6S (A) is the preferred substrate at both metal-nucleotide-binding sites (data not shown). Under the conditions of the assay with saturating (10 mM) free Mg2+ concentrations, the K values for MgATP and MgATP6S (A) were similar. Despite the wide variation in the concentrations of free Mn2+ and free Co2+, the K values for both Mn2+ and Co2+ complexed with ATP, ATP6S (A) and ATP6S (B) all fell within the range from 1.0 to 2.5 mM. It is evident, therefore, that substitution of the metal ion or substitution of ATP with ATP6S greatly alters the activity of the enzyme, but has a small effect on the apparent binding affinity of the metal-nucleotide to the enzyme. The loss of stereospecificity observed with MnATP6S and CoATP6S can be explained by the known ability of Mn2+ and Co2+ to bind to both oxygen and sulfur while Mg2+ binds preferentially to oxygen (20, 32).

In order to distinguish between interactions of metal-nucleotides at the two different metal-nucleotide-binding sites, a different kinetic approach had to be used. It is known from earlier studies that the overall apparent K of carbamylphosphate synthetase for MgATP2- is decreased with an increase of the free Mg2+ concentration (8, 13, 33). Consequently, the effect of varying the MgATP2- concentration on carbamylphosphate synthetase activity was studied in more detail under conditions when the free Mg2+ concentration was maintained constant at a value of 1 mM, which is thought to represent the free Mg2+ concentration in the matrix of normal rat liver mitochondria (34, 35). This value is five times higher than the apparent K of Mg2+ at the metal-binding site, so that formation of the E-Mg complex (see Fig. 1) should not be rate-limiting. Under these conditions, with saturating concentrations of bicarbonate, ammonia, and N-acetylglutamate, double reciprocal plots of initial rate versus MgATP2- over the range from 0.2 to 10 mM MgATP2- were nonlinear (Fig. 4). Extrapolation of the curve to the abscissa provided a value of 2.5 mM for the K of MgATP2-. However, since carbamylphosphate synthetase contains two metal-nucleotide-binding sites, the actual meaning of this value is unclear in relation to the relative affinities of MgATP2- at the two sites. This problem was approached by curve fitting the data to the rate Equation 3 which defines the K for the first MgATP2- binding site as K and the second as αK. Iterative nonlinear least squares fitting gave the curve shown in Fig. 4 and provided values for K of 0.14 ± 0.01 mM and 17.3 ± 1.1 for α, indicating that the first molecule of MgATP2- is bound to the enzyme with considerably higher affinity than the second.

The K for MgATP2- at the second site is thus 2.4 mM, which is similar to the single value obtained by extrapolating the linear portion of the double reciprocal plot of Fig. 4 to the abscissa. The present finding that the first metal-nucleotide site has a higher affinity for MgATP2- than the second site is in agreement with previous observations with the frog liver enzyme (25, 26). These authors reported values of 0.05 and 0.5 mM for the apparent dissociation constants of MgATP2- at the first and second sites, respectively. However, our findings contrast with those of Rubio et al. (10, 11) who obtained the reverse relative affinities (0.2 and 0.01 mM) using pulse-chase experiments with rat liver carbamylphosphate synthetase. Higher values in the region of 1–2 mM are normally reported for the apparent K of rat liver carbamylphosphate synthetase for MgATP2- with both the isolated enzyme and rat liver mitochondria (13, 33, 36), which can now be interpreted as reflecting mainly interaction of MgATP2- at the second metal-nucleotide-binding site.

**Competition of CaATP2- at the Metal-Nucleotide-binding Sites**—In order to design the inhibitory effects of CaATP2- on carbamylphosphate synthetase activity, it was necessary to design kinetic experiments that would allow a discrimination of CaATP2- inhibition between the two MgATP2- substrate sites so that the respective CaATP2- inhibitory constants could be calculated. Fig. 5 depicts the binding sequence of substrates and activators suggested by Elliott and Tipton (8) from kinetic studies with bovine liver carbamylphosphate synthetase. Mg2+ is bound first, followed by a random order addition of MgATP2- (at site 1) or N-acetylglutamate. This is followed by an ordered addition of bicarbonate, MgATP2- (at site 2), and finally ammonia. Since the affinities of the

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**Table I**

Summary of kinetic constants for rat liver carbamylphosphate synthetase using different metal activators and ATP6S diastereoisomers

| Metal nucleotide | $K_a$ (mM) | $V_{max}$ (nmol/mg min) |
|------------------|------------|-------------------------|
| MgATP            | 3.65       | 286E                   |
| MgATP6S (A)      | 1.40       | 0.2                     |
| MgATP6S (B)      | 1.90       | 0.10                    |
| MnATP            | 1.0        | 2400                    |
| MnATP6S (A)      | 2.5        | 0.27                    |
| MnATP6S (B)      | 1.9        | 0.10                    |
| CoATP            | 2.2        | 285                     |
| CoATP6S (A)      | 1.0        | 0.42                    |
| CoATP6S (B)      | 1.0        | 0.18                    |

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**Fig. 4.** Double reciprocal plot of rat liver carbamylphosphate synthetase activity versus MgATP2- concentration at a constant free Mg2+ concentration of 1 mM. The reaction velocity is calculated as per cent of $V_{max}$ and for graphical plotting reciprocal values $× 10^4$ are shown for the mean of two experiments. The line drawn represents the best fit to the data by nonlinear least squares analysis using Equation 3 under “Experimental Procedures.”

**Fig. 5.** Reaction scheme for mammalian carbamylphosphate synthetase showing the sequence of binding of substrates and activators. NAG, N-acetylglutamate. Subscripts 1 and 2 refer to binding of MgATP2- at the first and second metal-nucleotide-binding sites, respectively.
enzyme for binding of MgATP\(^{2-}\) (at site 1) and N-acetylglutamate are similar, either the upper or lower branch of the reaction pathway is expected to be favored by the relative concentrations of N-acetylglutamate or MgATP\(^{2-}\). If the lower branch of the reaction pathway is followed as suggested from other work (37), MgATP\(^{2-}\) binds to the first metal-nucleotide site before N-acetylglutamate, followed by bicarbonate, the second molecule of MgATP\(^{2-}\), and ammonia. Accordingly, it should be possible to monitor the CaATP\(^{2-}\) inhibition at each of the metal-nucleotide sites by selecting the appropriate conditions (38). Thus, using saturating concentrations of bicarbonate and ammonia and a high concentration of MgATP\(^{2-}\) relative to N-acetylglutamate, titrations of N-acetylglutamate concentration at a series of fixed calcium concentrations should allow CaATP\(^{2-}\) inhibition to be monitored at the first metal-nucleotide site. Similarly, using saturating concentrations of N-acetylglutamate and ammonia and limiting concentrations of bicarbonate in the absence and presence of different calcium concentrations should also monitor CaATP\(^{2-}\) inhibition at the first site. Finally, using saturating concentrations of bicarbonate and N-acetylglutamate, and limiting ammonia in the absence and presence of calcium should allow an analysis of CaATP\(^{2-}\) inhibition at the second metal-nucleotide site.

Fig. 6 shows the results of titrations of carbamylphosphate synthetase activity with variation of the N-acetylglutamate concentration in the range from 0.05 or 1 mM total calcium. The experiment was conducted using 50 mM bicarbonate, 10 mM ammonia, 5 mM total Mg\(^{2+}\), and 5 mM total ATP. The Lineweaver-Burk plot produced straight lines, which intersected at a single point on the abscissa, indicating a noncompetitive inhibition pattern with an apparent $K^*_n$ for N-acetylglutamate of 114 $\mu$M. This is similar to the values obtained by other workers (13, 25). Furthermore, it is apparent that Ca\(^{2+}\) or CaATP\(^{2-}\) do not affect the apparent $K^*_n$ for N-acetylglutamate. The concentration of CaATP\(^{2-}\) in the reaction mixture after addition of either 0.5 or 1 mM total calcium was calculated as in Fig. 2, and replots of the intercepts or the slopes of the lines of Fig. 6 versus [CaATP\(^{2-}\)] are shown as insets. Both replots intersected the abscissa at a value equivalent to 0.35 mM CaATP\(^{2-}\), which may be interpreted as an apparent $K^*_n$ for CaATP\(^{2-}\) at the first metal-nucleotide-binding site. This value was confirmed in further experiments (data not shown) in which seven different calcium concentrations up to 5 mM were used, each with N-acetylglutamate concentra-

![Fig. 6](image)

**Fig. 6.** Double reciprocal plot of rat liver carbamylphosphate synthetase activity versus N-acetylglutamate concentration with 0, 0.5, and 1 mM total calcium added. The insets show replots of the slope or intercept of the lines versus the calculated CaATP\(^{2-}\) concentrations of the reaction mixture.

Fig. 7 shows a Lineweaver-Burk plot with variation of the bicarbonate concentration from 1 to 20 mM with no calcium added and with 0.5 and 1 mM total calcium in the presence of 10 mM N-acetylglutamate, 5 mM total ATP, and 6 mM total Mg\(^{2+}\). As in Fig. 6, the inhibition pattern caused by calcium was noncompetitive, and the apparent $K^*_n$ for bicarbonate was 4 mM (cf. Refs. 8 and 13). Replots of the intercepts and the slope of the lines (see insets against [CaATP\(^{2-}\)]) were linear, and gave a value of 0.4 mM for the apparent $K^*_n$ for CaATP\(^{2-}\) inhibition at the first metal-nucleotide site. Thus, irrespective of whether the reaction pathway for mammalian carbamylphosphate synthetase I follows the upper or lower branch of Fig. 5, inhibition by CaATP\(^{2-}\) showed noncompetitive inhibition kinetics with variation of either the N-acetylglutamate or the bicarbonate concentration, and provided values of 0.35 and 0.4 mM, respectively, for the apparent $K^*_n$ for CaATP\(^{2-}\).

In the observed kinetic behavior favors a reaction mechanism consistent with the lower branch in which N-acetylglutamate binds after the first molecule of MgATP\(^{2-}\) (37).

In contrast, when the ammonia concentration was varied under the standard assay conditions with 50 mM bicarbonate, 10 mM N-acetylglutamate, 6 mM total Mg\(^{2+}\), and 5 mM total ATP, apparently parallel lines were produced with Lineweaver-Burk plots at total calcium concentrations of 0, 0.5, and 1 mM (Fig. 8). However, linear regression analysis showed that they were in fact weakly convergent. The apparent $K^*_n$ for ammonia was 1.7 mM (cf. Refs. 8 and 13). Earlier studies by Elliott and Tipton (8), which included ammonia titrations over a range of MgATP\(^{2-}\) concentrations, showed that at low concentrations of MgATP\(^{2-}\) (below 0.5 mM) the lines became convergent. These authors concluded, therefore, that ammonia binds to the enzyme after the second molecule of MgATP\(^{2-}\). The inset of Fig. 8 shows a replot of the intercept of the lines on the ordinate versus the calculated CaATP\(^{2-}\) concentrations after addition of 0.5 and 1 mM total calcium. The points on the replot fell on a straight line which intersected the abscissa to give an apparent $K^*_n$ for CaATP\(^{2-}\) of 0.12 mM. This value may be interpreted as representing the inhib-
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FIG. 8. Double reciprocal plots of rat liver carbamylphosphate synthetase activity versus ammonia concentration with 0, 0.5, and 1 mM total calcium added. The inset shows a replot of the intercept versus the calculated CaATP$^{2-}$ concentrations of the reaction mixture.

The inset shows a replot of the intercept versus the calculated CaATP$^{2-}$ concentrations of the reaction mixture.

In summary, and with reference to Fig. 1, the kinetic experiments described above using CaATP$^{2-}$ as an inhibitor of carbamylphosphate synthetase have allowed an assessment of the relative inhibitory constants at the two metal-nucleotide-binding sites. At the first MgATP$^{2-}$-binding site, the ratio of the apparent $K_a$ for MgATP$^{2-}$ to the apparent $K_i$ for CaATP$^{2-}$ ($K_a/K_i$) is 0.37, while at the second MgATP$^{2-}$-binding site the ratio of the constants ($\alpha K_i/\beta K_a$) is 19.5. Thus, the overall inhibitory effect of CaATP$^{2-}$ on the reaction velocity is expected to be exerted mainly at the second metal-nucleotide site.

Kinetic Model to Describe Calcium Inhibition of Carbamylphosphate Synthetase—From kinetic and binding studies with purified rat liver carbamylphosphate synthetase I, values have been obtained for the primary kinetic constants as follows: $K_a$ for free Mg$^{2+}$ ($K_a$), 180 $\mu$M; $K_i$ for free Ca$^{2+}$ ($K_i$), 193 $\mu$M; $K_a$ for MgATP$^{2-}$ at site 1 ($K_{a1}$), 140 $\mu$M; $K_a$ for MgATP$^{2-}$ at site 2 ($K_{a2}$), 2420 $\mu$M; $K_i$ for CaATP$^{2-}$ at site 1 ($K_{i1}$), 375 $\mu$M; $K_i$ for CaATP$^{2-}$ at site 2 ($K_{i2}$), 124 $\mu$M. Initially, a computer program was developed to fit the experimental data employing the above kinetic constants and a modification of Equation 2 in which the formation of mixed complexes (E·Mg·Mg$^{2+}$·CaATP$^{2-}$) were neglected. The simulated curve for data such as that of Fig. 2 gave a very poor fit, similar to the dotted line shown in Fig. 2A. It became obvious, therefore, that formation of mixed metal-nucleotide abortive complexes needed to be incorporated into the kinetic model. This required knowledge of two additional kinetic constants, namely $\gamma K_i$ and $\delta K_a$ (see Fig. 1).

These constants cannot readily be obtained from primary or secondary kinetic plots; hence, an iterative curve-fitting procedure was used to estimate the remaining unknown parameters. For this purpose, two additional sets of experimental data were used. In the first, the buffer contained 50 mM bicarbonate, 10 mM ammonia, 10 mM N-acetylglutamate as standard additions and four different concentrations of total ATP were used as follows: 0.5, 1, 2, and 5 mM. In each case, total Mg$^{2+}$ was added to give 1 mM excess free Mg$^{2+}$ concentration. To each set, different amounts of total calcium were added up to 5 mM (cf. Fig. 2). These conditions were chosen to provide a balanced distribution of points for a double reciprocal plot of velocity versus MgATP$^{2-}$. In the second experiment, similar conditions were used except that the MgATP$^{2-}$ concentration was varied with a constant free Mg$^{2+}$ concentration of 1 mM (cf. Fig. 4), and four sets of curves were generated by addition of 0, 0.5, 1, and 2 mM total calcium. These conditions allowed a balanced distribution of points for a double reciprocal plot of velocity versus MgATP$^{2-}$. In order to simplify the nonlinear least squares curve-fitting procedure using the BMDP program, the assumption was initially made that mixed complexes were formed primarily by addition of CaATP$^{2-}$ to the second metal-nucleotide site when the first site was occupied by MgATP$^{2-}$ (i.e. that $\delta >\gamma$; cf. Fig. 1). Consequently, the data from both sets of experiments were used to optimize for $\gamma$ using a modification of rate Equation 2.

The $V_{max}$ of the enzyme could not be precisely determined graphically from the Dixon plot data; hence, $\gamma$ was calculated for different values of $V_{max}$ until a minimum value was obtained for the sum of the least squares. In addition, 3 of the 28 data points gave very high nonrandom residuals and were eliminated from the analysis. The value for $\gamma$ was then very well determined with a $V_{max}$ of 1300 nmol/mg of protein·min and $\gamma = 0.309 \pm 0.003$. The process was repeated for the second experiment with all 32 data points, and again $\gamma$ was very well determined, at a value of 0.307 ± 0.002 ($V_{max} = 950$ nmol/mg of protein·min). Finally, the full rate Equation 2 was used to determine $\delta$. The BMDP program gave large and very poorly determined values for $\delta$; hence, it was assumed that the contribution of the E·Mg·CaATP$^{2-}$·MgATP$^{2-}$ complex (see Fig. 1) to the overall reaction velocity was negligible. Consequently, the kinetic constant for formation of mixed metal-nucleotide complexes ($\gamma K_i$) was considered to be 116 $\mu$M.

Fig. 9 shows simulations of plots of the first experiment referred to above expressed in the form of velocity versus [CaATP$^{2-}$] (Fig. 9A) or reciprocal velocity versus [CaATP$^{2-}$] (Fig. 9B), using a modification of rate Equation 2 with the term including $\delta$ eliminated and values for the kinetic constants determined as described above. Fig. 10 shows similar simulations of the second experiment using the same kinetic constants, except for $V_{max}$ with the data expressed in the form of velocity versus [MgATP$^{2-}$]. An excellent simulation of the nonlinear plots is obtained by the kinetic model, indicating that it provides a valid representation of the kinetic mechanisms for calcium inhibition of carbamylphosphate synthetase I.

In summary, the major effect of calcium in regulating the enzyme activity is by CaATP$^{2-}$-inhibition primarily at the second metal-nucleotide-binding site. Under conditions thought to mimic the ionic environment of the matrix space...
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**DISCUSSION**

A number of enzymes involved in phosphoryl transfer are known to be inhibited by calcium (39). Among those that have been extensively studied are pyruvate kinase (40), pyruvate carboxylase (41, 42), and bacterial carbamylphosphate synthetase (30). The present study shows that binding constants of free Mg$^{2+}$ and free Ca$^{2+}$ to the rat liver enzyme are considerably smaller than those for the bacterial enzyme (30), which is also inhibited by Ca$^{2+}$ and shares the same stereospecificity for the A diastereoisomer of ATP$^{2-}$ (31, 43). Since most enzymes that have a MgATP$^{2-}$-binding site also have an essential requirement for a free metal (usually Mg$^{2+}$ or Mn$^{2+}$), the question arises whether an observed inhibition of enzyme activity by calcium is caused by interaction of free Ca$^{2+}$ at the metal-binding site or by interaction of CaATP$^{2-}$ at the metal-nucleotide-binding site. Despite the importance of the question, very few studies have addressed this issue, with the exception of a kinetic study of inorganic pyrophosphatase (44).

Despite extensive kinetic studies with mammalian carbamylphosphate synthetase (8, 9, 33), the nonlinear nature of kinetic plots involving variations of MgATP$^{2-}$ concentration has made it difficult to obtain Michaelis constants by purely graphic procedures. However, from a knowledge of the kinetic mechanism, it is possible to generate a simplified rate equation by using concentrations of reactants and activators, other than MgATP$^{2-}$, well above their respective Michaelis constants. This rate equation can then be used to obtain solutions for the unknown kinetic constants by nonlinear least squares curve fitting of the experimental data in order to optimize for the apparent $K_a$ for MgATP$^{2-}$ reported by other workers using isolated carbamylphosphate synthetase (13), toluene-permeabilized rat liver mitochondria (45), or intact mitochondria (46). The fact that the affinity of the mammalian enzyme for Mg$^{2+}$ is high relative to that for the second molecule of MgATP$^{2-}$ and the finding that the ratio of $K_a$ (Mg)/$K_a$ (Ca) is about unity compared with a value of about 20 for the ratio of $K_a$ (MgATP$^2$)/$K_a$ (CaATP$^2$) at the second metal-nucleotide-binding site ($\alpha K_a/\gamma K_a$ in Fig. 1), largely accounts for the observation that the direct contribution of free Ca$^{2+}$ to overall inhibition of enzyme activity is relatively small. Thus, the interpretation of kinetic studies based on variations of MgATP$^{2-}$ and CaATP$^{2-}$ concentrations is aided by use of free Mg$^{2+}$ concentrations higher than the $K_a$ (Mg) range, so that despite inevitable variations of free Mg$^{2+}$ or free Ca$^{2+}$ concentrations due to their different binding constants to ATP, inhibitory interactions at the metal-binding site contribute little to the observed changes of enzyme reaction rate.

Addition of calcium to purified carbamylphosphate synthetase has, in fact, proved to be a useful inhibitory probe to extend previous studies related to the enzyme mechanism. Separate titrations of $N$-acetylglutamate, bicarbonate, and ammonia concentrations in the absence and presence of different total calcium concentrations gave linear kinetic plots consistent with binding of the first molecule of MgATP$^{2-}$ prior to addition of $N$-acetylglutamate and bicarbonate (37), and binding of the second molecule of MgATP$^{2-}$ prior to the addition of ammonia (8). These experiments also showed that Ca$^{2+}$ or CaATP$^{2-}$ did not affect the apparent $K_a$ for $N$-acetylglutamate (cf. Ref. 45), and allowed a quantitative evaluation of the inhibitory interactions of CaATP$^{2-}$ at both metal-nucleotide-binding sites. In addition, the present study provides excellent circumstantial evidence for the formation of mixed MgATP-CaATP complexes and an evaluation of their importance in accounting for the observed inhibition of enzyme activity by calcium. The value of combining purely kinetic studies with nonlinear curve fitting to computer models using different forms of the rate equation to describe the reaction velocity, when marked deviations of the kinetic plots from linearity are obtained, is well illustrated by the present study. Computer simulations are not able to prove reaction mechanisms except in exceptional cases, but they are useful in delineating a minimal mechanism consistent with the experimental data. The fact that the values obtained with the model could provide fits for two independent series of kinetic experiments with the same kinetic constants gives an added measure of confidence to the usefulness of the model, and may allow it to be utilized to study the regulation of carbamylphosphate synthetase activity in isolated mitochondria.

The experimental conditions chosen to evaluate the kinetic model of rat liver carbamylphosphate synthetase were designed to resemble the ionic conditions thought to be present in rat liver mitochondria, where rates of citrulline production have been studied as a function of the mitochondrial energy state or calcium content (6, 35, 36, 47). Thus, the free Mg$^{2+}$ concentration was maintained at about 1 mM and the MgATP$^{2-}$ concentration was varied up to 10 mM. Rat liver mitochondria as normally prepared in the absence of excess EGTA in the isolation medium contain 10-15 nmol of calcium/mg of protein (48), and the mitochondrial calcium content in the intact liver appears to be in the same range (49). Approximately 99.9% of this calcium is bound, and the free Ca$^{2+}$ concentration has been estimated to be in the region of 10 $\mu$M (7). The nature of the calcium-binding ligands has not been ascertained, although inorganic phosphate (50), phospholipid head groups (7) and proteins are reasonable candidates. Carbamylphosphate synthetase itself is present in liver.
mitochondria at a concentration of about 1 mM, and hence will contribute significantly to the overall binding sites for both metals and metal-nucleotides in the mitochondrial matrix. The possibility of significant concentrations of CaATP$^{-2}$ in the mitochondrial matrix has not been considered in most studies concerning metabolic regulation. However, the present work with purified carbamylphosphate synthetase suggests that the previously observed inhibition of citrulline production in isolated rat liver mitochondria with an increase of both metals and metal-nucleotides in the mitochondrial matrix is needed before more definitive conclusions can be reached concerning the physiological regulation of intramitochondrial enzymes by CaATP$^{-2}$.

One of the expectations on embarking on the present study (see Ref. 51) was that carbamylphosphate synthetase would prove to be a useful Ca$^{2+}$-sensitive indicator enzyme more in the range of the observed values for the matrix-free Ca$^{2+}$ concentration of rat liver mitochondria than other mitochondrial Ca$^{2+}$-sensitive enzymes such as isocitrate dehydrogenase, a-ketoglutarate dehydrogenase, and pyruvate dehydrogenase synthetase appears to have little usefulness as an enzyme indicator of the mitochondrial free Ca$^{2+}$ concentration, it is possible that measurements of citrulline production by rat liver mitochondria under defined conditions may be used as an intramitochondrial CaATP$^{-2}$ indicator.

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