# ANALYSIS OF A CALCIUM ION BINDING SYSTEM COMPOSED OF TWO DIFFERENT SITES

TSUYOSHI OHNISHI, EDWARD J. MASORO, HELEN A. BERTRAND, *and* BYUNG PAL YU

*From the Department of Physiology and Biophysics, The Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129*

ABSTRACT Using murexide (Mx), a metallochromic indicator, and a dual wavelength spectrophotometer with a high signal-to-noise ratio, the $Ca^{++}$ binding in a system containing two classes of binding sites was studied. Solutions with solute containing one or two classes of $Ca^{++}$ binding sites and without such solute were titrated with $Ca^{++}$ using Mx as an indicator of free $Ca^{++}$ concentration. Since curvilinear Scatchard plots are obtained from titration curves of solutes containing two classes of binding sites, a computer program was developed to resolve such plots into two linear partial plots, each corresponding to a single class of binding site. The validity of the procedure was examined with solutions of ethylene glycol bis($\beta$-aminoethyl)-*N*-*N*'-tetraacetic acid, adenosine triphosphate (EGTA, ATP), or a mixture thereof. The method was also applied to biological material and it was found that a protein fraction isolated from rat skeletal muscle sarcotubular membranes, termed Fraction-2 (Fr-2), has two classes of binding sites for $Ca^{++}$; the association constants of the high affinity site and low affinity site are $4.3 \times 10^5$ $M^{-1}$ and $9 \times 10^3$ $M^{-1}$, respectively. The advantages and limitations of this methodology are discussed.

## INTRODUCTION[1]

It is well established that the relaxation of muscle is regulated by the ATP dependent $Ca^{++}$ sequestration by sarcoplasmic reticulum (1–3). Since a minute amount of $Ca^{++}$ can cause significant physiological changes in muscle, it is essential to have a method of determining low concentrations of free $Ca^{++}$ quickly and easily. Ohnishi and Ebashi (4) developed the Mx method to follow the change of $Ca^{++}$ concentration in in vitro suspensions of isolated sarcoplasmic reticulum. By using Mx, a metallochromic indicator, together with a dual wavelength spectrophotometer and flow apparatus, it has been established that $Ca^{++}$ uptake by sarcoplasmic reticulum is fast enough to account for the speed of muscle relaxation (5).

[1] A preliminary report has been presented at the 7th Federation of European Biochemical Societies Meeting, Varna, Bulgaria, September, 1971.

Compared with methods of $Ca^{++}$ assay, such as the widely used atomic absorption method or $^{45}Ca$ isotope techniques, the Mx method has the advantage of being rapid and of eliminating tedious and laborious procedures. In this paper, applications of the Mx method for the study of equilibrium binding are presented. Determinations of the number of binding sites and the association constants of a system requires the accumulation of much data showing the relationship between free and bound $Ca^{++}$ over a wide range of free $Ca^{++}$ concentrations. In the other methods currently available, a series of samples is needed because a single datum (i.e., one point on a titration curve) is obtained from a single solution. Using the Mx method, however, a single sample can be used to obtain all required data. This is accomplished by increasing the concentration of $Ca^{++}$ progressively and recording the free $Ca^{++}$ concentration with each $Ca^{++}$ addition.

The data so obtained may be analyzed by a Scatchard plot (6). If a system contains a single class of binding sites, the Scatchard plot is linear permitting the determination of an association constant and the number of sites from the slope of the line and its intercept with the appropriate axis, respectively. When a system contains two classes of binding sites, the Scatchard plot becomes curvilinear (7, 8). A computer program was developed to break down such a curve into two linear partial plots, each corresponding to a single class of binding sites. The validity of this program was examined by using a solution containing EGTA and ATP, both Ca-chelating substances.

The method was also used to study a protein fraction, called Fr-2, solubilized from rat skeletal muscle sarcoplasmic reticulum (9, 10). Fr-2 was found to have two classes of $Ca^{++}$ binding sites, one with a high and the other with a low association constant.

## MATERIALS AND METHODS

All chemicals are of reagent grade and all solutions were prepared in deionized distilled water. Mx was purchased from Dojin Yakuchemical Company, Kumamoto, Japan; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) from Calbiochem, Los Angeles, Calif.; $CaCl_2$ from Orion Research, Inc., Cambridge, Mass. EGTA from Eastman Organic Chemicals, Rochester, N. Y.; and ATP from Sigma Chemical Co., St. Louis, Mo. The major protein component of rat sarcoplasmic reticulum called Fr-2 was prepared by the method of Yu and Masoro (10).

A dual wavelength spectrophotometer was constructed having the following characteristics: since the difference spectrum of a Mx solution with and without $Ca^{++}$ exhibits broad peaks (4), interference filters are used for obtaining monochromatic beams for the instrument. Such a filter system has two advantages, namely, (*a*) the optical system can be made very simple, and (*b*) it is possible to obtain high beam intensity in visible wavelength regions. These two features made it possible for us to build a stable instrument for $Ca^{++}$ assay. The optical system of a simple dual wavelength spectrophotometer is shown in Fig. 1. The scattering effect for turbid solutions is minimized by using a suitable light diffuser and a collecting lens. The sample cuvette is maintained at a constant temperature by circulating a thermo-regulated liquid through a jacket surrounding the sample cuvette. A small magnetic stirring device is

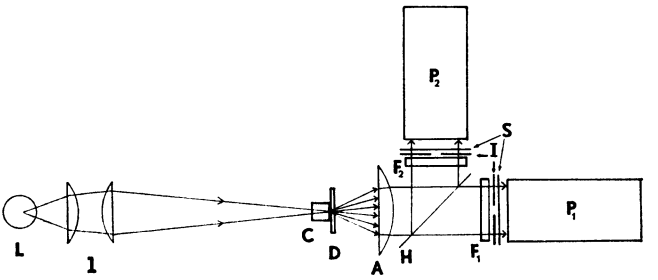


FIGURE 1 Optical system of a filter-type dual wavelength spectrophotometer. L, lamp; l, condenser lenses; C, cuvette; D, light diffuser; A, collecting lens; H, half mirror; $F_1$ and $F_2$, interference filters; I, adjustable slits; S, shutters; $P_1$ and $P_2$, photomultipliers (EMI 9524S). Output signals of $P_1$ and $P_2$ are fed into a difference amplifier, and the difference between the signals are recorded. When the difference is small, this is proportional to the optical density difference.

installed under the cuvette so that there is constant stirring during titration. When a sample solution gives an OD of 2.0 for a 10 × 10 mm standard cuvette, a 0.01 ΔOD can be expanded to full-scale with a noise level of ±0.0001 OD units and drift of ±0.0001 OD units/hr (the time constant of electronic circuit is 0.4 sec). The maximum sensitivity of $Ca^{++}$ assay in the Mx method with this system is 0.2 μM at low ionic strength.[2] Provided careful adjustments are made on commercially available dual wavelength spectrophotometers so that the signal-to-noise ratio is satisfactorily high, they should be adequate for this type of measurement.

## RESULTS

### *A System Containing a Single Class of $Ca^{++}$ Binding Sites*

The Mx method was used to analyze $Ca^{++}$ binding by EGTA and ATP; the data from these analyses are presented in Figs. 2, 3 A, and 3 B. These were done to test the methodology and to obtain the basic data needed for the subsequent analyses of systems containing two classes of $Ca^{++}$ binding sites.

Curve *J* in Fig. 2 represents the data obtained from the titration of an ATP solution with $Ca^{++}$ using Mx as an indicator of the free $Ca^{++}$ concentration. Curve *I* represents data obtained in the absence of chelating substances. The ordinate of this graph represents the ΔOD between 507 mμ and 535 mμ and the abscissa expresses total $Ca^{++}$ added to the sample.[3]

From a *j*th point *G* on the titration curve *J*, a horizontal line is drawn toward the ordinate and intersects curve *I* and the ordinate at *F* and *E*, respectively. The distance

[2] The sensitivity of the Mx method decreases with increasing ionic strength. According to unpublished data (Ohnishi), 5 mM and 40 mM HEPES solutions (pH 7.0) have ionic strengths of 0.002 and 0.015, respectively. The ionic strength of the experiment shown in Fig. 2 is 0.1, and the sensitivity of the Mx method at this ionis strength is about one-half that at a low ionic strength of 0.002.

[3] During the $Ca^{++}$ titration, the pH of the solution decreases as $H^+$ are released as a consequence of $Ca^{++}$ binding. Therefore, precalibrated amounts of N KOH were added several times during the titration to maintain a pH of 7.00 ±0.02. The volume of the solution is 2 ml and the total volume of added $Ca^{++}$ and KOH are less than 30 μl. The dilution effect caused by these additions is insignificant.

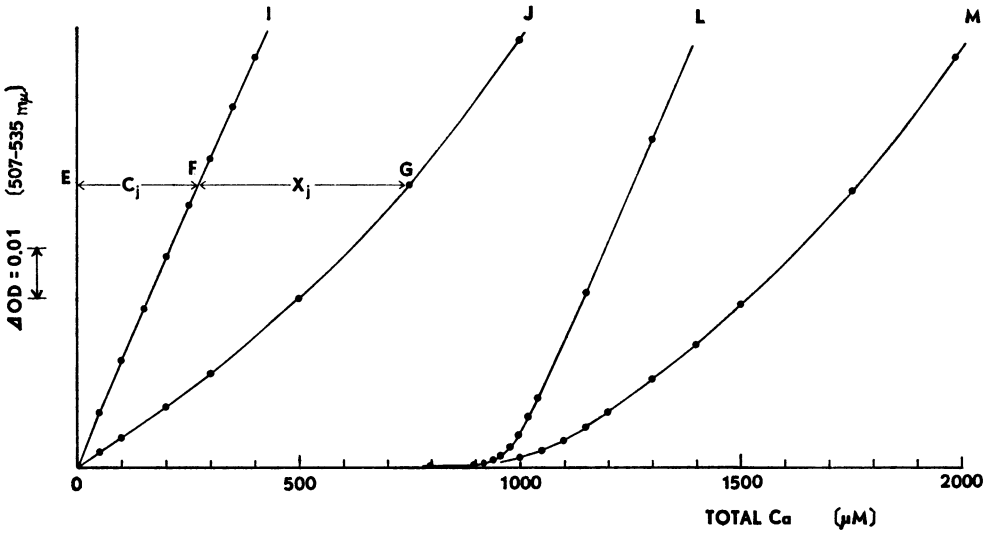


FIGURE 2 Titration curves. An interval along the ordinate shows 0.01 units in optical density difference (ΔOD) between 507 and 535 mμ, and the abscissa total $Ca^{++}$ added. Curve *I* represents results from a control solution without chelator; *J*, a 1 mM ATP solution; *L*, 1 mM EGTA solution; and *M*, 1 mM EGTA and 1 mM ATP solution. Experimental conditions are: 70 μM Mx, 40 mM HEPES (pH 7.00), and 85 mM KCl. Ionic strength of a solution is 0.1 (see footnote 2) and temperature 30°C.

*EG* represents the total $Ca^{++}$ concentration in the system $C_j^t$, the distance *EF* the free $Ca^{++}$ concentration in the system $C_j$, and the difference or the distance *FG* the concentration of bound $Ca^{++}$ $X_j$. The following expresses this relationship algebraically:

$$C_j^t = C_j + X_j. \tag{1}$$

If one-to-one binding between $Ca^{++}$ and ATP is assumed (11), the following is the equation of equilibrium:

$$K_2 = \frac{X_j}{C_j(N_2 - X_j)}, \tag{2}$$

where $K_2$ is the apparent binding constant and $N_2$ the concentration of ATP.

By defining $Y_j$ as

$$Y_j = X_j/C_j, \tag{3}$$

the following can be derived:

$$Y_j = K_2(N_2 - X_j). \tag{4}$$

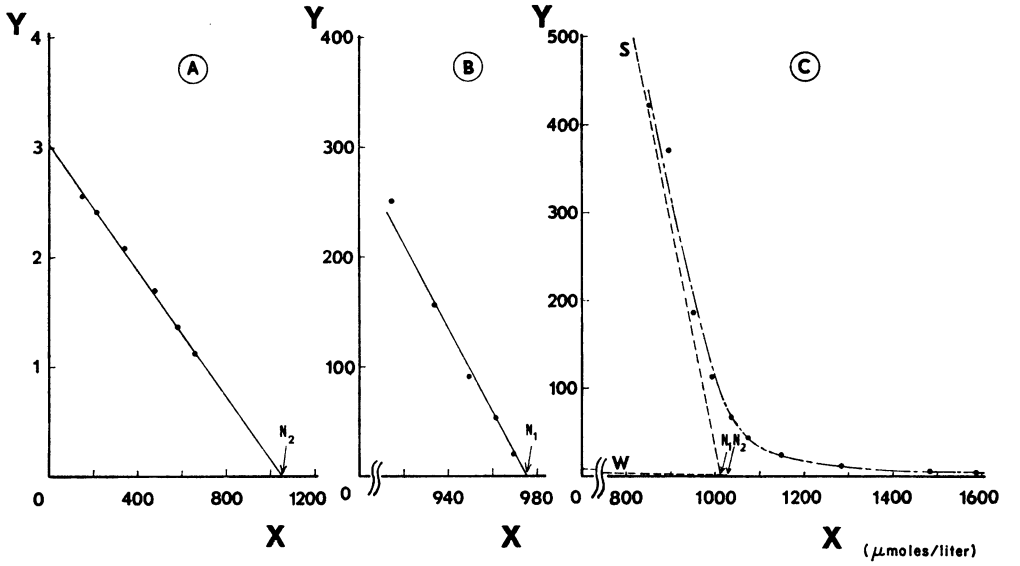


FIGURE 3 The Scatchard plots derived from the data reported in Fig. 2: the abscissa ($X$) represents the amount of bound $Ca^{++}$ expressed as micromoles per liter and the ordinate ($Y$) is a dimensionless value representing the ratio of bound $Ca^{++}$/free $Ca^{++}$. The three graphs are Scatchard plots resulting from the $Ca^{++}$ binding activities of solutions containing A, 1 mM ATP; B, 1 mM EGTA; and C, 1 mM EGTA and 1 mM ATP. In graph C the partial plots for EGTA and ATP are represented by the dashed lines $S$ and $W$, respectively. The broken line in graph C represents the theoretical Scatchard plot derived by reconstruction from the two partial plots $S$ and $W$. The $X$ intercept in each graph is labeled $N_1$ and/or $N_2$ denoting the number of binding sites for EGTA and ATP, respectively.

The Scatchard plot for ATP (as for any other substance) relates $Y_j$ vs. $X_j$; $K_2$ and $N_2$ are determined from the slope and the $X$ intercept of the line, respectively (Fig. 3 A). For convenience, $X_j$ is expressed as moles of bound $Ca^{++}$ per liter of solution. Therefore, both $X_j$ and $N_2$ have the dimension of concentration [M], while $Y_j$ becomes a dimensionless number representing the ratio of [bound $Ca^{++}$]/[free $Ca^{++}$]. Fig. 3 A represents the analysis of a 1 mM ATP solution. A similar analysis for 1 mM EGTA using curve $L$ of Fig. 2 is presented in Fig. 3 B; the apparent binding constant and number of binding sites are designated $K_1$ and $N_1$, respectively.

### *A System Containing Two Classes of Binding Sites*

The Mx method has been used to study the $Ca^{++}$ binding activity of systems containing two classes of $Ca^{++}$ binding sites (Figs. 2, 3 C, 5, and 9). Curvilinear Scatchard plots are derived from these analyses and resolution of such Scatchard plots into linear partial plots as well as reconstruction of such aSctchard plots from two linear plots has been accomplished. For theoretical convenience reconstruction of a Scatchard plot from two partial plots will be considered first.

*Reconstruction of the Scatchard Plot from Two Partial Plots.* Assume that two $Ca^{++}$ binding substances, with association constants and number of sites characterized by $K_1$, $K_2$, $N_1$, and $N_2$, coexist in solution and compete for $Ca^{++}$, then the following analysis can be made (in the case of the mixture of EGTA and ATP, suffixes 1 and 2 refer to EGTA and ATP, respectively):

$$K_1 = \frac{X_{1j}}{C_j(N_1 - X_{1j})}, \tag{5}$$

$$K_2 = \frac{X_{2j}}{C_j(N_2 - X_{2j})}, \tag{6}$$

where total bound $Ca^{++}$ $(X_j)$ is represented by

$$X_j = X_{1j} + X_{2j}. \tag{7}$$

By defining $Y_{1j}$ and $Y_{2j}$ as

$$\left.\begin{aligned} Y_{1j} &= \frac{X_{1j}}{C_j}, \\ Y_{2j} &= \frac{X_{2j}}{C_j}, \end{aligned}\right\} \tag{8 a}$$

the following relationship is derived by combining equations 3 and 8 *a*:

$$\frac{Y_j}{X_j} = \frac{Y_{1j}}{X_{1j}} = \frac{Y_{2j}}{X_{2j}} = \frac{1}{C_j}. \tag{8 b}$$

Therefore

$$Y_j = Y_{1j} + Y_{2j}, \tag{9}$$

and the two partial plots of the Scatchard plot are expressed by

$$\left.\begin{aligned} Y_{1j} &= K_1(N_1 - X_{1j}), \\ Y_{2j} &= K_2(N_2 - X_{2j}). \end{aligned}\right\} \tag{10}$$

If these two plots are known, the Scatchard plot ($Y_j$ vs. $X_j$) for the system can be reconstructed in two ways:

(*a*) A graphic method (7, 8) as in Fig. 4: draw a line from the origin toward the two partial plots *S* and *W*. The slope of this line represents the inverse of the free $Ca^{++}$ concentration (equation 8 *b*). Let the intersection of this line with the two partial plots be *P* and *Q*, and the distance from the origin to *P* and *Q* be $r_1$ and $r_2$, respectively. When the line *OQP* is extended, it intersects the Scatchard plot at point

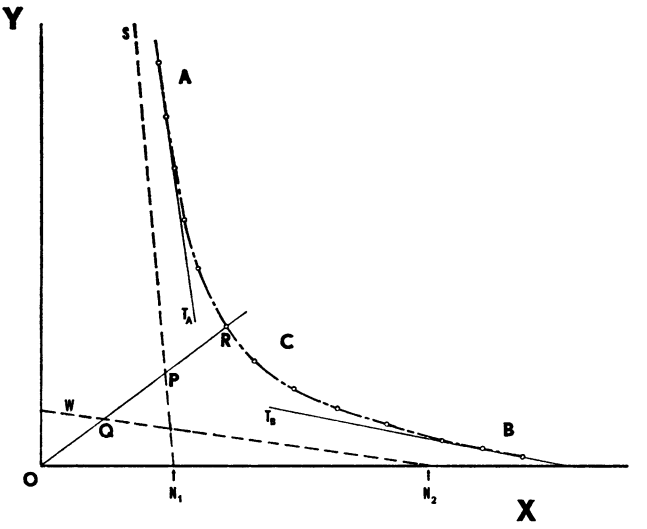


FIGURE 4 Relation between a Scatchard plot (—) and the two partial plots (---) referring to the strong $S$ and weak $W$ binding sites, respectively. $T_A$ and $T_B$ are tangents to the ends of the Scatchard plot. $A$ and $B$ are the linear portions of the plot.

$R$. The distance $r$ (i.e., the distance between the origin and $R$) can be determined as follows

$$r = X \sec \theta,$$
$$r_1 = X_1 \sec \theta,$$
$$r_2 = X_2 \sec \theta, \qquad (11)$$

where $\theta$ is the angle between the straight line ($OQPR$) and the abscissa. From equations 7 and 11, it is clear that $r$ is related to $r_1$ and $r_2$ by

$$r = r_1 + r_2. \qquad (12)$$

($b$) Numerical method: the following can be derived from equations 7, 8 $a$, and 10:

$$X_j = \frac{K_1 N_1 C_j}{1 + K_1 C_j} + \frac{K_2 N_2 C_j}{1 + K_2 C_j}. \qquad (13)$$

By eliminating $C_j$ from equations 13 and 3 the following relationship can be developed:

$$Y_j^2 - 2\beta Y_j - \gamma = 0, \qquad (14)$$

TABLE I

THE BINDING CHARACTERISTICS OF EGTA, ATP, AND MIXTURE THEREOF*

| Concentration of chelator(s) | Strong binding | | Weak binding | |
|---|---|---|---|---|
| | $K_1$ | $N_1$ | $K_2$ | $N_2$ |
| $\mu M$ | $\times 10^6\ M^{-1}$ | $\mu M$ | $\times 10^3\ M^{-1}$ | $\mu M$ |
| System *a*‡ | | | | |
| EGTA (250) | 3.3 | 240 | | |
| EGTA (1000) | 4.6 | 976 | | |
| ATP (250) | | | 5.8 | 240 |
| ATP (1000) | | | 2.9 | 1000 |
| System *b*‡ | | | | |
| EGTA (250) + ATP (250) | 3.5 | 240 | 5.3 | 250 |
| EGTA (250) + ATP (1000) | 2.6 | 230 | 2.7 | 1110 |
| EGTA (1000) + ATP (250) | — | — | — | —§ |
| EGTA (1000) + ATP (1000) | 2.6 | 1010 | 3.2 | 1024 |

* The apparent binding constants $K$'s and the concentration of binding sites $N$'s were determined by Scatchard plot as described in text. The ionic strength of the system is 0.1 (see footnote 2) and the temperature 30°C. Other conditions are the same as Fig. 2.

‡ System *a* is for a single chelator, and system *b* for a mixture of two chelators.

§ When $N_1$ is larger than $N_2$, reliable data cannot be obtained since the binding at the strong site masks the binding at the weak site.

where $\beta$ and $\gamma$ are functions of $X_j$ as given by

$$\left.\begin{aligned} \beta &= \frac{K_1N_1 + K_2N_2 - (K_2 + N_2)X_j)}{2}, \\ \gamma &= K_1K_2X_j(N_1 + N_2 - X_j). \end{aligned}\right\} \tag{15}$$

By solving equation 14 for $Y_j$, the following expression of the Scatchard plot is obtained:

$$Y_j = \beta + (\beta^2 + \gamma)^{1/2}. \tag{16}$$

*Breakdown of the Scatchard Plot into Two Partial Plots.* The computer program is designed as follows ($K_1 > K_2$ will be the convention followed): first draw a line (partial plot) for the weaker binding site by assuming approximate values for $K_2$ and $N_2$ from the tangent to that portion of the Scatchard plot farthest to the right (denoted by $T_B$ in Fig. 4); from this and equations 7 and 9, which are equivalent to the equation 12, a plot for the strong binding site can then be calculated. Another pair of $K_2$ and $N_2$ are then chosen in accord with the program and another plot for the strong binding site is calculated. Repeating this calculation through a set of iterations permits a plot to be selected for the strong binding site with the minimum least mean square deviation from a straight line.[4]

[4] The original nonlinear equation (equation 14) can be solved directly by computer, but it requires much more computer time than the method described here.

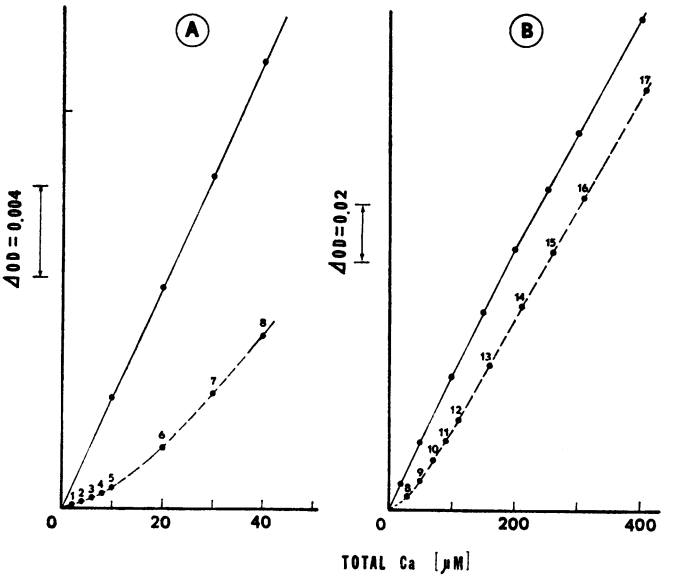


FIGURE 5 Titration curves of solutions containing Fr-2 at low ionic strength. Solid lines are in the absence of Fr-2 and dashed lines in the presence of Fr-2. A, at low $Ca^{++}$ concentration and B, at high $Ca^{++}$ concentration. Numbers on each point indicate the sequential order of titration. Protein concentration is 0.36 mg/ml. Other experimental conditions are 70 μM of Mx, 4 mM of HEPES (pH 7.00), and temperature 30°C.

Since determining $C_j$ and $X_j$ from the titration curves as in Fig. 2 is time consuming, this step is also computerized. Using an PDP-6 computer (Digital Equipment Corp., Maynard, Mass.) only 3 sec of computer time are required to obtain the two partial plots (i.e., to calculate the two $K$'s and two $N$'s) from the original titration data followed by the calculation of a reconstructed Scatchard plot from these partial plots. The broken line in Fig. 3 C for a solution containing EGTA and ATP were obtained from the curve $M$ in Fig. 2 in this fashion. In Fig. 3 C, open circles are data points; the dashed line denoted by $W$ is a partial plot for the weak binding site (ATP); $S$ is a partial plot for the strong site (EGTA); and the broken line is a theoretical Scatchard plot reconstructed from two dotted lines $W$ and $S$ according to equation 16. The validity of the method as tested by using known amounts of EGTA, ATP, and mixture thereof is established by the results shown in Table I.

### *$Ca^{++}$ Binding of a Soluble Protein Having Two Different Binding Sites*

The present method can be readily applied to the $Ca^{++}$ binding studies of a protein, if it is assumed that the $Ca^{++}$ binding at each site on a protein is independent.[5] The method was first applied to a soluble protein fraction from sarcotubular mem-

[5] It also must be assumed that protein does not interfere with the Mx-$Ca^{++}$ interaction. This assumption is reasonable because neither changing protein nor Mx concentration appreciably alter the results.

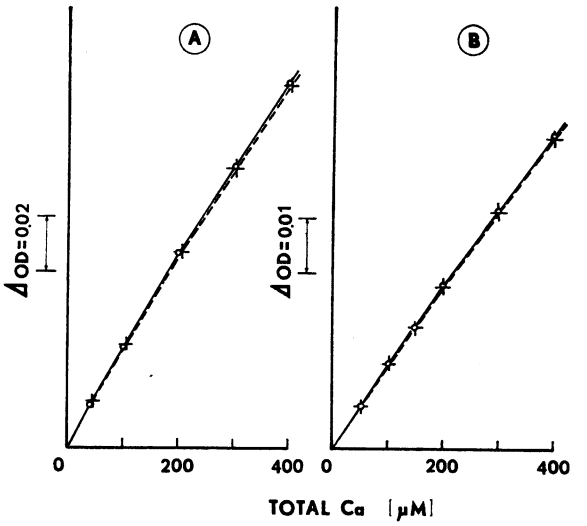


FIGURE 6 Titration curves of Fr-2 solution in the presence of solts. Solid lines (O——O) are in the absence of Fr-2 and dashed lines (+----+) are in the presence of Fr-2. Experimental conditions are A, 2.5 mM of $MgCl_2$, 0.36 mg/ml of Fr-2; and B, 150 mM of KCl, 0.2 mg/ml of Fr-2. Other conditions are the same as Fig. 5.

branes called Fr-2. Fr-2 was found to have two classes of $Ca^{++}$ binding sites. Fig. 5 is a curve for the titration of a solution containing Fr-2 with $Ca^{++}$. Unlike ATP-induced $Ca^{++}$ binding by isolated sarcoplasmic reticulum (12), $Ca^{++}$ binding by Fr-2 is inhibited by addition of $Mg^{++}$ (Fig. 6 A) or a large amount of monovalent cation such as $K^+$ (Fig. 6 B). Before transforming these titration curves into Scatchard plots, the question of how much $Ca^{++}$ is bound to Fr-2 before titration with $Ca^{++}$ must be determined. Naturally this amount should be as little as possible in order to obtain accurate results. $Ca^{++}$ bound to the protein may be removed by treating the protein-containing solution with chelex resin (Dowex A-100, Dow Chemical Co., Midland, Mich.), or by dialyzing it against EGTA followed by dialysis against a buffer solution to remove EGTA. From a practical point of view, however, it is not easy to establish that a protein so treated is free of chelating materials. Since even a small amount of such contamination would cause large error in the measurement of the $Ca^{++}$ binding properties of the protein, these methods have not been routinely used in our studies. Rather $Ca^{++}$ was removed from Fr-2 by treating it with 150 mM KCl (see Figs. 6 B and 7) followed by dialyzing it against buffer treated previously with chelex resin.[6]

The amount of $Ca^{++}$ bound to Fr-2 and the free $Ca^{++}$ concentration in the Fr-2 solutions were determined as follows: (*a*) the Mx-containing buffer solution is titrated with a known amount of $Ca^{++}$ in the presence and absence of 2.5 mM $Mg^{++}$

[6] Tests have established that treating buffer with chelex resin does not result in contamination, i.e. the buffer solution so treated does not show any $Ca^{++}$ binding capacity on Mx-$Ca^{++}$ titration. In contrast, protein treated directly with chelex resin sometimes show very high binding constants ($10^6 \sim 10^7$) suggesting that a small amount of very fine resin particles might be bound by the protein.

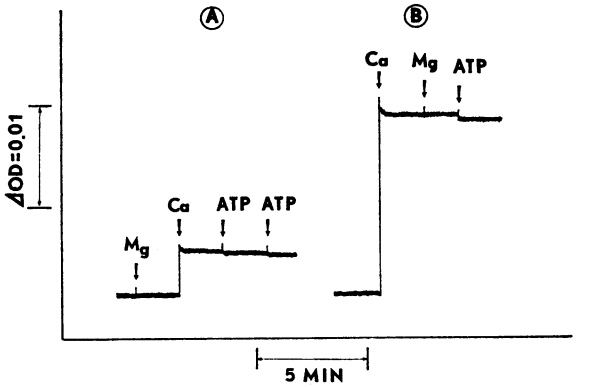


FIGURE 7 Effect of KCl on the $Ca^{++}$ binding by Fr-2. Fr-2 was dissolved in 150 mM KCl and then addition of $Mg^{++}$ in the absence of previous addition of $Ca^{++}$ (A) or presence of a previous addition of $Ca^{++}$ (B) does not release $Ca^{++}$ from Fr-2. Therefore 150 mM KCl probably prevents $Ca^{++}$ binding by Fr-2. Moreover unlike suspensions of isolated sarcoplasmic reticulum (4), ATP (each arrow indicates increase of 100 $\mu$M in ATP concentration) does not induce $Ca^{++}$ binding by Fr-2. The small decrease of free $Ca^{++}$ concentration observed on addition of ATP is probably caused by ATP-$Ca^{++}$ binding in the presence of $Mg^{++}$, since the same amount of decrease is observed in control experiments containing no Fr-2. Amounts of reagents were added in the figure to result in A, $MgCl_2$, 2.5 mM; $Ca^{++}$, 20 $\mu$M; and B, $Ca^{++}$, 80 $\mu$M; $MgCl_2$, 2.5 mM. Other conditions are the same as Fig. 6 B. Length of arrow under the abscissa shows time interval of 5 min.

to obtain the relationship between free $Ca^{++}$ concentration and $\Delta OD$ under these conditions (Fig. 8 A). (b) A Fr-2 solution prepared in the above Mx buffer (containing no $Mg^{++}$) is titrated with EGTA to determine the amount of free $Ca^{++}$ present, $C_0$ (Fig. 8 B). (*c*) Another solution of Fr-2 is titrated to 2.5 mM $Mg^{++}$ to expel $Ca^{++}$ bound to the protein molecule thus enabling $X_0$ to be estimated (Fig. 8 C). Normally each of these correction values is less than a few micromolars of $Ca^{++}$. These values of $Ca^{++}$ (i.e., $C_0$ and $X_0$) are used to correct each titration point as follows:

$$\left.\begin{aligned} C_j' &= C_j + C_0, \\ X_j' &= X_j + X_0, \\ Y_j' &= X_j'/C_j', \end{aligned}\right\} \qquad (17)$$

where the prime designates corrected values. A Scatchard plot of Fr-2 after such a correction ($Y_j'$ vs. $X_j'$) is shown by the open circles in Fig. 9. Two dashed lines in the Fig. 9, $S$ and $W$, are partial plots and the broken line a theoretical plot reconstructed from the two partial plots according to equations 15 and 16. As described previously, $N_1$ and $N_2$ have a dimension of [moles per liter of solution]. Therefore, the dividing of these values by the amount of (grams) of protein in liter solution converts the data to moles of $Ca^{++}$ bound per grams of protein. In this paper, $n_1$ and $n_2$ are used instead of $N_1$ and $N_2$ when such a conversion is made. From these results, Fr-2 was shown to have two $Ca^{++}$ binding sites, one with high affinity ($K_1 = 4.3 \times 10^5$

$M^{-1}$ and $n_1$ = 68 $\mu$moles/g protein) and one with low affinity ($K_2$ = 9 × $10^3$ $M^{-1}$ and $n_2$ = 190 $\mu$moles/g protein).[7]

## DISCUSSION

The Mx method permits the $Ca^{++}$ concentration of a sample to be continuously assayed without stopping the reaction. This is a major advantage since unlike other methods, all needed data can be obtained from a single solution both for kinetic studies of $Ca^{++}$ concentration changes (4, 5) and for equilibrium binding studies. Moreover, because it only involves direct spectrophotometry, the method is simple and rapid.

That Mx is not specific for $Ca^{++}$, however, does present problems. Most divalent and trivalent cations except $Mg^{++}$ can cause appreciable spectral change in Mx solutions. Therefore, amounts of such divalent or trivalent cations which can interfere must not be present in the assay medium when applying the Mx method for $Ca^{++}$ studies.

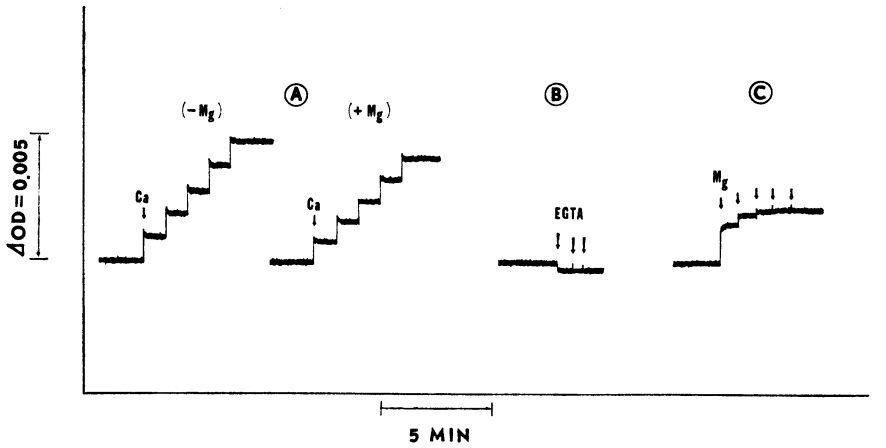


FIGURE 8 Methods of determining amount of $Ca^{++}$ in Fr-2 solutions before titration. A, Calibration of buffer system without Fr-2 in the absence and presence of 2.5 mM $MgCl_2$. Each step shows the effect of 2 $\mu$M increase in $Ca^{++}$ concentration (because of the effect of the ionic strength of the $MgCl_2$, the sensitivity is slightly smaller in the latter case). B, Titration of Fr-2 solution by EGTA in the absence of $Mg^{++}$ to determine free $Ca^{++}$ concentration (each arrow shows the addition of EGTA to increase its concentration by 25 $\mu$M), C, Release of $Ca^{++}$ bound to Fr-2 adding $MgCl_2$ to a 2.5 mM concentration. (Each arrow shows an increase of 0.5 mM $MgCl_2$. A very small change in Mx spectrum is caused by $Mg^{++}$ itself,[8] but this effect has been subtracted in drawing this figure.) Solutions for B and C contain 0.36 mg/ml of Fr-2. An arrow under the abscissa shows time interval of 5 min.

[7] The value of 68 $\mu$moles/g protein for the strong binding site means that 1 mole $Ca^{++}$ is bound per 15,000 g of Fr-2 with high affinity. It is quite possible that two (or more than two) subunit molecules are cooperatively involved in the $Ca^{++}$ binding by sarcoplasmic reticulum. Further studies on the $Ca^{++}$ binding of Fr-2 have been reported elsewhere (13).

[8] The sensitivity of the Mx method for $Mg^{++}$ is about 1/2000 that of $Ca^{++}$ at neutral pH.

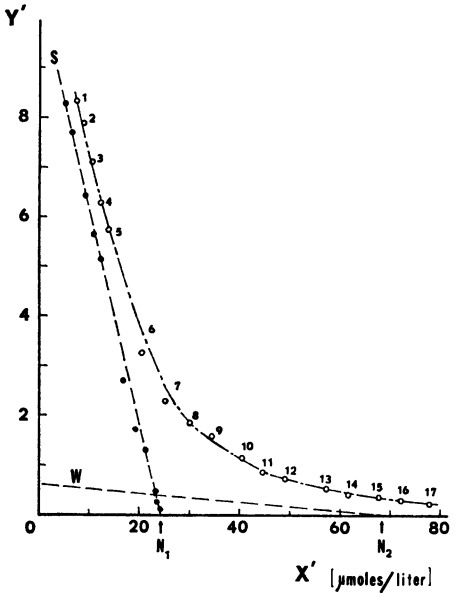


FIGURE 9 Scatchard plot for Fr-2 from data of Figs. 5 and 8. Open circles are experimental data. Closed circles are so determined from these data and a partial plot for the weak binding site *W* as to give the minimum least mean square deviation from a line. This line is equivalent to the partial plot for the strong binding site *S*. A broken line is the theoretical plot which is reconstructed from two partial plots *W* and *S* according to a principle described in the text. *X* and *Y* axes are expressed by the same units as in Fig. 3.

Fortunately, the fact that $Mg^{++}$ does not produce a significant spectral change[8] in the Mx method makes it most useful for $Ca^{++}$ assays in physiological systems, since $Mg^{++}$ is the major divalent cation in biological systems. This insensitivity to $Mg^{++}$ can be used to extend the applicability of the Mx method; e.g. (*a*) the interfering effect of ATP on the $Ca^{++}$ assay by Mx can be suppressed by adding excess $Mg^{++}$ (4) and (*b*) $Ca^{++}$ bound to protein can in many cases be released by adding an excess of $Mg^{++}$.

The spectral changes in Mx solutions caused by monovalent cations are much smaller than those with divalent cations. The primary effect of monovalent cations is to decrease the $Ca^{++}$-induced spectral change in Mx solutions.[2] Therefore, as long as the concentration of monovalent cations is kept relatively constant during the reaction, the effect of monovalent cations on the Mx method can be neglected.

The apparent binding constant of EGTA or ATP for $Ca^{++}$ measured by the Mx method (Table I) agrees well with that obtained by other methods. For EGTA, it can be calculated to be $4 \times 10^6$ M$^{-1}$ (at pH 7) from the data of Holloway and Reilley (14). For ATP, the present values are comparable with $4.0 \times 10^3$ M$^{-1}$ (15) and $5.9 \times 10^3$ M$^{-1}$ (16) reported in the literature.

From the present studies, the following guiding principles have been established

TABLE II

EFFECT OF NUMBER AND DISTRIBUTION OF DATA POINTS ON THE RESULTS OF SCATCHARD PLOT ANALYSIS*

| Data used for computer analysis‡ | Total number of data points | Strong binding | | Weak binding | |
|---|---|---|---|---|---|
| | | $K_1$ | $N_1$ | $K_2$ | $N_2$ |
| | | $\times 10^5\ M^{-1}$ | $\mu M$ | $\times 10^3\ M^{-1}$ | $\mu M$ |
| Control 1, 2, ..., 16, 17 | 17 | 4.27 | 24.4 | 8.98 | 68.2 |
| Test analysis *a* | | | | | |
| 2, 3, ..., 16, 17 | 16 | 4.16 | 24.7 | 8.93 | 67.8 |
| 3, 4, ..., 16, 17 | 15 | 3.87 | 25.3 | 8.69 | 67.4 |
| 1, 2, ..., 15, 16 | 16 | 4.14 | 25.0 | 8.30 | 69.6 |
| 1, 2, ..., 14, 15 | 15 | 4.51 | 23.5 | 10.2 | 65.6 |
| 3, 4, ..., 14, 15 | 13 | 4.11 | 24.4 | 9.58 | 65.6 |
| 5, 6, ..., 12, 13 | 9 | 3.81 | 24.1 | 10.9 | 62.0 |
| Test analysis *b* | | | | | |
| 1, 3, 5, ..., 15, 17 | 9 | 4.28 | 24.5 | 9.18 | 67.4 |
| 1, 5, 9, 13, 17 | 5 | 3.72 | 27.2 | 8.32 | 65.2 |
| 1, 2, 3, 9, 15, 16, 17 | 7 | 4.00 | 26.1 | 9.59 | 65.3 |

* In the computer analysis of data for Fig. 9, some of data are omitted in test analyses *a* and *b* in order to see whether there are enough data to give an accurate result. The amount of binding site *N*'s are expressed by (micromoles per liter) so that one can compare these results with plots shown in Fig. 9.

‡ The numbers in this column correspond to those in Figs. 5 and 9.

in regard to the analysis of Scatchard plots: (*a*) the widely employed method of using tangents to the two ends of the Scatchard plot as the two partial plots for two classes of binding sites has been shown not to be a valid procedure (see also reference 17). Clearly, simply drawing tangents (for example, $T_A$ and $T_B$ in Fig. 4) to both ends of the plot gives erroneous results. (*b*) In order to obtain accurate results, as many points as possible in the linear portions of the curve (denoted by *A* and *B* in Fig. 4) must be measured; if not enough points at *A* and *B* are measured, the plots will give a falsely low value for $K_1$ and a falsely high value for $K_2$. To learn if enough data in the linear portion have been obtained, it is necessary to change the number of points used for the computer calculation; Table II shows an example of this kind of examination in calculating $Ca^{++}$ binding by Fr-2. The set of numbers in the left column shows the data used for the computer analysis (these numbers correspond to those in Figs. 5 and 9). Part *a* of Table II shows that omitting one or two points at both ends does not greatly change the values for *K*'s and *N*'s suggesting that there are enough points at the linear portion. Part *b* of Table II shows that it is more important to have a certain number of points at both ends of the curve than to have an abundance of data throughout the curve. In order to increase the number of points in the portion *A* (see Fig. 4) of the curve, the sensitivity of measurement at low $Ca^{++}$ concentration (as low as 0.2 $\mu$M) must be increased. This can only

be achieved by improving the signal-to-noise ratio of the dual wavelength spectrophotometer; this has been accomplished with spectrophotometer built in this laboratory.

The advantages of computer analysis of curvilinear Scatchard plots relate not only to the rapidity of analysis and removal of tedious human labor, but also permit the analysis of systems having large differences between $K_1$ and $K_2$. For example, if $K_1$ and $K_2$ differ by a factor of $10^3$ as is the case with the data in Table I, it is impossible successfully to analyze these data graphically, whereas good results may be obtained by computer analysis.

From the results presented here on the $Ca^{++}$ binding by Fr-2, we may numerically consider the possible role of Fr-2 in $Ca^{++}$ binding by sarcoplasmic reticulum. From data on $n$'s, 68 + 190 = 258 $\mu$moles total binding sites per g Fr-2 were found. Assuming that Fr-2 occupies 90% of total membrane protein (10) and that it is the only protein fraction which can bind $Ca^{++}$, it can be calculated that 258 × 0.9 = 232 $\mu$moles $Ca^{++}$ will be found per g of sarcoplasmic reticular membrane protein. This value is in good agreement with the value obtained for $Ca^{++}$ binding by sarcoplasmic reticular vesicles in vitro (18).

We wish to thank Dr. Bernard E. Pennock of The Medical College of Pennsylvania and the staff of The Medical School Computer Facility of the University of Pennsylvania for their instructions in developing the computer program.
We also thank Miss Mary C. Stom for preparing soluble protein fraction from sarcoplasmic reticulum.

Computer calculations were made at The Medical School Computer Facility of the University of Pennsylvania (National Institutes of Health grant RR-15).
This work was supported in part by grant GB-12145 from the National Science Foundation and grant AM-09458 from the National Institute of Arthritis and Metabolic Diseases.
Dr. Bertrand is a postdoctoral fellow on National Institutes of General Medical Sciences training grant GMO2008.

*Received for publication 1 March 1972.*

## REFERENCES

1. EBASHI, S. 1960. *J. Biochem.* **50**:236.
2. EBASHI, S. 1961. *Progr. Theor. Phys. Suppl.* **17**:35.
3. EBASHI, S., and M. ENDO. 1968. *Prog. Biophys. Mol. Biol.* **18**:123.
4. OHNISHI, T., and S. EBASHI. 1963. *J. Biochem.* **54**:506.
5. OHNISHI, T., and S. EBASHI. 1964. *J. Biochem.* **55**:599.
6. SCATCHARD, G. 1949. *Ann. N. Y. Acad. Sci.* **51**:660.
7. ROSENTHAL, H. E. 1967. *Anal. Biochem.* **20**:525.
8. DANCHIN, A., and M. GUERON. 1970. *Eur. J. Biochem.* **16**:532.
9. YU, B. P., F. D. DEMARTINIS, and E. J. MASORO. 1968. *Anal. Biochem.* **24**:523.
10. YU, B. P., and E. J. MASORO. 1970. *Biochemistry.* **9**:2909.
11. BURTON, K. 1959. *Biochem. J.* **71**:388.
12. COHEN, A., and Z. SELINGER. 1969. *Biochim. Biophys. Acta.* **183**:27.
13. BERTRAND, H. A., E. J. MASORO, T. OHNISHI, and B. P. YU. 1971. *Biochemistry.* **10**:3679.
14. HOLLOWAY, J. H., and C. N. REILLEY. 1960. *Anal. Chem.* **32**:249.
15. MARTELL, A. E., and G. SCHWARZENBACH. 1956. *Helv. Chim. Acta.* **39**:653.
16. WALAAS, E. 1958. *Acta Chem. Scand.* **12**:528.
17. KLOTZ, I. M., and D. L. HUNSTON. 1971. *Biochemistry.* **10**:3065.
18. CARVALHO, A. P., and B. LEO. 1967. *J. Gen. Physiol.* **50**:1327.