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# A STUDY OF THE RATE OF CHELATION OF MAGNESIUM BY CDTA AND EDTA IN THE ATP ($Na^+ + K^+$)-ATPase SYSTEM

EDWARD S. HYMAN

*Touro Research Institute, 1401 Foucher Street, New Orleans, LA 70115 (U.S.A.)*

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## Summary

1. Using Jørgensen's preparation (Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36—52) of ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) from the outer medulla of the rabbit kidney the observations of Klodos and Skou (Klodos, I. and Skou, J.C. (1977) Biochim. Biophys. Acta 481, 667—679) have been confirmed. At equimolar concentrations, CDTA, which is known to associate with $Mg^{2+}$ more slowly than EDTA, interrupts phosphorylation more slowly than EDTA. This interruption is greatly delayed if the free $Mg^{2+}$ is buffered by MgEDTA.

2. Using the phosphorylation of ($Na^+ + K^+$)-ATPase in 140 mM NaCl at pH 7.5 as a rapid assay of free $Mg^{2+}$ at 0°C and at 37°C, the fall in free $Mg^{2+}$ was measured as a function of time after adding each of three concentrations of CDTA to $MgCl_2$ and to a MgEDTA metal-buffer system.

3. The data for the fall in free $Mg^{2+}$ upon adding CDTA to $MgCl_2$ at 0°C were fitted to the equation for a quasi first order reaction assuming a constant concentration of the chelating agent. The rate constant for the fall in free $Mg^{2+}$ at 0°C was 1.8 $s^{-1}$ with 5 mM CDTA, 2.4 $s^{-1}$ with 10 mM CDTA, and 3.8 $s^{-1}$ with 20 mM CDTA. At 37°C the rate was too fast for the assay.

4. When 10 mM CDTA was added to the metal-buffer system containing 1 mM $Mg^{2+}$ and 3 mM EDTA, the free $Mg^{2+}$ fell from the level of the initial equilibrium with EDTA alone to a final level in which free $Mg^{2+}$ is in equilibrium with both chelating agents. The fall is not instantaneous. There was an initial brief induction period of rapid fall in free $Mg^{2+}$ followed by a slower, steady, semilogarithmic fall.

5. The slowed fall in free $Mg^{2+}$ observed in the MgEDTA-CDTA system is a phenomenon in the reversible transfer of an ion or a ligand from one strong association to another. The change in concentration of free ions with time can

Abbreviation: CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid or its salt.

be fitted to rate equations for two reversible quasi first order reactions in series, and from these equations the rate constants for both associations can be derived. Conversely, given the rate constants, the equations may be used to predict the concentration of the free ion and of each chelate as a function of time.

6. In the presence of MgEDTA buffer the rate constants found for the association of free $Mg^{2+}$ at 0°C were 1.4 $s^{-1}$ with 5 mM CDTA, 2.1 $s^{-1}$ with 10 mM CDTA, and 3.5 $s^{-1}$ with 20 mM CDTA. These values are comparable to the above rate constants for the fall in free $Mg^{2+}$ without the EDTA metal-buffer. The rate constant for association of free $Mg^{2+}$ with 2 mM EDTA was 3.2 $s^{-1}$, about 8 times the estimated rate constant for equimolar CDTA. The rate constants for dissociation were $1 \cdot 10^{-4}$ $s^{-1}$ for 5 mM CDTA, $8 \cdot 10^{-5}$ $s^{-1}$ for 10 mM CDTA, and $6.6 \cdot 10^{-5}$ $s^{-1}$ for 20 mM CDTA. For 2 mM EDTA it was $6 \cdot 10^{-3}$ $s^{-1}$.

7. In the presence of MgEDTA at 37°C the values derived for the rate constants for association with free $Mg^{2+}$ were 2600 $s^{-1}$ for 5 mM CDTA, 4200 $s^{-1}$ for 10 mM CDTA, and 7500 $s^{-1}$ for 20 mM CDTA. For 2 mM EDTA the rate constant for association with $Mg^{2+}$ was 2475 $s^{-1}$, about twice the estimated rate constant for equimolar CDTA. The rate constants for dissociation were 0.024 $s^{-1}$ for 5 mM CDTA, 0.019 $s^{-1}$ for 10 mM CDTA, and 0.017 $s^{-1}$ for 20 mM CDTA. For 2 mM EDTA it was 0.55 $s^{-1}$.

8. The slowed fall of the concentration of free ion upon adding a second chelating agent is largely due to reassociation of the ion with the initial chelating agent. The slow fall is expected whenever addition of a second chelating agent lowers the concentration of the free ion, except when the rate of dissociation of the first chelate times the total concentration of the ion divided by the sum of the rates of association of the ion with the two chelating anions is not significantly greater than the concentration of the free ion at the final equilibrium. Then the fall in free ion concentration is very rapid, approximating that of the unbuffered (fully dissociated) ion species. These equations should be applicable to other chelating anions and to binding sites of macromolecules. As another example of the retarded fall in free $Mg^{2+}$, it was found to be slow upon adding CDTA to a magnesium citrate equilibrium.

9. No chelating agent removes free $Mg^{2+}$ instantly or stops phosphorylation instantly. The delay in stopping phosphorylation depends upon the minimal level of free $Mg^{2+}$ necessary to sustain phosphorylation at a given temperature, and it depends upon the delay in reducing the free $Mg^{2+}$ to that level. The latter delay depends upon the initial concentration of free $Mg^{2+}$, of chelated $Mg^{2+}$, and of free chelating anions; and it depends upon the rate constants for each ion association which in turn are a function of temperature and pH. For the most rapid interruption of phosphorylation by a chelating agent minimal $Mg^{2+}$ should be used, and if a metal buffer must be present the same anion or one with a faster rate of association should be used to interrupt phosphorylation.

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## Introduction

Strong ion association, or chelation, of magnesium has an integral function in the phosphorylation of $(Na^+ + K^+)$-ATPase, and its interruption by foreign

chelating agents such as EDTA is a standard method of studying partial reactions of the enzyme. This interruption by EDTA is the transfer of magnesium from one chelating agent, ATP or the enzyme, to a second one which is at a higher concentration and which has a higher association constant. Association constants are a ratio at equilibrium of the rate of association to the rate of dissociation. Although there are extensive tables of association constants for magnesium with many polyanions [1], there is very little data for the rates of association even for common chelating agents, and such data is not in physiologic ranges [2]. The reaction rates are difficult to obtain unless there is a rapid spectrophometric, NMR, or other method of measuring the bound or the free state of one of the ion species involved. No such method exists for $Mg^{2+}$.

Klodos and Skou [3] called attention to the rate of chelation of $Mg^{2+}$ when they found that CDTA, which chelates cations more slowly than EDTA [4], removes free $Mg^{2+}$ too slowly to be useful in the study of $(Na^+ + K^+)$-ATPase. They found that CDTA took several seconds to remove free $Mg^{2+}$ at 0°C, and the removal of free $Mg^{2+}$ was progressively slowed by small increments of EDTA added prior to the CDTA. They concluded that MgEDTA serves as a reservoir which continues to dissociate free $Mg^{2+}$ faster than the CDTA associates the free $Mg^{2+}$, and that the transfer continues for an hour. Moreover, evidence for the continued presence of free $Mg^{2+}$ persisted even when they believed enough CDTA had been added to chelate all the remaining free $Mg^{2+}$ at least down to 2 nM.

But, if the pH of solutions of $MgCl_2$ and Tris-EDTA are adjusted so that, upon mixing, the last 10% of the $H^+$ liberated by chelation will change the color of an indicator dye, the chelation of free $Mg^{2+}$ appears instantaneous at room temperature and at 0°C. Moreover, if the presence of a metal-buffer can delay removal of free $Mg^{2+}$ by CDTA for an hour, do other chelating agents remove buffered $Mg^{2+}$ in less than one second for proper study of the partial reactions of $(Na^+ + K^+)$-ATPase? After confirming the basic observations of Klodos and Skou in this laboratory, and after confirming the rapid color change of the pH indicator by microsecond flash photographs delayed at intervals of 0.01 s, this study of the rate of chelation of $Mg^{2+}$ was undertaken to better define the transfer of $Mg^{2+}$ from one chelating agent (or anionic site) to another. The $(Na^+ + K^+)$-ATPase system was used as a rapid probe to assay free $Mg^{2+}$ in the micromolar range. The results confirm the slow fall in free $Mg^{2+}$ when CDTA is added to a MgEDTA buffer system. These results can be fitted to a mathematical expression for the $Mg^{2+}$ exchange between two chelating agents, and reasonable values for the rate constants for association and dissociation of $Mg^{2+}$ with EDTA and with CDTA can be derived. The rate constants for association with CDTA derived are comparable to those determined for the simple reaction of $MgCl_2$ with CDTA. They show that EDTA binds $Mg^{2+}$ only a few times faster than does CDTA, and that MgEDTA liberates $Mg^{2+}$ less rapidly than it is bound by CDTA. The buffering of free $Mg^{2+}$ with respect to time may have a more general importance than the EDTA-Mg-CDTA system.

## Materials and Methods

The $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) was prepared from the outer medulla of a rabbit kidney by the method of Jorgensen [5]. Under standard conditions

at 37°C in 10 mM imidazole buffer at pH 7.5 it assayed at about 3 units per mg protein.

Unless stated otherwise, all reactions were carried out in a medium containing 140 mM NaCl, and 10 mM imidazole buffer at pH 7.5.

*Reagents*

ATP labeled in the gamma position with $^{32}P$([$\gamma$-$^{32}P$]ATP) was obtained from the Amersham Corporation, Arlington Heights, IL, U.S.A. Vanadium-free, non-radioactive Tris-ATP was obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. CDTA was obtained from Sigma Chemical Company. EDTA and sodium citrate were Analyzed Reagent Grade obtained from J.T. Baker Chemical Company. By atomic absorption the CDTA contained 1 mmol $Mg^{2+}$ per mol CDTA, and the EDTA contained less.

*Apparatus*

All reactions were carried out in an automatic apparatus which includes a thermostatically controlled mixing chamber to which reagents can be added from any number of electrically driven Teflon burettes that are timed electronically. The time interval between burettings was recorded on a quartz-stopwatch triggered photoelectrically. In this apparatus delivery of reagents and mixing is complete in 0.03 s as measured by photographing the color change of a pH indicator using a time delayed photoflash of 1 $\mu$s duration. Observations were made at 0°C and 37°C.

After repeating the initial observations of Klodos and Skou the enzyme was used as a means of rapidly assaying $Mg^{2+}$. In a typical experiment a given amount of CDTA was added to a solution of $MgCl_2$ or to a mixture of $MgCl_2$ and EDTA at time zero. After a controlled variable time ATP and enzyme were added simultaneously but from separate burettes to minimize non-specific phosphorylation. Then, after 0.31 or 5.0 s of phosphorylation, trichloroacetic acid was added from another burette.

Equilibrium results were calculated using the stability constants compiled by Martel [1] calculated for the temperature and pH by standard equations [6]. The association constants used at 37°C were $2.25 \cdot 10^6$ for MgEDTA, $2.17 \cdot 10^7$ for MgCDTA, and 16840 for MgATP; and at 0°C they were $2.67 \cdot 10^5$, $2.64 \cdot 10^6$, and 8320 respectively. Equilibrium ion concentrations for $Mg^{2+}$ in the presence of two or more chelating agents were calculated using a published program on a Texas Instruments SR-59 hand calculator [7].

## Results and Discussion

*The relationship between MgATP and formation of $^{32}P$-labeled ($Na^+ + K^+$)-ATPase, a calibration curve*

In previous work in this laboratory formation of ($Na^+ + K^+$)-[$^{32}P$]ATPase has repeatedly been found to be a sigmoid function of the log MgATP concentration. At 37°C phosphorylation is complete in a little more than 0.3 s. The curves in Fig. 1 were determined using MgEDTA buffers to fix free $Mg^{2+}$ in the same range as in the experiments to follow. Throughout the work the enzyme was phosphorylated with 40 $\mu$M ATP bearing the $^{32}P$ label. The curve at 37°C

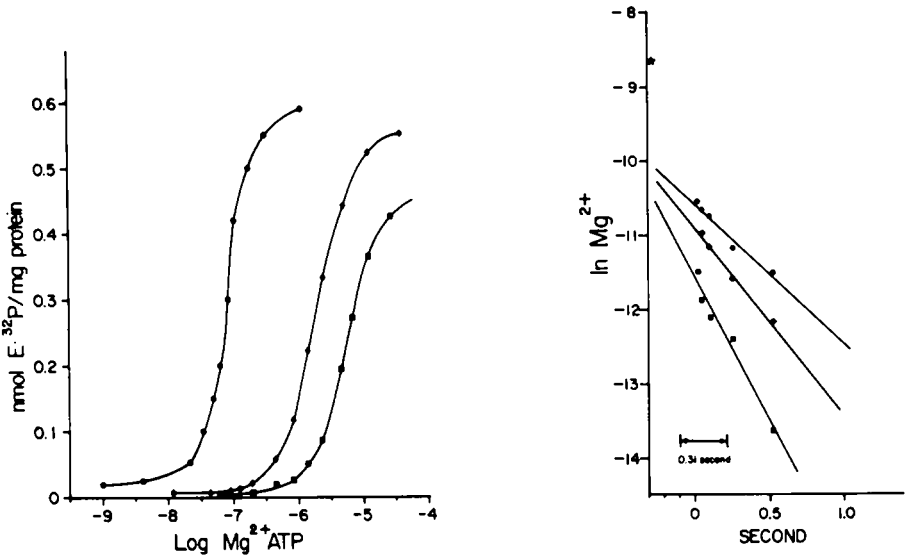


Fig. 1. Phosphorylation of $(Na^+ + K^+)$-ATPase (E) as a function of log ([MgATP]in M) at 0°C and 37°C. The medium contained 7.5 mM EDTA, 140 mM NaCl, 40 μM [γ-$^{32}$P]ATP, and 33 μg E in 10 mM imidazole buffer at pH 7.5. The free $Mg^{2+}$ and the MgATP were varied by adding increasing concentrations of $MgCl_2$. ●——●, 37°C with 0.31 s phosphorylation time; ◆——◆, 0°C with 5 s phosphorylation time, and ■——■, 0°C with 0.31 s phosphorylation time. These curves were used to determine free $Mg^{2+}$ from the $(Na^+ + K^+)$-[$^{32}$P]ATPase (E · $^{32}$P) value.

Fig. 2. The fall in free $Mg^{2+}$ with time at 0°C upon adding CDTA to $MgCl_2$. At time zero 5 mM (●——●), 10 mM (◇——◇), or 20 mM (■——■) CDTA was added to 0.02 mM $MgCl_2$ in the standard medium buffered at pH 7.5. At the time on the $x$ axis 40 μM ATP and 33 μg $(Na^+ + K^+)$-ATPase were simultaneously added from separate burettes. After 0.31 s trichloroacetic acid was added. Free $Mg^{2+}$ was calculated from the $(Na^+ + K^+)$-[$^{32}$P]ATPase (E · $^{32}$P) using the appropriate curve in Fig. 1. The slopes of the regression lines of the least sum of squares best fits are $-1.8\ s^{-1}$ for 5 mM, $-2.4\ s^{-1}$ for 10 mM, and $-3.8\ s^{-1}$ for 20 mM CDTA. The calculated final equilibria are at −17.96 for 5 mM, −18.68 for 10 mM, and −19.38 for 20 mM. ln $Mg^{2+}$ at the initial equilibrium (*) is placed at −0.31 s because each data point represents the value derived from phosphorylation over a 0.31-s interval beginning at the time of the data point. Thus the point at 0.05 s represents the integral of phosphorylation from 0.05 s to 0.36 s.

has been repeated many times in this laboratory over a period of 3 years with many batches of $(Na^+ + K^+)$-ATPase. At 0°C phosphorylation requires a little more than 5 s, but the results shown by the curve in Fig. 1 are reproducible at 5 s. In this study the 5-s curve was reproduced 3 times using 2 batches of enzyme. The curve at 0.31 s at 0°C was constructed in order to have a reference for a more rapid assay of $Mg^{2+}$. These curves were used to translate $(Na^+ + K^+)$-[$^{32}$P]ATPase formed into log MgATP from which free $Mg^{2+}$ could be calculated.

The sigmoid curves in Fig. 1 differ from the linear relationships used by Klodos and Skou for $(Na^+ + K^+)$-[$^{32}$P]ATPase vs. log $Mg^{2+}$ using $(Na^+ + K^+)$-ATPase from brain. The difference is not explained by calculation of MgATP from their data. The slope of the near linear portion of the sigmoid curve is slightly greater than twice the slope found by Klodos and Skou.

*Rate of reaction of CDTA with free $Mg^{2+}$*

In Fig. 2 the ln $Mg^{2+}$ is plotted against time after the addition of 5 mM,

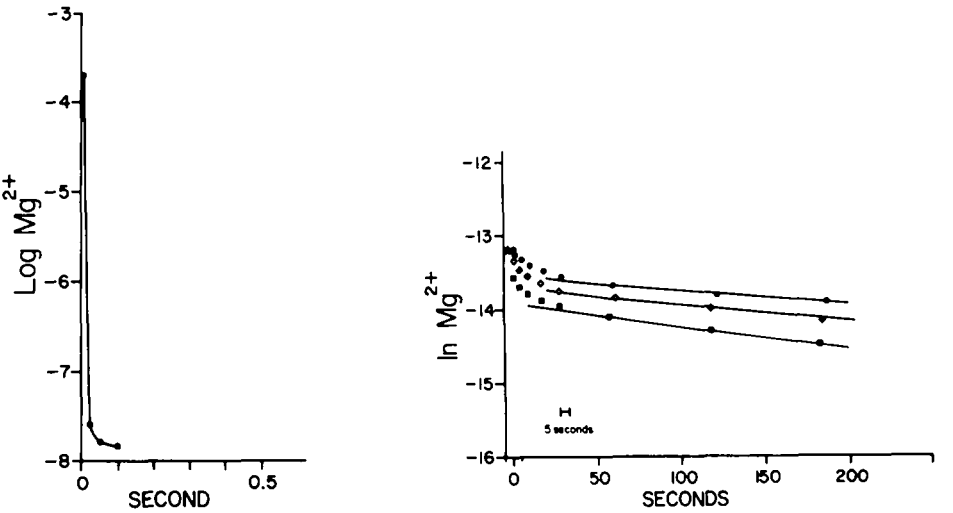


Fig. 3. The fall in free $Mg^{2+}$ with time upon adding 10 mM CDTA to 0.2 mM $MgCl_2$ at 37°C. Other than the temperature the experimental conditions were the same as in Fig. 2. The free $Mg^{2+}$ fell 4 orders of 10 by the time of the first assay at 0.02 s.

Fig. 4. The fall in free $Mg^{2+}$ upon adding 5 mM (●———●), 10 mM (◇———◇), and 20 mM (■———■) CDTA to metal buffered $Mg^{2+}$ at 0°C. The standard medium contained 140 mM NaCl, 1 mM $MgCl_2$ and 3 mM EDTA. At time zero CDTA was added. At the time on the $x$ axis [$\gamma$-$^{32}$P]ATP and ($Na^+ + K^+$)-ATPase were added from separate burettes. Five seconds later trichloroacetic acid was added. Free $Mg^{2+}$ was calculated from the ($Na^+ + K^+$)-[$^{32}$P]ATPase formed. After a period of rapid fall (induction period) there is a steady semilogarithmic fall. At the final equilibria the ln free $Mg^{2+}$ are −16.26 for 5 mM CDTA, −17.02 for 10 mM CDTA, and −17.75 for 20 mM CDTA. For reasons given under Fig. 2, ln $Mg^{2+}$ at the initial equilibrium (*) is placed at −5 s.

10 mM, and 20 mM CDTA to 0.2 mM $MgCl_2$ using the 0.31-s phosphorylation time at 0°C. The results show a semilogarithmic relationship. The slopes calculated by least squares best fit are −1.8, −2.4, and −3.8 $s^{-1}$. The intercepts with the initial magnesium concentration are in negative time. The interval 0.31 s is marked on the graph to demonstrate that the change in free $Mg^{2+}$ is very rapid even with respect to the abbreviated phosphorylation time, and thus the phosphorylation measured was a summation over a time interval during which the $Mg^{2+}$ had significantly fallen. Since a measurement recorded at 0.05 s is actually a summation of phosphorylation over the interval 0.05 to 0.36 s, the intercept at the ln of the initial magnesium concentration would be expected to be in negative time. The values for $Mg^{2+}$ in this graph were derived from the near linear portion of the calibration curve in Fig. 1. Extension beyond that time would involve integration of formation of ($Na^+ + K^+$)-[$^{32}$P]-ATPase in the toe of the calibration curve which would be far less reliable.

At 37°C the binding of $Mg^{2+}$ was entirely too fast to be measured even when 5 mM CDTA was added. In Fig. 3, using 10 mM CDTA, the apparent magnesium concentration fell 4 orders of 10 in the first time interval, 0.02 s.

*The rate of reaction of a MgEDTA buffer with CDTA*

In Fig. 4, at 0°C, CDTA was added to a solution containing 1 mM $MgCl_2$

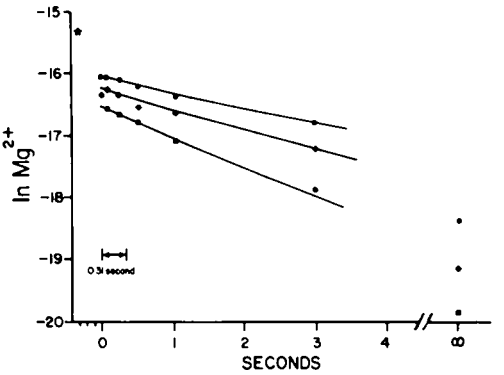


Fig. 5. Same as Fig. 4 but at 37°C with a phosphorylation time of 0.31 s. As in Fig. 2, the initial equilibrium (*) was placed at −0.31 s. ●———●, 5 mM CDTA; ◇———◇, 10 mM CDTA; and ■———■, 20 mM CDTA. The initial fall in ln $Mg^{2+}$ was too rapid to measure. Final equilibria are shown at time = ∞.

and 3 mM EDTA. In the initial equilibrium free $Mg^{2+}$ is 1.87 μM (or 1.86 μM with 40 μM ATP added) plotted at −13.19 on the ln scale. At zero time 5 mM, 10 mM, or 20 mM CDTA was added. After the time shown in the $x$ axis the enzyme and ATP were simultaneously added from separate burettes. Five seconds later the phosphorylation reaction was interrupted with trichloroacetic acid. The precipitated enzyme was washed and $^{32}P$ was counted. MgATP was determined from the reference curve in Fig. 1 and free $Mg^{2+}$ was calculated. At each concentration of CDTA there is an initial rapid fall in ln $Mg^{2+}$, an induction period, followed by a slow semilogarithmic fall. During the induction period the fall and the rate of fall is greater with higher concentration of CDTA. The ln of calculated free $Mg^{2+}$ levels predicted for the final equilibrium involving 1 mM $Mg^{2+}$, 3 mM EDTA, 40 μM ATP are −16.16 for 5 mM CDTA, −17.02 for 10 mM, and −17.15 for 20 mM.

The same experiment was repeated at 37°C using a 0.3 s phosphorylation time as shown in Fig. 5. At 37°C the fall in ln $Mg^{2+}$, during the induction period, like the fall of unbuffered $Mg^{2+}$ in Fig. 3, is too rapid for the assay. Thus the data for the three curves do not approach the ln of the initial equilibrium value for free $Mg^{2+}$ at −15.32, at time zero. The values for ln $Mg^{2+}$ at the final equilibrium are shown by the appropriate symbol at time = infinity.

*Mathematical approximations*

In the absence of EDTA a mathematical approximation for the fall in free $Mg^{2+}$ is a relatively simple expression for the approach to equilibrium involving a second order reaction forward and a first order reaction in reverse. The expression may be further simplified by assuming that the concentration (activity) of free CDTA remains constant, thus converting the forward reaction to a quasi first order reaction with an equilibrium association constant, $K'_a$, equal to the molar association constant, $K_a$, times molarity of CDTA. At the lowest concentration there is 25 times as much CDTA as $Mg^{2+}$, and this approximation is close. If only the initial part of the curve is considered then the back

reaction causes only a small deviation towards horizontal which is hardly apparent and which is readily corrected by subtracting the equilibrium value from all other values. The equation now becomes a simple first order forward reaction with a slope as given. The slope found upon adding 10 mM CDTA may be expected to be twice the slope for 5 mM, and the slope for 20 mM twice that after 10 mM, but this was not observed in two sets of experiments. The most likely reason is that the effect of ionic strength was not considered. An increase in ionic strength would probably lower the stoichiometric association constant affecting the rate of association more than the rate of dissociation. In this laboratory an increase in ionic strength has also been found to lessen phospho-enzyme formation under otherwise standard conditions. The mixture containing 20 mM CDTA has an ionic strength of 0.26 M.

The equations for the calculation of the exchange of magnesium between two metal chelates is far more complicated. They can be simplified with approximations, primarily by assuming that the concentration of the free anion remains constant and then representing both second order reactions as quasi first order reactions as above. Again the molar equilibrium association constant, $K_a$, is replaced by $K'_a$ which equals $K_a$ times the molarity of the anion. The approximations to follow were made according to the scheme.

$$A_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A_2 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} A_3$$

with $A_1$ representing $Mg^{2+}$ as EDTA complex, $A_2$ representing free $Mg^{2+}$, and $A_3$ representing $Mg^{2+}$ as the CDTA complex. The equilibrium association constant $K'_a$ is the rate of association divided by the rate of dissociation. For MgEDTA $K'_a = k_{21}/k_{12}$ and for MgCDTA the $K'_a = k_{23}/k_{32}$. To approximate a first order reaction, $K'_a$ for MgEDTA was set equal to $K_a$ times 0.002 M, which is essentially the molarity of the excess of EDTA at the beginning of the reaction, at time equals 0. With continuation of the reaction this value for free EDTA rises to approach the final equilibrium as determined by the calculator program [7], which is 0.00293 M for 5 mM MgCDTA, to 0.00297 for the 10 mM CDTA, and to 0.00298 for the 20 mM CDTA. No mathematical correction was made for this rise, but the experiment was continued only long enough for free EDTA to rise to about 2.3 mM. Since the use of these approximations in the first order equations to follow predicts a final equilibrium which is an acceptable approximation of the calculated final equilibrium, the approximations are reasonable. Even simplified to two quasi first order reactions in series, the integrated equations for concentration of $Mg^{2+}$ versus time are not simple [8].

$$[A_1] = [A_1^0]\left\{\frac{k_{21}k_{32}}{\lambda_2\lambda_3} + \frac{k_{12}(\lambda_2 - k_{23} - k_{32})}{\lambda_2(\lambda_2 - \lambda_3)}e^{-\lambda_2 t} + \frac{k_{12}(k_{23} + k_{32} - \lambda_3)}{\lambda_3(\lambda_2 - \lambda_3)}e^{-\lambda_3 t}\right\}$$

$$[A_2] = [A_1^0]\left\{\frac{k_{12}k_{32}}{\lambda_2\lambda_3} + \frac{k_{12}(k_{32} - \lambda_2)}{\lambda_2(\lambda_2 - \lambda_3)}e^{-\lambda_2 t} + \frac{k_{12}(\lambda_3 - k_{32})}{\lambda_3(\lambda_2 - \lambda_3)}e^{-\lambda_3 t}\right\}$$

$$[A_3] = [A_1^0]\left\{\frac{k_{12}k_{23}}{\lambda_2\lambda_3} + \frac{k_{12}k_{23}}{\lambda_2(\lambda_2 - \lambda_3)}e^{-\lambda_2 t} - \frac{k_{12}k_{23}}{\lambda_3(\lambda_2 - \lambda_3)}e^{-\lambda_3 t}\right\} \quad (1)$$

where $[A_1^0]$ is $[A_1]$ at time = 0, which in this case nearly equals the total $Mg^{2+}$,

$$\lambda_2 = \tfrac{1}{2}(p + q)$$
$$\lambda_3 = \tfrac{1}{2}(p - q)$$

where $p = (k_{12} + k_{21} + k_{23} + k_{32})$ and $q = [p^2 - 4(k_{12}k_{23} + k_{21}k_{32} + k_{12}k_{32})]^{1/2}$.

These equations assume that all of the $Mg^{2+}$ begins as $A_1$, but in the experiments all the $Mg^{2+}$ begins as an equilibrium between $A_1$ and $A_2$ in the absence of $A_3$. Thus a plot of $\ln[A_2]$ versus time, according to the above equation, begins at minus infinity at time zero and very rapidly rises to the semilogarithmic slopes drawn in Figs. 4 and 5.

As an alternate to the above equations for the explanation of the experimental data, the experimental curves in Fig. 4 are noted to resemble the composite of two semilogarithmic curves which could somehow represent two rate components for the removal of free $Mg^{2+}$, perhaps a fast rate for the removal of free $Mg^{2+}$ during the induction period and a slower rate for the removal of the free $Mg^{2+}$ liberated from the EDTA complex during the subsequent steady state. This possibility is easily excluded. The slower steady fall in free $Mg^{2+}$ at 0°C is semilogarithmic. Extrapolation of this semilogarithmic slope to time zero yields projected values for $\ln[A_2]$, or $\ln[A_2']$, and thus value for $[A_2']$ during the induction period. A plot of ln (free $Mg^{2+} - [A_2']$) vs. time in Fig. 6

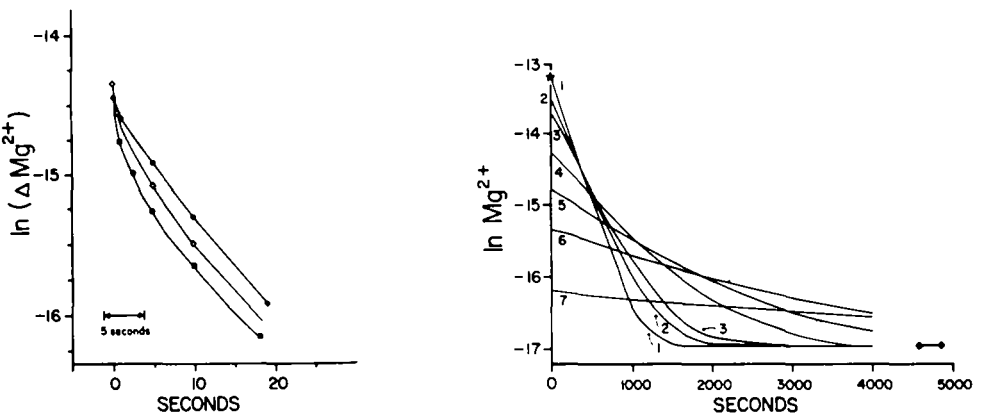


Fig. 6. The rapid fall in $Mg^{2+}$ during the induction phase at 0°C. The semilogarithmic slopes in Fig. 4 were extrapolated to zero time and appropriate values for $Mg^{2+}$ (derived from the extrapolated ln $Mg^{2+}$) were subtracted from each experimental value for $Mg^{2+}$ during the induction phase. This plot of ln ($\Delta Mg^{2+}$) against time shows that the initial fall at each concentration of CDTA (devoid of the later semilogarithmic fall) is curved in a semilogarithmic plot and thus cannot be described by another rate constant. At time = 0 each curve approaches the slope found using unbuffered $Mg^{2+}$. •———•, 5 mM CDTA; ◇———◇, 10 mM CDTA; and ▪———▪, 20 mM CDTA.

Fig. 7. A plot of a family of curves for ln $Mg^{2+}$, or $[A_2]$, as a function of $k_{12}$ to illustrate the effects of reducing $k_{12}$ below a critical value. $K_a'$ for MgEDTA was set at 535, $k_{23}$ at 2.1 $s^{-1}$, and $k_{32}$ at 0.00008 $s^{-1}$. These are the values found for 10 mM CDTA. Except for the zero time intercept these curves were derived from Eqn. 1. Because that equation was written for all the $Mg^{2+}$ starting as $A_1$ at zero time, the zero intercept was calculated from Eqn. 5. ln $Mg^{2+}$ at the initial equilibrium (without CDTA) is marked by the star (*) and at the final equilibrium by ◇———◇. All curves must start at * and end at ◇———◇. Only curve 1, with $k_{12}$ greater than 0.1 $s^{-1}$, extrapolates to the initial equilibrium value at zero time. Curve 1 also falls more rapidly to the final equilibrium. In curve 2, $k_{12} = 0.01$ $s^{-1}$, in curve 3 $k_{12} = 0.006$ $s^{-1}$, in curve 4 $k_{12} = 0.002$ $s^{-1}$, in curve 5 $k_{12} = 0.001$ $s^{-1}$, in curve 6 $k_{12} = 0.0005$ $s^{-1}$, and in curve 7 $k_{12} = 0.0001$ $s^{-1}$. Curve 3 with $k_{12} = 0.006$ $s^{-1}$ fits the experimental data.

gives a curved line for the remaining fast component during the induction period for each of the concentrations of CDTA used. This demonstrates that upon mathematically removing the slower semilogarithmic fall in free $Mg^{2+}$ the remaining early fast fall during the induction period is more complex than a simple semilogarithmic function. Each of the curves in Fig. 6 appears to approach the slope of the fall in unbuffered free $Mg^{2+}$ in Fig. 2 as time approaches zero.

Fitting the experimental data to the mathematical expression for $[A_2]$ results in a determination of the reaction rates for both EDTA and CDTA as follows. At time = 0 the free $Mg^{2+}$ is fixed by the initial equilibrium devoid of CDTA. Since $K'_a$ is constant equal to $k_{21}/k_{12}$, any value assumed for $k_{12}$ will fix the value of $k_{21}$, and similarly any value assumed for $k_{23}$ will fix $k_{32}$. In Fig. 7, using values for 0°C, $K'_a$ is fixed at 535 for the first equilibrium and at 26400 for the second. Then with $k_{23}$ fixed at 2.1 $s^{-1}$ (the value for 10 mM CDTA), a family of curves was plotted as $k_{12}$ is varied. From $k_{12}$ of about 0.1 to $k_{12}$ = infinity the curves for ln $Mg^{2+}$ are almost identical (see below). A greater $k_{12}$ is accompanied by a greater $k_{21}$ and a more rapid return of $Mg^{2+}$ from $A_2$ to $A_1$. When $k_{12}$ is less than 0.1 $s^{-1}$ the extrapolated zero time intercept falls and the slope is lessened as shown. The lesser slope is due to a reduced rate of fall of $A_1$ as the smaller $k_{12}$ itself limits the transfer of $A_1$ to $A_2$. Since the curve that starts lower due to a lower $k_{12}$ is associated with slower depletion of $A_1$, that curve will take longer to reach the same equilibrium value, and it must eventually cross all curves which start higher. Using the same values of $K'_a$ another family of curves (not illustrated) is generated by fixing $k_{12}$ and varying $k_{23}$. By trial and error, there is only one set of $k_{12}$ and $k_{23}$ that fit the experimental data at each concentration of CDTA.

From inspection of the experimental data at 0°C and at 37°C in Figs. 4 and 5 it is clear that $k_{12}$ for MgEDTA must be relatively small because the zero intercept of the near semilogarithmic slope is well below the initial equilibrium. All 3 curves at 0°C were fitted to the equation using $k_{12}$ = 0.006 $s^{-1}$, and at 37°C

TABLE I

SUMMARY OF RATE CONSTANTS FOUND WITH $Mg^{2+}$

$k^*$, the rate in the system without the EDTA metal buffer. $k$(molar) = $k_{23}$ ÷ molarity of CDTA.

| | Assocation ($s^{-1}$) | | | | Dissociation ($s^{-1}$) | |
|---|---|---|---|---|---|---|
| | $k^*$ | $k_{23}$ | $k_{21}$ | $k$(molar) | $k_{32}$ | $k_{12}$ |
| 0°C | | | | | | |
| 5 mM CDTA | 1.8 | 1.4 | | 280 | $1.06 \times 10^{-4}$ | |
| 10 mM CDTA | 2.4 | 2.1 | | 210 | $0.80 \times 10^{-4}$ | |
| 20 mM CDTA | 3.8 | 3.5 | | 175 | $0.66 \times 10^{-4}$ | |
| 2 mM EDTA | | | 3.2 | 1600 | | 0.006 |
| 30 mM citrate | | | 11 | 360 | | 0.6 |
| 37°C | | | | | | |
| 5 mM CDTA | | 2600 | | $5.2 \times 10^{-5}$ | 0.024 | |
| 10 mM CDTA | | 4200 | | $4.2 \times 10^{-5}$ | 0.019 | |
| 20 mM CDTA | | 7500 | | $3.8 \times 10^{-5}$ | 0.017 | |
| 2 mM EDTA | | | 2475 | $12.4 \times 10^{-5}$ | | 0.55 |

using $k_{12} = 0.55\ s^{-1}$. The values for other constants are given in Table I.

At 0°C the values of $k_{23}$ determined by fitting the equation for $[A_2]$ to the experimental data in Fig. 4 are quite close to those determined directly using unbuffered $Mg^{2+}$ (Fig. 2). At 0°C the rate of association for MgEDTA using 2 mM EDTA, $k_{21}$, is $3.2\ s^{-1}$ which is greater than the $1.4\ s^{-1}$ rate of association of $Mg^{2+}$ with 5 mM CDTA, $k_{23}$. It is estimated that for the same molarity the rate of association for EDTA would be about 6 to 8 times that for CDTA. The estimated dissociation rate for MgEDTA, $k_{12} = 0.006\ s^{-1}$, is much faster than that for MgCDTA, $k_{32} = 0.0001\ s^{-1}$ at 5 mM.

At 37°C the same relationship holds but the rates are much more rapid. The rate of association of $Mg^{2+}$ with 2 mM EDTA was found to be $2475\ s^{-1}$ and with 5 mM CDTA $2600\ s^{-1}$. The rate constant for the dissociation of 2 mM MgEDTA was found to be $0.55\ s^{-1}$ which is considerably greater than $0.012\ s^{-1}$ for MgCDTA at 5 mM.

In their description of the slow fall in free $Mg^{2+}$ upon adding CDTA, Klodos and Skou concluded that the binding of free $Mg^{2+}$ by CDTA was slow; and that the yet slower fall in free $Mg^{2+}$ in the presence of MgEDTA required the rate of dissociation of MgEDTA to be faster than the rate of association of $Mg^{2+}$ with CDTA, so that the association equilibrium for MgEDTA would only be slightly distorted as $Mg^{2+}$ is drained off by CDTA. In Fig. 7 this is the case in curve No. 1 when $k_{12}$ exceeds $0.1\ s^{-1}$ which would be the curve when $k_{12}$ was greater than $k_{23} = 2.1\ s^{-1}$ (see equation to follow). There would be no induction period. However, the condition that $k_{12} > k_{23}$ is not necessary for the slowed fall in free $Mg^{2+}$, and in the concentration ranges studied, $k_{12}$ is more than 100 times less than $k_{23}$.

*A simpler approximation of the early part of the curve*

Simple approximations are useful to understand the slowed fall of free $Mg^{2+}$. The simplest concept for the slow fall in buffered free $Mg^{2+}$ comes from the realization that for a total of 1 mM of $Mg^{2+}$ at 0°C, only 1.86 $\mu$M is free.

$$[A_2] = \frac{[A_1]}{K'_a} = \frac{k_{12} \cdot [A_1]}{k_{21}} \tag{2}$$

and the instantaneous rate of removal by CDTA is proportional to the free fraction only. As the free $Mg^{2+}$ is removed it is replenished from a reservoir of $Mg^{2+}$ which is 535 times as large. If replenishment is instantaneous, the rate of fall of free $Mg^{2+}$ is about 1/535 that of the unbuffered,

$$\frac{d[A_2]}{dt} = \frac{k_{23}}{K'_a} \cdot [A_2]$$

and

$$[A_2] = [A_2^0] \cdot e^{(-k_{23} \cdot t)/K'_a} = [A_1^0] \cdot \frac{k_{12}}{k_{21}} \cdot e^{(-k_{12} \cdot k_{23} \cdot t)/k_{21})} \tag{3}$$

In the EDTA-CDTA system where the free $Mg^{2+}$ ($A_2$) is low, the nearly linear portion of the curves for free $Mg^{2+}$ may be approximated by a set of equations omitting $k_{32}$

$$A_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A_2 \overset{k_{23}}{\rightarrow} A_3$$

and by assuming a quasi steady state for $A_2$ so that

$$\frac{d[A_2]}{dt} = k_{12} \cdot [A_1] - k_{21} \cdot [A_2] - k_{23} \cdot [A_3] = 0$$

$$[A_2] = \frac{k_{12}}{k_{21} + k_{23}} \cdot [A_1] \tag{4}$$

$$-\frac{d[A_1]}{dt} = \frac{d[A_3]}{dt} = \frac{k_{12} \cdot k_{23}}{k_{21} + k_{23}} \cdot [A_1]$$

$$[A_1] = [A_1^0] \cdot e^{(-k_{12} \cdot k_{23} \cdot t)/k_{21}+k_{23}}$$

substituting in Eqn. 4

$$[A_2] = [A_1^0] \cdot \frac{k_{12}}{k_{21} + k_{23}} \cdot e^{(-k_{12} \cdot k_{23} \cdot t)/k_{21}+k_{23}} \tag{5}$$

The zero intercept of the near straight line semilogarithmic phase is

$$\text{Free } Mg^{2+} = \frac{k_{12}}{k_{21} + k_{23}} \cdot \text{total } Mg^{2+} \tag{6}$$

When $k_{21}$ is much greater than $k_{23}$ this equation reduces to Eqn. 3 above. This happens in Fig. 7 when the value of $k_{12}$ exceeds 0.1 $s^{-1}$. At $k_{12}$ = 0.1 $s^{-1}$, $k_{21}$ = 0.1 × 535 = 53.5 $s^{-1}$ which greatly exceeds the $k_{23}$ of 2.1 $s^{-1}$. The free $Mg^{2+}$ at the extrapolation to time = 0 falls from the equilibrium value of 1.86 $\mu$M only to 1.80 $\mu$M at the zero extrapolation.

Eqn. 5 gives a reasonable approximation of the straight line portion of the curves in Figs. 4 and 5, but a semilog plot of this equation is a simple straight line which does not approach the final equilibrium because there is no $k_{32}$.

These equations explain Klodos and Skou's observation of a progressive enhancement of effective free $Mg^{2+}$ at a constant 100 $\mu$M free $Mg^{2+}$ when progressive amounts of MgEDTA are present in the initial equilibrium before CDTA is added. The total $Mg^{2+}$, or $[A_1^0]$ in the equation, was progressively increased by the added MgEDTA.

*Discussion of the retarded fall in free $Mg^{2+}$*

The retarded fall in free $Mg^{2+}$ when $Mg^{2+}$ transfers from one chelate to another is not limited to the Mg-EDTA-CDTA system. In Fig. 8 a similar delay in the fall of free $Mg^{2+}$ is evidenced by the ATPase assay when CDTA is added to the magnesium-citrate complex. In Fig. 9 the data for the $(Na^+ + K^+)$-[$^{32}$P]-ATPase formed are fitted to the equation for $[A_2]$ above using $K_a$ = 600, $k_{12}$ = 0.6 $s^{-1}$, and the same $k_{23}$ = 1.4 $s^{-1}$ found in the MgEDTA-CDTA system at 0°C. The $K_a$ for magnesium citrate is well below that for MgEDTA, and the $k_{12}$ is again less than $k_{23}$. Using the ATPase detection system no delay was found in the fall of free $Mg^{2+}$ upon adding CDTA to magnesium succinate, magnesium maleate, or magnesium tartrate, each at 20 mM, probably because their $K_a$ values are low and the delay would be too brief to be detected.

According to the equations above the fall in free $Mg^{2+}$ will be retarded to some degree whenever $Mg^{2+}$ is drained from a $Mg^{2+}$ buffer, whether by a second chelating agent or by an absolute electrochemical sink. The metal buffer can involve simple polyvalent anions or it can involve anionic sites on macromolecules. The metal buffer resists not only a change in the equilibrium value of the

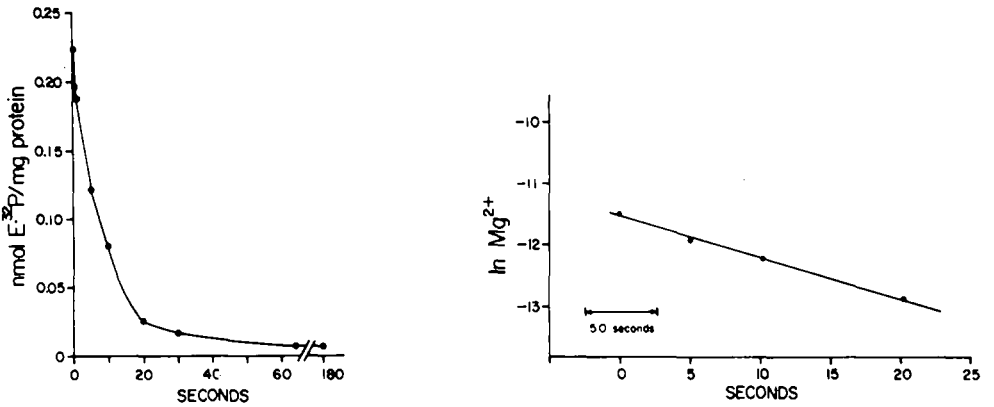


**Fig. 8. Assay for $Mg^{2+}$ as the fall in free $Mg^{2+}$ is delayed by a magnesium citrate buffer at 0°C. The initial medium containing 30 mM trisodium citrate, 50 mM NaCl, and 0.2 mM $MgCl_2$ was brought to pH 7.5 and 10 mM imidazole buffer at pH 7.5 was added. At time zero 5 mM CDTA was added. At the time on the $x$ axis 40 $\mu$M ATP and $(Na^+ + K^+)$-ATPase were simultaneously added from separate burettes. Five seconds later trichloroacetic acid was added. $(Na^+ + K^+)$-[$^{32}$P]ATPase ($E \cdot {}^{32}P$) is plotted against time.**

**Fig. 9. The $E \cdot {}^{32}P$ values in Fig. 8 were translated into log MgATP using a calibration curve in magnesium citrate metal buffers assuming $K_a = 600$ at 0°C, pH 7.5. Then free $Mg^{2+}$ was calculated from MgATP. The curve is fitted with $k_{12} = 0.6$, $k_{21} = 11$, and $k_{23} = 1.4$, the same that was found for 5 mM CDTA in the EDTA-Mg-CDTA system.**

free metal ion, but it also resists the rapidity of a change, a buffering with respect to time.

The time curves of the concentrations of $A_1$, $A_2$, and $A_3$ are predictable if the reaction rates are known. Using the data above an interesting difference can be predicted between adding EDTA to a MgEDTA buffer and adding CDTA to the same MgEDTA buffer, and the prediction is easily confirmed by experiment. If an equal excess (another 2 mM) of EDTA is added to a MgEDTA buffer containing 1 mM $Mg^{2+}$ and 3 mM EDTA, the equations predict that free $Mg^{2+}$ ($A_2$) will drop almost instantly to the level of the final equilibrium, to about 1/2 of the concentration of the free $Mg^{2+}$ present in the initial equilibrium. In the initial equilibrium free $Mg^{2+}$ is fixed by the association constant $K'_a$, where $k_{12}/k_{21} = 1/K'_a$. When another 2 mM EDTA is added as the second chelating agent, $k_{23}$ equals $k_{21}$, and by Eqn. 6 the free fraction of $Mg^{2+}$ at the zero time extrapolation is $k_{12}/(k_{21} + k_{23}) = k_{12}/2 \cdot k_{21}$. Thus the free $Mg^{2+}$ rapidly falls to half that of the initial equilibrium. Then by the calculator program the free $Mg^{2+}$ in final equilibrium of the mixture of 1 mM $Mg^{2+}$ and 5 mM EDTA is also almost exactly half that of the initial equilibrium. Thus the free $Mg^{2+}$ would rapidly fall to the final equilibrium level and remain at that level as if there had been no delay in the fall. But if one could measure the initial MgEDTA complex ($A_1$) separate from $Mg^{2+}$ complex of the added EDTA ($A_3$), the longer equation shows that $A_3$ would slowly rise and approach equilibrium as $A_1$ fell to approach that equilibrium. In this way the case of adding more of the same chelating agent is unique. However, if this same transfer involved similar anionic sites on nearby macromolecules or resin beads, the transfer from $A_1$ to $A_3$ would be clear.

If instead of EDTA an equal molarity of CDTA is added, $k_{23}$ would be about

1/6 to 1/8 of $k_{21}$ and there would be only a small (11—14%) initial fall in free $Mg^{2+}$. After the initial fall, the free $Mg^{2+}$ would then slowly fall to the final equilibrium as in Figs. 4 and 5. If, however, the molarity of the added CDTA were increased 8- to 10-fold to about 20 mM so that $k_{23} = k_{21}$, then the concentration of free $Mg^{2+}$ would rapidly fall to half that of the initial equilibrium (as in the case with 2 mM EDTA), but thereafter the concentration of free $Mg^{2+}$ would continue to fall to the final equilibrium almost 100-fold below initial equilibrium. The difference between adding 2 mM EDTA and adding the greater concentration of CDTA (which would give the same $k_{23}$) is in the $k_{32}$ which is about 100-fold less with the CDTA. Thus as $[A_3]$ increases with added EDTA the rate of release of its bound $Mg^{2+}$ is unchanged from $A_1$, but as $[A_3]$ increases with CDTA its bound $Mg^{2+}$ is released much more slowly than it is from $A_1$, and $[A_2]$ continues to fall as $[A_3]$ increases.

If $k_{23}$ of a second chelating agent is of the same magnitude or larger than $k_{21}$ so that there is a significant early fall in free $Mg^{2+}$, and if $K'_a$ of that second chelating agent is large enough so that a measurable quantity of free $Mg^{2+}$ will become $A_3$, then it is possible for $[A_2]$ to fall rapidly and then to more slowly rise to the final equilibrium. A hypothetical example would be a second chelating agent with $K'_a$ equal to that of 2 mM EDTA, but with $k_{23}$ and thus $k_{32}$ several times larger. A practical example of a fall and then a rise in $[A_2]$ would occur in the transfer of $Mg^{2+}$ from one anionic resin bead ($A_1$) to another ($A_3$).

ATP and the enzyme itself also compete for $Mg^{2+}$ with both chelating agents in the above time-buffer systems. The molar rate of association of $Mg^{2+}$ with ATP is not known. With its rigid structure and dense charges ATP probably associates with $Mg^{2+}$ faster than does equimolar EDTA. Although EDTA is usually assumed to bind $Mg^{2+}$ faster than does ATP, EDTA is usually added at 250 times the concentration of ATP, and the multiple of the molar $K_a$ is that much greater. At a higher concentration of ATP, perhaps 50 mM, it is conceivable that ATP would cause a dip and rise in $[A_2]$ predicted above. Preliminary data in this laboratory suggest that the rate of association of MgATP is measurable with ATP in the micromolar range, and in that range this rate could be limiting.

In this study the rate of association of MgATP is assumed only to be constant from the time-buffering experiment to the equilibrium reference using the same metal buffer in the same concentration range. The present rapid assay of free $Mg^{2+}$ is limited in accuracy and in scope. For this reason, and because all solutions are more concentrated than 0.1 M, theoretical treatment of this data is limited. A more exact and more general method would be useful. Perhaps a polarographic system could be devised to measure $k_{12}$ as a direct function of voltage, current, and time by selectively gating $Mg^{2+}$ at the cathode or by replacing interfering alkali cations with quarternary amines. The $Mg^{2+}$ current would be proportional to $k_{23}$, $k_{32}$ would be nearly zero, and $[A_2]$ would be a function of voltage.

*Interruption of phosphorylation of ($Na^+ + K^+$)-ATPase by adding chelating agents*

Clearly no chelating agent will remove $Mg^{2+}$ instantly. EDTA at 250 times

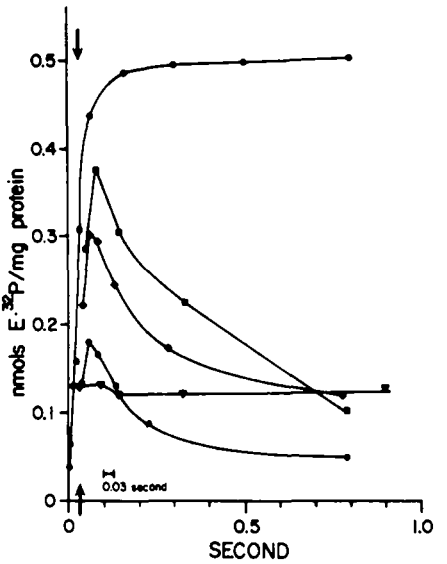


Fig. 10. Interruption of phosphorylation at 37°C. At time zero 40 μM ATP is added to $(Na^+ + K^+)$-ATPase in the standard medium containing 1 mM $MgCl_2$, 140 mM NaCl, and 10 mM imidazole at pH 7.5. The uninterrupted top curve (●——●) reached equilibrium in about 0.2 s. In each of the other 4 curves an agent was added at 0.03 s, during the rapid rise in $(Na^+ + K^+)$-[$^{32}$P]ATPase (E · $^{32}$P). From top to bottom the agent is 10 mM CDTA (■——■), 10 mM EDTA (◇——◇), 10 mM ATP (○——○), and the control 100 μl of 50% trichloroacetic acid to give a final concentration of 10% (▼——▼). At the time on the $x$ axis trichloroacetic acid was added to stop phosphorylation and dephosphorylation. A delay in interrupting phosphorylation is shown by a rise of E · $^{32}$P above the control. The slight rise after adding 10 mM ATP could be due to slow desorption and utilization of the bound [$\gamma$-$^{32}$P]ATP. The rise is significantly greater with EDTA and yet a little greater with CDTA. Neither CDTA nor EDTA interrupts phosphorylation instantly.

the concentration of the ATP used is not instantaneous as shown in Fig. 10. In that experiment at 37°C, $(Na^+ + K^+)$-ATPase was phosphorylated under standard conditions. At time zero phosphorylation is started by adding 40 μM of [$\gamma$-$^{32}$P]ATP to the medium containing $(Na^+ + K^+)$-ATPase, 1 mM $MgCl_2$, 140 mM NaCl, and 30 mM imidazole buffer at pH 7.5. In the top curve phosphorylation is not interrupted, and $(Na^+ + K^+)$-[$^{32}$P]ATPase reaches a steady state plateau in about 0.2 s. In the other four curves an agent was added at 0.03 s. From above downward they are 10 mM CDTA, 10 mM EDTA, 10 mM ATP, and 100 μl of 50 % trichloroacetic acid (which gives a final concentration of 10% trichloroacetic acid). Because of the rapid rate of rise of $(Na^+ + K^+)$-[$^{32}$P]ATPase at 0.03 s, a small delay in interrupting phosphorylation will result in a rise in $(Na^+ + K^+)$-[$^{32}$P]ATPase after 0.03 s. Trichloroacetic acid destroys the enzyme and immediately prevents further phosphorylation or dephosphorylation. The 250-fold excess of unlabeled ATP does not interrupt $(Na^+ + K^+)$-[$^{32}$P]ATPase formation instantly (by comparison to trichloroacetic acid), and the difference could be due to slow dissociation of the labeled ATP bound to $(Na^+ + K^+)$-ATPase. There is a significant rise after the addition of EDTA and a slightly greater rise after the CDTA. Fresh sodium tripolyphosphate (not shown) is as rapid or more rapid than EDTA.

The rate of removal of $Mg^{2+}$ by a chelating agent may be increased by

increasing the concentration of the chelating agent with the limitation that the ionic strength of the medium will rapidly increase and may interfere with some other parameter of the experiment.

The rate of removal of free $Mg^{2+}$ is also a function of any metal buffer present, of temperature, and of the total concentration of $Mg^{2+}$ present. Cessation of phosphorylation depends upon the sensitivity of $(Na^+ + K^+)$-ATPase to MgATP, which in this laboratory has been found to be a function of temperature and of the free $Mg^{2+}$ present. In most experiments at 0°C 10 mM EDTA or even 10 mM CDTA is adequate to stop phosphorylation in an acceptable time. At 37°C the difference between the two is less than at 0°C. Optimal removal of $Mg^{2+}$ occurs when the least $Mg^{2+}$ is present and there is no metal buffer. With 40 $\mu$M ATP, phosphorylation at 0°C is as complete and is almost as rapid with 0.1 mM $Mg^{2+}$ as it is with 1 mM, and at 37°C 0.01 mM is as effective as 1 mM. Use of the 0.01 mM instead of 1 mM will obviate the need for the concentration of free $Mg^{2+}$ to fall through two orders of ten, and it would also result in equilibrium level of free $Mg^{2+}$ almost 100-fold lower.

**Note added in proof** (Received April 22nd, 1980)

*Integrated general rate equations for the concentration of three components of two first order reversible reactions in series*

In the above manuscript the equations used [10] to predict the concentration of $A_1$, $A_2$ and $A_3$ at any given time in the reaction:

$$A_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A_2 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} A_3$$

were limited to the case in which $[A_2]$ and $[A_3]$ were each zero at time zero, and all of the substance ($Mg^{2+}$) begins in the form $A_1$.

The following equations were derived for the more general case in which any values may be assigned to $[A_1]$, $[A_2]$ and $[A_3]$ at time 0. In the above example, $[A_1]$ and $[A_2]$ may be assigned the values of the initial equilibrium of the dissociation of MgEDTA at time 0, and $[A_3]$, representing MgCDTA, may be set to 0. The general equation then predicts $[A_2]$ to fall rapidly from its value in the initial equilibrium with EDTA alone, the induction period, and then to fall semilogarithmically in a quasi steady state.

Should $[A_2]$ and $[A_3]$ be each set to zero at time zero, then the general equation reduces to that of the special case [10].

The general equation also predicts a rapid fall and subsequent rise of $[A_2]$ when the second chelating agent (or binding site) introduced is low in capacity (so that the final equilibrium value of $[A_2]$ is not much lower than the initial value) and $k_{23}$ is large.

$$[A_1] = Q_1 + Q_2 e^{-\lambda_2 t} + Q_3 e^{-\lambda_3 t}$$

$$[A_2] = \frac{Q_1(k_{12})}{k_{21}} + \frac{Q_2(k_{12} - \lambda_2)\, e^{-\lambda_2 t}}{k_{21}} + \frac{Q_3(k_{12} - \lambda_3)\, e^{-\lambda_3 t}}{k_{21}}$$

$$[A_3] = \frac{Q_1 k_{23} k_{12}}{k_{21} k_{32}} + \frac{Q_2 k_{23}(k_{12} - \lambda_2)\, e^{-\lambda_2 t}}{k_{21}(k_{32} - \lambda_2)} + \frac{Q_3 k_{23}(k_{12} - \lambda_3)\, e^{-\lambda_3 t}}{k_{21}(k_{12} - \lambda_3)}$$

$$Q_1 = \begin{vmatrix} [A_1^0] & 1 & 1 \\ [A_2^0] & (k_{12} - \lambda_2)/k_{21} & (k_{12} - \lambda_3)/k_{21} \\ [A_3^0] & \dfrac{k_{23}(k_{12} - \lambda_2)}{k_{21}(k_{32} - \lambda_2)} & \dfrac{k_{23}(k_{12} - \lambda_3)}{k_{21}(k_{32} - \lambda_3)} \end{vmatrix} \div D$$

$$Q_2 = \begin{vmatrix} 1 & [A_1^0] & 1 \\ k_{12}/k_{21} & [A_2^0] & (k_{12} - \lambda_3)/k_{21} \\ \dfrac{k_{23} k_{12}}{k_{32} k_{21}} & [A_3^0] & \dfrac{k_{23}(k_{12} - \lambda_3)}{k_{21}(k_{32} - \lambda_3)} \end{vmatrix} \div D$$

$$Q_3 = \begin{vmatrix} 1 & 1 & [A_1^0] \\ k_{12}/k_{21} & (k_{12} - \lambda_2)/k_{21} & [A_2^0] \\ \dfrac{k_{23} k_{12}}{k_{32} k_{21}} & \dfrac{k_{23}(k_{12} - \lambda_2)}{k_{21}(k_{32} - \lambda_2)} & [A_3^0] \end{vmatrix} \div D$$

$$D = \begin{vmatrix} 1 & 1 & 1 \\ k_{12}/k_{21} & (k_{12} - \lambda_2)/k_{21} & (k_{12} - \lambda_3)/k_{21} \\ \dfrac{k_{23} k_{12}}{k_{32} k_{21}} & \dfrac{k_{23}(k_{12} - \lambda_2)}{k_{21}(k_{32} - \lambda_2)} & \dfrac{k_{23}(k_{12} - \lambda_3)}{k_{21}(k_{32} - \lambda_3)} \end{vmatrix}$$

$$\lambda_2 = \tfrac{1}{2}(p + q), \quad \lambda_3 = \tfrac{1}{2}(p - q)$$

$$p = k_{12} + k_{21} + k_{23} + k_{32}$$

$$q = [p^2 - 4(k_{12} k_{23} + k_{21} k_{32} + k_{12} k_{32})]^{1/2}$$

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