Binding of Two Sr$^{2+}$ Ions Changes the Chemical Specificities for Phosphorylation of the Sarcoplasmic Reticulum Calcium ATPase through a Stepwise Mechanism*

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Taro Fujimori and William P. Jencks
From the Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254-9110

The sequential binding of Sr$^{2+}$ and Ca$^{2+}$ to the cytoplasmic transport sites of the sarcoplasmic reticulum calcium ATPase allows the formation of two different mixed complexes: $E\cdot Sr-Ca$, with Sr$^{2+}$ bound to the "inner" site and Ca$^{2+}$ bound to the "outer" site, and $E\cdot Ca-Sr$, with Ca$^{2+}$ bound to the inner site and Sr$^{2+}$ bound to the outer site (pH 7.0, 25°C, 10 mM MgCl$_2$, 100 mM KCl). Both $E\cdot Sr-Ca$ and $E\cdot Ca-Sr$ react with ATP to internalize one $46^{Ca}$-phosphoenzyme. The value of $K_{0.5}$ = 83 $\mu$M Sr$^{2+}$ for activation of the enzyme for phosphorylation by ATP is much larger than $K_{0.5}$ = 28 $\mu$M Sr$^{2+}$ for inhibition of phosphoenzyme formation from inorganic phosphate ($n_H$ = 1.0–1.3). These results are consistent with the sequential binding of two strontium ions with negative cooperativity and dissociation constants of $K_{Sr1} = 35$ $\mu$M and $K_{Sr2} = 55$ $\mu$M. The species $E\cdot Sr_2$ and $E\cdot Ca_2$ react rapidly with ATP but not inorganic phosphate. However, enzyme with one strontium bound, $E\cdot Sr$, does not react with either inorganic phosphate or ATP. Therefore, the conformational changes in the enzyme that alter the chemical specificity for phosphorylation by ATP and by inorganic phosphate are different. This requires the existence of at least three forms of the unphosphorylated enzyme with three different chemical specificities for catalysis.

The binding of two calcium ions to the cytoplasmic transport sites of the calcium ATPase from sarcoplasmic reticulum is the initial step in the transport of two calcium ions from the cytoplasm into the lumen of the SR.[1] The binding of calcium acts as a switch that changes the catalytic specificities of the enzyme: free enzyme without calcium bound to the cytoplasmic transport sites is phosphorylated by inorganic phosphate, whereas enzyme with two calcium ions bound is phosphorylated by ATP (1, 2). The binding of two calcium ions occurs with a high degree of positive cooperativity (3–9), which prevents the formation of a large quantity of enzyme bound calcium, $E\cdot Ca$, at equilibrium. Therefore, the chemical specificity of $E\cdot Ca$ has not been fully characterized; however, $E\cdot Ca$, which was formed transiently, does not react with ATP (9). The positive cooperativity for the binding of two calcium ions makes it difficult to determine if the changes in the chemical specificities for phosphorylation by ATP and inorganic phosphate occur concurrently or independently.

The binding of strontium to the cytoplasmic transport sites also acts as a switch that changes the catalytic specificity of the enzyme: the binding of strontium permits the formation of phosphoenzyme from ATP and inhibits the formation of phosphoenzyme from inorganic phosphate (10). Strontium is transported by the calcium ATPase at a rate that is comparable to the rate of calcium transport (11–14). Incubation of the enzyme with calcium for several seconds is known to cause a conformational change from the $E$ species to the $E'$ species, which is defined as the stable form of the enzyme in the presence of calcium. This conformational change is accompanied by an increase in the rate of phosphorylation by ATP: $E\cdot ATP$–Ca$_2$ forms phosphoenzyme with a rate constant of $k = 220$ s$^{-1}$ compared with $k = 70$ s$^{-1}$ for $E\cdot ATP$–Sr$_2$. Strontium is also apparently able to catalyze the $E$ to $E'$ conformational change since the stable species of enzyme with bound strontium is also phosphorylated by ATP with $k = 220$ s$^{-1}$ (15). The binding of strontium and calcium to the cytoplasmic transport sites also causes similar changes in the intrinsic fluorescence of the enzyme and the fluorescence of 1-anilino-8-naphthalenesulfonate covalently bound to the enzyme (13, 16). At pH 7.4, the Hill slope of $n_H$ = 1.64 observed with strontium for the change in the intrinsic fluorescence of the enzyme is slightly lower than the value of $n_H$ = 1.83 observed with calcium (13). However, at pH 7.0, the Hill slope of $n_H$ = 0.93 observed with strontium for the change in the fluorescence of ANS$^-$ bound to enzyme is significantly lower than the value of $n_H$ = 1.8 observed with calcium (16).

The value of 1:1 for the stoichiometry of Sr$^{2+}$ uptake per ATP hydrolyzed, which was calculated from a comparison of the rate of $^{46}$Sr uptake and ATP hydrolysis, is lower than the value of 2:1 for Ca$^{2+}$ uptake (12, 13). However, a value of 2:1 for the stoichiometry of Sr$^{2+}$ uptake per ATP hydrolyzed was determined recently by using the pulsed pH-stat method, which allows this stoichiometry to be measured in a single assay (14).

Results reported here indicate that strontium binds to both cytoplasmic transport sites but with little or no positive cooperativity; this conclusion is consistent with Hill slopes of $n_H$ = 1.0–1.3 and a stoichiometry of 2:1 for Sr$^{2+}$ uptake per ATP hydrolyzed. The changes in chemical reactivity toward phosphorylation by either ATP or P, caused by the binding of strontium do not occur with the high degree of positive cooperativity that is observed for the binding of calcium; Hill slopes of $n_H$ = 1.0–1.3 were observed with strontium, whereas
Hill slopes of $n_H = 1.6-2.0$ have been reported for calcium (9, 17, 18). Therefore, enzyme with one strontium ion bound to the cytoplasmic transport sites can be formed at equilibrium; however, enzyme with one calcium ion bound cannot be formed at equilibrium in large quantities.

Three different chemical specificities were observed in the presence of strontium: free enzyme reacts only with inorganic phosphate, enzyme with one strontium ion bound does not react with either inorganic phosphate or ATP, and enzyme with two strontium ions bound reacts only with ATP. Therefore, the changes in chemical specificities caused by strontium occur in a stepwise manner. The binding of one strontium ion prevents phosphorylation by inorganic phosphate but does not activate the enzyme for phosphorylation by ATP. This agrees with the finding that enzyme with one calcium ion bound, which was formed transiently, does not react with ATP (9); however, it is not known if this species can react with inorganic phosphate. The binding of the second strontium ion or the second calcium ion activates the enzyme for phosphorylation by ATP. Although strontium binds to the cytoplasmic transport sites with much weaker affinity than calcium, the changes in catalytic specificity caused by strontium appear to be identical to those caused by calcium.

It is known that the dissociation of two calcium ions from the cytoplasmic transport sites is sequential: the calcium ion bound to the “inner” transport site cannot dissociate unless the “outer” transport site is unoccupied (9, 19–23). Two different species of enzyme with one strontium ion and one calcium ion bound to the cytoplasmic transport sites, $E$-$Sr$-$Ca$ and $E$-$Ca$-$Sr$, were formed; these mixed species are able to react with ATP and internalize one calcium ion and, presumably, one strontium ion. The formation of two different mixed species of enzyme with calcium and strontium demonstrates that strontium can bind to both the inner and the outer transport sites.

**EXPERIMENTAL PROCEDURES**

**Materials**—Na$_5$ATP (Sonderqualität), NADH, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were purchased from Boehringer Mannheim. MOPS and A23187 were purchased from Calbiochem. KCl and EGTA were purchased from Fluka. SrCl$_2$-6H$_2$O (99.96%) was purchased from Aldrich. $[^3P]$H$_2$PO$_4$, $[^32P]$ATP (>90% pure), and $[^4C]$Cl$_2$ were purchased from Du Pont-New England Nuclear. Tris (Ultra pure) was purchased from Schwarz/Mann. All other solutions were at 0–4°C. Bovine serum albumin and ATP were added to the acid-quenched reaction mixtures to give final concentrations of $0.03$ mg/ml total protein and $0.1$ mM ATP. After $2$ h at 0°C, the samples were centrifuged at $1,500 	imes g$ for $15$ min, and the pellets were resuspended in $0.5$ mM HClO$_4$ and $15$ mM KH$_2$PO$_4$. The protein was collected on Whatman GF/C glass fiber filters by vacuum filtration and rinsed with $15$ ml of the re suspension solution. The radioactivity was measured by liquid scintillation counting in glass vials containing $7$ ml of Aquasol-2.

**RESULTS**

The Binding of $[^4C]$Ca to $E$-$Sr$-$Ca$—The binding of calcium to the cytoplasmic transport sites was assayed by measuring the amount of $[^4C]$Ca internalized after the addition of ATP and EGTA (Fig. 1). It is known that the addition of ATP to enzyme with two calcium ions bound results in the formation of phosphoenzyme and the internalization of both calcium ions into the vesicles (37, 38). The simultaneous addition of ATP and EGTA to $E$-$[^4C]$Ca results in the rapid binding of ATP to form $E$-$[^4C]$ATP, which partitions between the formation of phosphoenzyme, with $k = 220$ s$^{-1}$, and dissociation of $[^4C]$Ca, with $k = 80$ s$^{-1}$; therefore, the addition of ATP and EGTA to $E$-$[^4C]$Ca results in a single turnover of the enzyme with the internalization of $220/(220 + 80) = 0.7$ of the $[^4C]$Ca bound to the cytoplasmic transport sites and provides a measure of the amount of $[^4C]$Ca that was bound prior to the addition of ATP and EGTA (9, 39).

The open circles and open squares in Fig. 1 show that the addition of $0.4$ mM ATP and $15$ mM EGTA to enzyme that had been phosphorylated by $[^32P]$ATP was quenched with acid to give a final concentration of $0.5$ mM HClO$_4$ and $15$ mM KH$_2$PO$_4$. Enzyme that had been phosphorylated by $[^32P]$Pi was quenched with acid to give a final concentration of $0.5$ mM HClO$_4$. All subsequent manipulations and solutions were at 0–4°C. Bovine serum albumin and ATP were added to the acid-quenched reaction mixtures to give final concentrations of $0.03$ mg/ml total protein and $0.1$ mM ATP. After $2$ h at 0°C, the samples were centrifuged at $1,500 	imes g$ for $15$ min, and the pellets were resuspended in $0.5$ mM HClO$_4$ and $15$ mM KH$_2$PO$_4$. The protein was collected on Whatman GF/C glass fiber filters by vacuum filtration and rinsed with $15$ ml of the re suspension solution. The radioactivity was measured by liquid scintillation counting in glass vials containing $7$ ml of Aquasol-2.

![binding diagram](image_url)

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2 Petihory and Jencks (9) refer to the first calcium ion to dissociate from the cytoplasmic transport sites as the outer calcium ion and to the second calcium ion as the inner calcium ion.
The binding of calcium to enzyme incubated with strontium is biphasic. All reaction mixtures contained 10 mM MgCl₂, 100 mM KCl, and 40 mM MOPS, pH 7.0, at 25°C. Enzyme incubated with either 730 μM free strontium (○), 39 μM free calcium (C), or 100 μM EGTA (●) was mixed with 230 μM ⁴⁰CaCl₂ for varying times, followed by the addition of 0.3 mM ATP and 15 mM EGTA. Syringe A contained 0.52 mg/ml intact SR vesicles and either 0.1 mM EGTA (C), 0.8 mM SrCl₂ and 0.1 mM EGTA (●), or 40 μM unlabeled Ca²⁺ and 1 μM EGTA (○). Syringe B contained 460 μM ⁴⁰CaCl₂. Syringe C contained 0.9 mM ATP, 45 mM EGTA, 90 μM phosphoenolpyruvate, and 5 μg/ml pyruvate kinase. Mixing of the contents of syringes A and B resulted in the following conditions: 190 μM free ⁴⁰Ca (C), 250 μM free ⁴⁰Ca (●), or 400 μM free Sr²⁺ and 190 μM free ⁴⁰Ca (○). The SRV were dialyzed overnight at 4°C in the presence of 5 mM MgCl₂, 100 mM KCl, 40 mM MOPS, and 250 mM sucrose, pH 7.0. The solid line is drawn for the binding of 1.8 nmol of ⁴⁰Ca/mg with a rate constant of 1.5 nmol of ⁴⁰Ca/mg with a rate constant of 0.1 s⁻¹. The dotted line is drawn for the binding of 2.2 nmol of ⁴⁰Ca/mg with a rate constant of 75 s⁻¹ and the binding of 1.4 nmol of ⁴⁰Ca/mg with a rate constant of 3 s⁻¹.

2.0 μM Ca²⁺ was measured by Petithory and Jencks* (9) for the formation of ¹⁸⁴⁷S₄Ca under the conditions used for this work; therefore, >99% of the enzyme will be present as ¹⁸⁴⁷S₄Ca in the presence of ~200 μM ⁴⁰Ca. The addition of ATP and EGTA to ¹⁸⁴⁷S₄Ca results in the internalization of ~70% of the ⁴⁰Ca bound to the cytoplasmic transport sites (9). A value of 5.2 nmol of ⁴⁰Ca bound per mg of protein was calculated from the observed value of 3.6 nmol of ⁴⁰Ca/mg of protein internalized after the addition of ATP and EGTA, and the value of 70% for the kinetic partitioning toward phosphorylation under these conditions. A value of E₅₀ = 2.6 nmol/mg was calculated from the total concentration of ⁴⁰Ca bound and the ratio of two calcium ions bound per E₅₀ (9); this value is within the range of E₅₀ = 1.7–4.0 nmol/mg that was observed for different preparations of enzyme. Therefore, 3.6 nmol of ⁴⁰Ca/mg of protein is internalized after the addition of ATP and EGTA when 2 mol of ⁴⁰Ca are bound per mol of enzyme and 70% of the enzyme undergoes phosphorylation, as indicated on the right axis of Fig. 1.

The filled circles (●) in Fig. 1 show that the addition of 190 μM ⁴⁰Ca to enzyme that had been incubated with 730 μM Sr²⁺ results in the binding of ⁴⁰Ca with biphasic kinetics: one ⁴⁰Ca/²⁺ binding rapidly within 50 ms, followed by much slower binding of a second ⁴⁰Ca/²⁺ over several seconds. The solid line is calculated for rapid binding of one ⁴⁰Ca/²⁺, with kₗ = 150 s⁻¹, and slow binding of 0.83 ⁴⁰Ca/²⁺, with kₗ = 0.7 s⁻¹. The value of 3.3 nmol of ⁴⁰Ca/mg observed at t = 5 s is slightly lower than the value of 3.6 nmol of ⁴⁰Ca/mg observed after the addition of 190–250 μM ⁴⁰Ca to enzyme that had been incubated in the presence of either EGTA or unlabeled calcium. This decrease of 8% in the end point value may represent either competition at equilibrium between the binding of Sr²⁺ and ⁴⁰Ca for the cytoplasmic transport sites or competitive inhibition by Sr²⁺ at the Mg²⁺ binding site.

The open circles (○) show that the addition of 250 μM ⁴⁰Ca to enzyme that had been incubated with 39 μM ⁴⁰Ca also results in the binding of ⁴⁰Ca with biphasic kinetics: one ⁴⁰Ca binds rapidly within 50 ms, followed by much slower binding of a second ⁴⁰Ca to enzyme incubated with 730 μM Sr²⁺ and ⁴⁰Ca for the cytoplasmic transport sites or competitive inhibition by Sr²⁺ at the Mg²⁺ binding site.

It is known that calcium binds to and dissociates from the cytoplasmic transport sites in a sequential mechanism (9, 19, 21). The rate-limiting step in the exchange of ⁴⁰Ca into ¹⁸⁴⁷S₄Ca is the dissociation of ⁴⁰Ca from the cytoplasmic transport sites. The dissociation of calcium from the outer binding site is rapid, with kₗ Ca ≈ 50 s⁻¹, and is not affected by the presence of calcium in the external medium, but the dissociation of calcium from the inner binding site is slower and is inhibited by the presence of calcium in the external medium (9). This inhibition is caused by occupancy of the outer calcium binding site: the outer calcium binding site must be empty before the inner calcium ion may dissociate. This sequential mechanism for the dissociation of calcium has been proposed to explain the biphasic kinetics observed for the binding of ⁴⁰Ca to ¹⁸⁴⁷S₄Ca (9, 21).

The biphasic kinetics that are observed for the binding of ⁴⁰Ca to ¹⁸⁴⁷S₄Ca (○, Fig. 1) are consistent with an ordered and sequential mechanism for the dissociation of strontium from the cytoplasmic transport sites, as shown in Scheme 1. A rate constant of kₗ S₄Ca = 115 ± 15 s⁻¹ was observed for the dissociation of strontium from ¹⁸⁴⁷S₄Ca in the presence of 15 mM EGTA (15); this value agrees within experimental error with the value of kₗ S₄Ca = 150 ± 30 s⁻¹ observed for the dissociation of one strontium ion in the presence of 190 μM calcium. Therefore, the dissociation of the outer strontium ion is not inhibited by the presence of 190 μM calcium in the external medium and results in the rapid formation of ¹⁸⁴⁷S₄Ca, with strontium bound to the inner binding site. The binding of calcium to the outer binding site of ¹⁸⁴⁷S₄Ca results in the formation of ¹⁸⁴⁷S₄Ca. The dissociation of the inner strontium ion can occur only when the outer binding site is unoccupied. Therefore, the dissociation of the inner strontium ion is very slow in the presence of 190 μM calcium and occurs over several seconds. The solid line in Fig. 1 is drawn for dissociation of the outer and inner strontium ions with rate constants of 150 s⁻¹ and 0.7 s⁻¹, respectively.

Scheme 1

*Petithory and Jencks (9, 27) reported values of K₉₅ = 3.6 μM Ca²⁺ for the formation of ¹⁸⁴⁷S₄Ca and K₉₅ = 1.4 μM; however, they used a value of kₗ Ca = 3.4 × 10⁻¹⁰ M⁻¹ s⁻¹ for the dissociation constant of the calcium-EGTA complex at pH 7.0 (47), which is ~2-fold larger than the value of 3.9 × 10⁻⁷ M used in this paper (32). Hael and Jencks (29) calculated a value of K₉₅ = 2.0 μM Ca²⁺ for the formation of ¹⁸⁴⁷S₄Ca by using the data of Petithory and Jencks and the dissociation constant of 3.9 × 10⁻¹⁰ M for the calcium-EGTA complex, as described in the Appendix.
The open squares (○) in Fig. 1 show that the addition of 190 μM 45Ca to enzyme that had been incubated with EGTA results in the formation of E·45Ca2 with biphasic kinetics. The dotted line is calculated for the formation of 0.6·E·45Ca2/E\textsubscript{total} with k = 75 s\textsuperscript{-1}, followed by the formation of 0.4·E·45Ca2/E\textsubscript{total} with k = 3 s\textsuperscript{-1}. Pettitroy and Jencks (27) measured a faster overall rate for the reaction of 120 μM 45Ca with enzyme that had been incubated in the presence of EGTA and 5 mM Mg\textsuperscript{2+} and showed that two calcium ions must be bound to the transport sites before phosphorylation by ATP can occur. It is possible that the slower rate for the reaction of 190 μM 45Ca with enzyme that had been incubated in the presence of EGTA and 10 mM Mg\textsuperscript{2+} (○, Fig. 1) results from binding of magnesium to the cytoplasmic transport sites and slow dissociation of Mg\textsuperscript{2+} in the presence of 190 μM Ca\textsuperscript{2+}.

The relatively rapid binding of the second 45Ca ion to enzyme that had been incubated with EGTA (○) demonstrates that the slow binding of the second calcium ion to enzyme that had been incubated with strontium is not caused by slow association of 190 μM calcium with E·Ca. We conclude that it is caused by the slow dissociation of strontium from E·Sr·45Ca, which is required before 45Ca can bind to the inner transport site. The plateau in the binding of 45Ca that is observed between 0.03 and 0.25 s demonstrates that E·Sr·45Ca is a kinetically stable intermediate that can be phosphorylated by ATP, which results in the occlusion of one 45Ca and, presumably, one strontium ion.

The addition of ATP and EGTA to E·Sr·45Ca results in the internalization of 1.8 nmol of 45Ca/mg of protein. This value is the same as the value observed after the addition of ATP and EGTA to E·45Ca2·4Ca and is equivalent to one-half of the value of 3.6 nmol of 45Ca2 that was observed after the addition of ATP and EGTA to E·45Ca2. These data indicate that the reactivity of E·Sr·45Ca after the simultaneous addition of ATP and EGTA is identical to that of E·Ca, which is known to form 70% phosphoenzyme because of the kinetic partitioning between phosphorylation and dissociation of calcium under these conditions, as described above (39). This result suggests that the replacement of Ca\textsuperscript{2+} for Sr\textsuperscript{2+} at the inner transport site has little or no effect on the rate constant for the dissociation of Ca\textsuperscript{2+} from the outer transport site, with k = 80 s\textsuperscript{-1}, in spite of the much lower affinity of Sr\textsuperscript{2+} for the cytoplasmic binding sites.

The Dissociation of 45Ca from E·45Ca2 in the Presence of Strontium—The rate of dissociation of 45Ca from E·45Ca2 was measured in the presence of 50 μM Mg\textsuperscript{2+}, 300 μM Sr\textsuperscript{2+}, or 5 mM EGTA as shown in Fig. 2. The amount of calcium bound to the cytoplasmic transport sites was assayed by measuring the internalization of 45Ca after the addition of ATP and EGTA as described above (Fig. 1). The addition of 0.5 mM ATP and 15 mM EGTA to enzyme that had been incubated for 15 s in the presence of a saturating concentration of 45Ca, 40 μM, results in the internalization of 3.0 nmol of 45Ca/mg. A value of 4.3 nmol of 45Ca bound per mg of protein was calculated from the observed value of 3.0 nmol of 45Ca/mg internalized and the fraction of the bound 45Ca that is internalized after the addition of ATP and EGTA, 70%. A value of E\textsubscript{total} = 2.1 nmol/mg was calculated from the total concentration of 45Ca bound, 4.3 nmol/mg, and the ratio of two calcium ions bound per E\textsubscript{total} (9); this value of E\textsubscript{total} is within the range of E\textsubscript{total} = 1.7–4.0 nmol/mg that is observed for these preparations of enzyme. Therefore, 3.0 nmol of 45Ca/mg is internalized when 2 mol of 45Ca are bound per mol of enzyme, as indicated on the left and right axes of Fig. 2, and 70% of the enzyme undergoes phosphorylation. The filled circles (●) in Fig. 2 show that the dissociation of 45Ca from E·45Ca in the presence of 300 μM Sr\textsuperscript{2+} is biphasic. The solid line is calculated for the rapid dissociation of one 45Ca/E\textsubscript{total} with k = 50 s\textsuperscript{-1}, followed by slow dissociation of the second 45Ca/E\textsubscript{total} with k = 4 s\textsuperscript{-1}. The open circles (○) in Fig. 2 show that the dissociation of 45Ca from E·45Ca2 in the presence of 50 μM Ca\textsuperscript{2+} is biphasic, which is consistent with previous results (9, 21). The dashed line is drawn for a biphasic reaction with two sequential rate constants of 50 s\textsuperscript{-1} and 4 s\textsuperscript{-1}. Therefore, the dissociation of 45Ca from E·45Ca2 is qualitatively similar in the presence of 300 μM strontium or 50 μM 45Ca.

The biphasic dissociation of 45Ca in the presence of 50 μM 45Ca or 300 μM Sr\textsuperscript{2+} is consistent with an ordered sequential mechanism for the dissociation of 45Ca (9, 19, 21). The rapid dissociation of the first 45Ca/E\textsubscript{total} represents the dissociation of 45Ca from the outer binding site, which is not inhibited by the presence of calcium in the medium (9, 19, 21). The slow dissociation of the second 45Ca/E\textsubscript{total} represents the dissociation of 45Ca from the inner binding site. The dissociation of calcium from E·Ca is known to occur with k = 30 s\textsuperscript{-1} in the absence of calcium and strontium (27). The slow dissociation of the inner calcium ion in the presence of 50 μM 45Ca or 300 μM Sr\textsuperscript{2+} indicates that Ca\textsuperscript{2+} and Sr\textsuperscript{2+} can bind to E·45Ca to form E·45Ca·Ca and E·45Ca·Sr, respectively. The 45Ca ion bound to the inner transport site cannot dissociate when either Ca\textsuperscript{2+} or Sr\textsuperscript{2+} is bound to the outer transport site.

The dotted line in Fig. 2 is drawn for the dissociation of 45Ca from E·45Ca in the presence of 5 mM EGTA, with k = 50 s\textsuperscript{-1}. This first-order reaction corresponds to the dissociation of 45Ca from the outer binding site, which results in the formation of E·45Ca. The addition of ATP and EGTA to enzyme with one 45Ca ion bound, E·45Ca, does not result in the formation of phosphoenzyme and, therefore, does not result in the internalization of 45Ca under these conditions (9). Therefore, both 45Ca ions of the species E·45Ca lose their reactivity to ATP after the dissociation of one 45Ca ion, with k = 50 s\textsuperscript{-1}, under these conditions.

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**Fig. 2.** The dissociation of calcium from E·45Ca2 is biphasic in the presence of strontium. All reaction mixtures contained 10 mM MgCl\textsubscript{2}, 100 mM KCl, and 40 mM MOPS, pH 7.0, at 25 °C. Syringes A contained 0.58 mg/ml SRV, 40 μM 45CaCl\textsubscript{2}, and 5 μM EGTA. Syringes B contained either 10 mM EGTA (□), 1.05 mM CaCl\textsubscript{2} and 1 mM EGTA (●), or 2 mM SrCl\textsubscript{2} and 2 mM EGTA (○). Syringe C contained 0.9 mM ATP, 45 mM EGTA, 0.09 mM phosphoenolpyruvate, and 5 μg/ml pyruvate kinase. The reaction mixture contained either <0.01 μM free Ca\textsuperscript{2+} (□), 50 μM free Ca\textsuperscript{2+} (○), or 300 μM free Sr\textsuperscript{2+} and 0.07 μM free Ca\textsuperscript{2+} (●) after the mixing of the contents of syringes A and B. The SRV were dialyzed overnight at 4 °C in the presence of 5 mM MgSO\textsubscript{4}, 100 mM KCl, 40 mM MOPS, and 200 mM sucrose, pH 7.0. The solid line is drawn for a biphasic reaction with two sequential rate constants of 50 s\textsuperscript{-1} and 4 s\textsuperscript{-1}.
The filled circles (●) in Fig. 2 show that only one 45Ca ion of the species E-45Ca loses its reactivity to ATP with a rate constant of k ≈ 50 s⁻¹ in the presence of 300 μM Sr²⁺. This result indicates that one strontium ion binds to the outer transport site of E-45Ca to form E-45Ca-Sr and activates the enzyme for phosphorylation by ATP, as shown in Scheme 2.

The dissociation of the inner calcium ion is 87% slower in the presence of 300 μM Sr²⁺ with kₜ₉ = 4 ± 2 s⁻¹, compared with the dissociation of calcium from E-Ca in the presence of 5 mM EGTA, with kₜ₉ = 30 s⁻¹ (9). This suggests that in the presence of 300 μM Sr²⁺ the ratio of E-Ca to E-Ca-Sr is 13%/87%, which corresponds to a value of Kₛ₉ = 45 ± 20 μM for inhibition of calcium dissociation by binding of Sr²⁺ to E-Ca to form E-Ca-Sr.

The Binding of Strontium to the Cytoplasmic Transport Sites at Equilibrium: Inhibition of EP Formation from Inorganic Phosphate—The SR-calcium ATPase can react with inorganic phosphate to form phosphoenzyme if the cytoplasmic transport sites are unoccupied (17). Phosphorylation of the enzyme by inorganic phosphate at equilibrium is inhibited by calcium with Kₛ₉ = 1.8 μM and a Hill slope of n₉ = 2, which indicates that two Ca²⁺ ions bind to the cytoplasmic transport sites with a high degree of positive cooperativity and that this binding prevents reaction of the enzyme with inorganic phosphate (17). This inhibition by calcium of phosphoenzyme formation from inorganic phosphate is a measure of the affinity of the cytoplasmic transport sites for calcium.

The affinity of the cytoplasmic transport sites for strontium was determined by measuring the inhibition of the formation of phosphoenzyme from inorganic phosphate at equilibrium, as shown in Fig. 3, A and B. The value of Kₛ₉ = 28 μM for inhibition by Sr²⁺ is much larger than the value of Kₛ₉ = 1.8 μM for inhibition by calcium under similar conditions and confirms that strontium binds to the cytoplasmic transport sites with a much weaker affinity than calcium. The Hill plot in Fig. 3B shows that the inhibition by strontium is consistent with a Hill slope of n₉ = 1.0 (dashed line); the data are not consistent with a Hill slope of n₉ = 2.0 (dotted line). The simplest model consistent with the data shown in Fig. 3, A and B, consists of one strontium ion-binding to the cytoplasmic transport sites. The dashed lines in Fig. 3, A and B are drawn for inhibition by a single strontium ion, as shown in Scheme 3, with values of n₉ = 1.0 and Kₛ₉ = 24 μM. However, this model was rejected on the basis of the following evidence.

5 Pickart and Jencks reported a value of Kₛ₉ = 3.2 μM for the inhibition by Ca²⁺ of phosphoenzyme formation from 10 mM inorganic phosphate at equilibrium (18); however, this value was calculated by using an apparent dissociation constant of 7.4 × 10⁻⁷ M for the calcium-EGTA complex (47). The concentrations of calcium reported in this work were calculated by using an apparent dissociation constant for calcium-EGTA of 3.9 × 10⁻⁷ M at pH 7.0 (32). A value of Kₛ₉ = 1.8 μM for the inhibition of phosphoenzyme formation from 10 mM Pi, was calculated from the reported value of Kₛ₉ = 3.2 μM and the apparent dissociation constant of 3.9 × 10⁻⁷ M.

![Fig. 3. A](image1)

**Scheme 2**

**Scheme 3**

1) Two strontium ions bind to the cytoplasmic transport sites (13).
2) Two moles of strontium are transported per mol of ATP hydrolyzed (14).
3) Strontium is able to bind to both the inner and the outer
transport sites as shown in Figs. 1 and 2.

4) Increasing the concentration of Sr^{2+} above the value of \( K_{0.5} = 28 \mu M \) causes a second conformational change with \( K_{0.5} = 83 \mu M \), which activates the enzyme for phosphorylation by ATP, as shown in Fig. 4; this second conformational change must involve the binding of a second strontium ion, as discussed below.

\[
\frac{[E]}{[E_{\text{total}}]} = \frac{K_{\text{eq1}} K_{\text{eq2}} [P_i]}{[S] K_{\text{eq}}} \left( \frac{[P_i]}{K_{p} + [P_i]} + \frac{[P]}{K_p + [P]} + 1 \right)
\]  

(1)

The data shown in Fig. 3, A and B are consistent with the binding of two strontium ions to the enzyme with negative cooperativity; the solid lines are drawn for the sequential binding of two strontium ions as shown in Scheme 4, with values of \( K_{S_{\text{eq}}} = 35 \mu M \) and \( K_{S_{\text{eq}}} = 55 \mu M \). In this scheme, the strontium ions bind to the cytoplasmic transport sites in a sequential mechanism analogous to the mechanism for the binding of two calcium ions (9, 19, 21, 41) or the binding of one calcium ion and one strontium ion to form a mixed complex as shown in Figs. 1 and 2. The dissociation constants for the binding of strontium would be slightly different for a random binding mechanism. The solid lines were calculated by using Equation 1, which is derived in the Appendix. The denominator of this equation contains both a quadratic term, \([S] - [S]_{2}\), and a linear term, \( K_{S_{\text{eq}}} [S] \). The value of the Hill slope is determined by the ratio of these two terms, \( [S]_{2}/(K_{S_{\text{eq}}} [S]) \)

\[
= \frac{[S]}{[S]_{2} + [S]_{2} + [S]_{2}} \left( \frac{[P_i]}{K_{p} + [P_i]} + \frac{[P]}{K_p + [P]} + 1 \right).
\]  

Equation 2 describes the relationship between the Hill slope, \( n_H \), and \([S]_2\) for Scheme 4 and is derived in the Appendix.

\[
n_H = 1 + \frac{[S]_2}{[S] + K_{S_{\text{eq}}} = 1 + \frac{[E_{\text{eq1}}]}{[E_{\text{eq2}}} + \frac{[E]}{[E_{\text{eq2}}}}
\]  

(2)

A Hill slope of \( n_H \sim 2 \) is predicted when the quadratic term in Equation 1 is much larger than the linear term, \( [S]_{2} \gg K_{S_{\text{eq}}} \). At these high concentrations of strontium, the binding of the second strontium ion is highly favorable, and the ratio of \([E - \text{Sr}]_{2}\) to \([E - \text{Sr}]_{2}\) will be low. These two forms of enzyme, \( E - \text{Sr} \) and \( E - \text{Sr} \), do not react with inorganic phosphate. If the concentration of \( E - \text{Sr} \) is much greater than the concentration of \( E - \text{Sr} \), then most of the enzyme that is unreactive toward inorganic phosphate must dissociate two strontium ions to become reactive to inorganic phosphate; this is indicated by the Hill slope of \( n_H \sim 1.0 \). The Hill plot shown in Fig. 3B is most accurate near the half-maximal concentration of \( K_{0.5} = 28 \mu M \) for strontium. Data outside the range \( -1 < \log [E]/[E_{\text{total}}] - E \) \(< 1 \) have large errors associated with them. The values of \( K_{S_{\text{eq}}} = 35 \mu M \) and \( K_{S_{\text{eq}}} = 55 \mu M \) result in a Hill slope of \( n_H \sim 1.3 \) at the half-maximal concentration of \( 28 \mu M \)Sr^{2+}, according to Equation 2. The inhibition by strontium of phosphoenzyme formation from inorganic phosphate is consistent with Hill slopes of \( n_H = 1.0 - 1.5 \) and is best fit by \( n_H \sim 1.3 \) (solid line) in the range \( -1 < \log [E]/[E_{\text{total}}] - E \) \(< 1 \).

The levels of phosphoenzyme in this experiment were measured by incubating the enzyme with 2.5 mM [32P]Pi, 10 mM MgCl₂, 1 mM EGTA, and varying concentrations of SrCl₂. In the presence of 2.5 mM [32P]Pi, and 0 mM Sr^{2+}, ~10% of the enzyme is phosphorylated under these conditions (18). The value of \( E_{\text{eq2}} = 0.3 \) nmol/mg observed in the presence of 2.5 mM [32P]Pi, and 0 mM Sr^{2+} indicates that the concentration of active sites is \( E_{\text{total}} = 3.0 \) nmol/mg. This value agrees with values of \( E_{\text{eq2}} = 2 - 4 \) nmol/mg which were usually observed after the addition of saturating [Ca^{2+}] and [γ-32P]ATP to these preparations of enzyme.

The apparent affinity of the enzyme for strontium is decreased in the presence of phosphate by competition between the formation of phosphoenzyme and the binding of strontium. Equation 3, which is derived in the Appendix, describes the effect of phosphate concentration on the value of \( K_{0.5} \), for the inhibition of EP formation from inorganic phosphate according to Scheme 4. Values of \( K_{0.5} = 28 \mu M \) in the presence of 2.5 mM P, and \( K_{0.5} = 24 \mu M \) in the presence of 0 mM P, were obtained from Equation 3, the equilibrium constants in Table I, and the values of \( K_{S_{\text{eq}}} = 35 \mu M \) and \( K_{S_{\text{eq}}} = 55 \mu M \). Therefore, the presence of 2.5 mM P, is predicted to decrease the apparent affinity of the enzyme for strontium by 17%, according to the model shown in Scheme 4.

\[
K_{0.5} = \frac{K_{S_{\text{eq}}}}{2} \left( \sqrt{1 + 4K_{S_{\text{eq}}} \left( \frac{[E_{\text{eq}}]}{[E_{\text{eq}}]} + \frac{[E]}{[E_{\text{eq}}]} \right)} - 1 \right)
\]  

(3)

The inhibition of EP formation from inorganic phosphate by calcium and strontium shows that phosphate cannot react with the enzyme when calcium or strontium is bound to the transport site. However, it is not known whether the binding of these divalent ions to the transport sites inhibits the binding of inorganic phosphate, as shown in Scheme 4, or whether this binding inhibits the formation of inorganic phosphoenzyme. The concentrations of free ions as shown in Table I, and the values of \( K_{S_{\text{eq}}} = 35 \mu M \) and \( K_{S_{\text{eq}}} = 55 \mu M \) result in a Hill slope of \( n_H = 1.3 \) at the half-maximal concentration of \( 28 \mu M \)Sr^{2+}, according to Equation 2. The inhibition by strontium of phosphoenzyme formation from inorganic phosphate is consistent with Hill slopes of \( n_H = 1.0 - 1.5 \) and

\[
E^p \xrightarrow{K_{E_{\text{eq2}}}} E_{\text{eq1}} \xrightarrow{K_{E_{\text{eq2}}}} E_{\text{eq2}} \xrightarrow{K_{E_{\text{eq2}}}} E_{\text{eq2}}.
\]  

SCHEME 4

\[
E^p \xrightarrow{K_{E_{\text{eq2}}}} E_{\text{eq1}} \xrightarrow{K_{E_{\text{eq2}}}} E_{\text{eq2}} \xrightarrow{K_{E_{\text{eq2}}}} E_{\text{eq2}} \xrightarrow{K_{E_{\text{eq2}}}} E_{\text{eq2}}.
\]  

SCHEME 5
and the equilibrium constants in Table I. Thus, the presence of 2.5 mM P, is calculated to decrease the apparent affinity of the enzyme for strontium by 8%, according to the model shown in Scheme 5. Therefore, the presence of 2.5 mM P, is predicted to have only a small effect, 8–17%, on the affinity of the enzyme for Sr2+ according to either of the mechanisms shown in Schemes 4 and 5.

\[
K_{o,3} = \frac{K_{o,2}^2}{2} \left( \sqrt{1 + 4 \frac{K_{o,2}}{K_{o,1}} \frac{[P_i] + K_{o,2}}{K_{o,1} + K_{o,3} + K_{o,2}}} - 1 \right)
\]  

(4)

The Binding of Strontium to the Cytoplasmic Transport Sites at Equilibrium: Formation of 'E-Sr'—The affinity of the cytoplasmic transport sites for strontium was also determined by incubating enzyme with different concentrations of strontium and then measuring the concentration of phosphoenzyme formed after the addition of [γ-32P]ATP and EGTA, as shown in Fig. 4, A and B. The addition of ATP and EGTA to 'E-Sr' results in the rapid formation of 'E-ATP-Sr', which partitions between the formation of phosphoenzyme with \( k \approx 220 \text{ s}^{-1} \) and the irreversible dissociation of Sr2+ with \( k_{-S} \approx 115 \text{ s}^{-1} \) (15). The fraction of 'E-Sr', that undergoes phosphorylation in a single turnover after the addition of ATP and EGTA depends on the rate constants for the formation of phosphoenzyme, \( k_p \), and for the dissociation of strontium, \( k_{-S} \), as shown in Equation 5.

\[
\frac{EP}{E_{total}} = \frac{k_p}{k_p + k_{-S}}
\]  

(5)

Values of \( k_p \) and \( k_{-S} \) are determined in 65% phosphorylation of 'E-Sr'. The addition of EGTA prevents the binding of additional strontium to the transport sites; therefore, the amount of phosphorylation formed is proportional to the concentration of 'E-Sr', that is present prior to the addition of ATP and EGTA.

The activation of phosphoenzyme formation from ATP by strontium is consistent with the solid lines in Fig. 4, A and B, which were drawn for a value of \( K_{o,3} = 83 \text{ mM} \). The value of \( K_{o,3} = 83 \text{ mM} \) Sr2+ is much larger than the value of \( K_{o,2} = 2.0 \text{ mM} \) Sr2+ for activation of phosphoenzyme formation from ATP, which confirms that strontium binds to the cytoplasmic transport sites with a much weaker affinity than calcium. The concentration of free Sr2+ that is present after the addition of Sr2+ for reaction with ATP is consistent with a Hill slope of 2.5 and EGTA depends on the rate constants for the binding of strontium, \( k_p \), and for the dissociation of strontium, \( k_{-S} \), as shown in Scheme 5.

The Hill plot in Fig. 4B shows that activation of the enzyme by Sr2+ for reaction with ATP is consistent with a Hill slope of \( n_H = 1.0 \) (dashed line) but is not consistent with a Hill slope of \( n_H = 2.0 \), shown by the dotted line. Therefore, the binding of two strontium ions does not occur with the high degree of positive cooperativity that is observed for the binding of two calcium ions, with \( n_H = 2 \) (6, 9, 17–19).

The data shown in Fig. 4, A and B, are consistent with the binding of two strontium ions with negative cooperativity.

The solid lines are in good agreement with the data and are drawn for the sequential binding of two strontium ions as shown in Scheme 6 with \( K_{o,3} = 35 \text{ mM} \) and \( K_{o,3} = 35 \text{ mM} \). The dashed line is drawn for a Hill slope of 1.0 and \( K_{o,3} = 87 \text{ mM} \). The dotted line is drawn for a Hill slope of 1.0 and \( K_{o,3} = 87 \text{ mM} \).
Binding of Strontium to the SR Calcium-ATPase

\[
E^p \rightleftharpoons E^{in} \rightleftharpoons E \rightleftharpoons E_{Sr} \rightleftharpoons E_{Sr^2+} \rightleftharpoons E_{Sr^3+} \rightleftharpoons E_{Sr^2+ATP} \rightleftharpoons E_{Sr^3+ATP}
\]

Scheme 6

The value of \( K_{Sr^2+} = 83 \mu M \) for the formation of "E-Sr^2+" was measured in the presence of 2.5 mM unlabeled inorganic phosphate, which was added to allow comparison with the experiment shown in Fig. 3, as described below. This value of \( K_{Sr^2+} \) was calculated by using equilibrium constants from Table I and Equation 7, which is derived in the Appendix.

\[
K_{Sr^2+} = \frac{K_{Sr^2+}}{2} \left( 1 + \sqrt{1 + \frac{4 K_{Sr^2+} [Sr^2+] [P_i] + [P_i] + 1}{K_{Sr^2+}}} \right)
\]  (7)

A value of \( K_{Sr^2+} = 79 \mu M \) was calculated from Equation 7 for the formation of "E-Sr^2+" in the absence of inorganic phosphate; therefore, the presence of 2.5 mM inorganic phosphate is predicted to have only a small effect, 5%, on the apparent affinity of the cytoplasmic transport sites for Sr^2+. No significant difference (±10%) was observed in the value of \( K_{Sr^2+} = 83 \mu M \) Sr^2+ for phosphorylation of enzyme by [γ-32P]ATP in the presence (○) or absence (△) of 2.5 mM unlabeled inorganic phosphate.

Prior to the addition of [γ-32P]ATP and EGTA, the incubation medium was identical to that used for the experiment shown in Fig. 3, except that the inorganic phosphate was unlabeled. The addition of [γ-32P]ATP and EGTA measures the concentration of "E-Sr^2+" that is present before the addition of ATP and EGTA; therefore, a direct comparison can be made between the results shown in Figs. 3 and 4, as described below (Fig. 5).

Equation 8, which is derived in the Appendix, describes the relationship between the Hill slope for the formation of "E-Sr" and [Sr^2+] for Scheme 6:

\[
n_{Hill} = 2 - \frac{[Sr]}{[Sr] + K_{Sr^2+} \left( \frac{K_{Sr^2+} [P_i] + [P_i]}{K_p} + 1 \right)}
\]  (8)

\[
n_{Hill} = 2 - \frac{[E_{Sr^2+}]}{[E_{Sr^2+}] + [E] \left( \frac{K_{Sr^2+} [P_i] + [P_i]}{K_p} + 1 \right)}
\]

A Hill slope of \( n_{Hill} \sim 2.0 \) is predicted when the concentration of strontium is much less than the value of \( K_{Sr^2+} \). At these low concentrations of strontium, the binding of the first strontium ion is unfavorable, and the ratio of [E] to ["E-Sr"] is high. These two forms of enzyme, E and "E-Sr", do not react with ATP; only enzyme with two strontium ions bound, "E-Sr^2+", can react with ATP. If the concentration of E is much larger than the concentration of "E-Sr", then most of the enzyme that is unreactive to ATP must bind two strontium ions to become reactive to ATP, and this is reflected in the Hill slope of \( n_{Hill} \sim 2.0 \). A Hill slope of \( n_{Hill} \sim 1 \) is predicted when the concentration of strontium is much greater than \( K_{Sr^2+} \) at low concentrations of inorganic phosphate, [P_i] ≤ K_p. At these high concentrations of strontium, the binding of the first strontium ion is highly favorable, and the ratio of [E] to ["E-Sr"] will be very low. If the concentration of "E-Sr" is much greater than the concentration of E, then most of the enzyme that is unreactive to ATP must bind only one strontium ion to become reactive to ATP, and this is reflected in the Hill slope of \( n_{Hill} \sim 1.0 \). The Hill plot shown in Fig. 4B is most accurate near the half-maximal concentration of \( K_{Sr^2+} = 83 \mu M \). Values outside the range \(-1 < \log([E]/(E_{max} - E_P)) < 1\) have large errors associated with them. The values of \( K_{Sr^2+} = 35 \mu M \) and \( K_{Sr^2+} = 55 \mu M \) were calculated to result in a Hill slope of \( n_{Hill} = 1.3 \) at the half-maximal concentration of 83 mM Sr^2+ in the presence of 2.5 mM inorganic phosphate from Equation 8 and the equilibrium constants in Table I.

A value of \( E_P = 1.9 \) nmol/mg was observed 8 ms after the addition of 0.4 mM [γ-32P]ATP and 15 mM EGTA to enzyme that had been incubated in the presence of 50 μM Ca^2+ (data not shown). The level of phosphoenzyme observed under these conditions is known to be representative of 70% phosphorylation of the enzyme (39). This fraction of phosphorylation is the result of kinetic partitioning of "E-ATP-Ca^2+" between the dissociation of Ca^2+, with \( k_{Ca} = 80 \) s⁻¹, and the formation of phosphoenzyme, with \( k_p = 220 \) s⁻¹, as shown in Equation 9 (39).

\[
\frac{E_P}{E_{total}} = \frac{k_p}{k_p - k_{Ca} - k_p} \left( e^{-k_p t_{max}} - e^{-k_p t} \right)
\]  (9)

The total concentration of active sites was calculated from this ratio and the observed level of phosphoenzyme: \( E_{total} = (1.9 \) nmol/mg)/0.6 = 3.4 nmol/mg. This value of \( E_{total} \) is within the range of values that were usually observed for these preparations of enzyme, \( E_{total} = 2-4 \) nmol/mg.

A value of \( E_P = 1.5 ± 0.2 \) nmol/mg was observed after the addition of 0.4 mM [γ-32P]ATP and 15 mM EGTA to enzyme that had been incubated in the presence of a saturating concentration of strontium (Fig. 4A). The lower concentration of phosphoenzyme observed with strontium, 1.5 nmol/mg, compared with calcium, 1.9 nmol/mg, can be explained by the faster dissociation of strontium from "E-ATP-Sr^2+" with \( k_{Sr^2+} = 80-180 \) s⁻¹, and the faster hydrolysis of phosphoenzyme formed by the addition of strontium and ATP to vesicles made leaky with ionophore, \( k_{ion} = 23-33 \) s⁻¹ (15). A value of \( E_P/E_{total} = 0.44 \) was calculated from the value of \( E_P = 1.5 \) nmol/mg observed in the presence of a saturating concentration of strontium and the value of \( E_{total} = 1.3 \) nmol/mg, which was determined as described above. This value agrees with the theoretical value of \( E_P/E_{total} = 0.44-0.59 \), which was calculated from Equation 10 and values of \( k_p = 220 \) s⁻¹, \( k_{Sr^2+} = 80-180 \) s⁻¹, and \( k_{ion} = 23-33 \) s⁻¹ (15).

Fig. 5 shows the difference in the dependence on strontium concentration for inhibition of phosphoenzyme formation from inorganic phosphate (○) and for activation of the enzyme for phosphorylation by ATP (△). The open circles show that 50% of the enzyme is unreactive to inorganic phosphate in the presence of 28 μM Sr^2+. This inhibition results from the binding of at least one strontium ion to the cytoplasmic transport sites of 50% of the enzyme at this concentration of Sr^2+. The open squares show that after incubation in the presence of 28 μM Sr^2+ for several seconds, only 15% of the enzyme is activated for phosphorylation by ATP. The observed value of \( K_{Sr^2+} = 83 \mu M \) for activation of the enzyme for phosphorylation by ATP is 3-fold higher than the value of
Calcium ions bound reacts with ATP, whereas the free enzyme transports sites with positive cooperativity. Enzyme with two ATP (42-46). The chemical specificity of the enzyme is al-reacts with inorganic phosphate. However, a third form of the enzyme by inorganic phosphate. The binding of the second strontium ion causes a higher affinity than the binding of the first strontium ion occurs with higher affinity than the binding of the second strontium ion.

The solid lines are drawn for the sequential binding of two strontium ions, as shown in Scheme 6, with negative cooperativity, $n_t \approx 1.3$, and values of $K_{Sr1} = 35 \mu M$ and $K_{Sr2} = 55 \mu M$ (Equations 1 and 6). The value of $K_{Sr1} = 28 \mu M$ Sr$^{2+}$ for the inhibition of the reaction with inorganic phosphate and the value of $K_{Sr1} = 83 \mu M$ Sr$^{2+}$ for activation of the reaction with ATP indicate that the binding of the first strontium ion occurs with higher affinity than the binding of the second strontium ion.

Fig. 5 demonstrates that the unphosphorylated enzyme has three distinct chemical reactivities. The area to the left of both curves represents enzyme with no bound strontium ions, which reacts with inorganic phosphate to form phosphoenzyme. The area between the two curves represents enzyme with one bound strontium ion, which does not react with either inorganic phosphate or ATP. The area to the right of both curves represents enzyme with two bound strontium ions, which reacts with ATP to form phosphoenzyme but does not react with inorganic phosphate. These results demonstrate that the binding of the first strontium ion causes a conformational change, which inhibits phosphorylation of the enzyme by inorganic phosphate. The binding of the second strontium ion causes a different conformational change, which activates the enzyme for phosphorylation by ATP.

**DISCUSSION**

Two Different Chemical Specificities for Phosphorylation Have Been Observed with Calcium, but Three Different Chemical Specificities for Phosphorylation Are Observed with Strontium—The sarcoplasmic reticulum calcium ATPase can undergo phosphorylation by either inorganic phosphate or ATP (42-46). The chemical specificity of the enzyme is altered by the binding of two calcium ions to the cytoplasmic transport sites with positive cooperativity. Enzyme with two calcium ions bound reacts with ATP, whereas the free enzyme reacts with inorganic phosphate. However, a third form of the enzyme, in addition to these two chemical reactivities, was observed in the presence of strontium. The novel form of the unphosphorylated enzyme has one strontium ion bound to the cytoplasmic transport sites, ‘E-Sr, and is unreactive to both ATP and inorganic phosphate, as shown in Scheme 7. The species with one bound calcium ion, ‘E-Ca, which was generated transiently, is also known to be unreactive to ATP (9). However, it is not known if ‘E-Ca is able to react with inorganic phosphate because two calcium ions bind with positive cooperativity to the transport sites, as shown in Scheme 8, so that only a small fraction of the enzyme is present as ‘E-Ca at equilibrium. The maximum concentration of ‘E-Ca is calculated to be 21% of $E_{total}$ from Equation 11 and the values of $K_{Ca1} = 3.0 \mu M$ and $K_{Ca2} = 0.8 \mu M$.4

$$\frac{[E_M]}{[E_{total}]} = \frac{[E] + [E_M] + [E_{Mn}]}{[E]} = \frac{1}{K_{Pi}} + \frac{1}{K_{Pi} + 1} \frac{[P_i]}{[E]}$$

A much larger fraction of enzyme with one bound ion can be formed with strontium because strontium binds with little or no positive cooperativity. The maximum concentration of ‘E-Sr is predicted to be 39% of $E_{total}$ from Equation 11 and the values of $K_{Sr1} = 35 \mu M$ and $K_{Sr2} = 55 \mu M$. Therefore, strontium was used to determine the chemical reactivity of enzyme with one ion bound to the transport sites because a significant concentration of ‘E-Sr can exist at equilibrium.

The two forms of chemical specificity that were previously identified in the presence of calcium also exist in the presence of strontium: enzyme with no ions bound to the cytoplasmic transport sites, E, reacts with inorganic phosphate but does not react with ATP. Phosphorylation by 10 mM inorganic phosphate is inhibited by the binding of calcium to the cytoplasmic transport sites with $K_{Ca} = 1.8 \mu M$ (18), whereas phosphorylation by 2.5 mM inorganic phosphate is inhibited by a much higher concentration of strontium, with $K_{Sr1} = 28 \mu M$ (Fig. 3). Phosphorylation of the enzyme by ATP under these conditions requires that two ions are bound to the cytoplasmic transport sites. The species ‘E-Ca$^2$ and ‘E-Sr$^2$ are both phosphorylated by ATP but do not react with inorganic phosphate. The corrected value of $K_{Ca} = 2.0 \mu M$ Ca$^{2+}$ for the formation of ‘E-Ca$^2$ (9) is much smaller than the value of $K_{Sr1} = 83 \mu M$ Sr$^{2+}$ for the formation of ‘E-Sr$^2$ (Fig. 4). Although two strontium ions bind with much weaker affinity than calcium and with little or no cooperativity ($n_t$
The three different chemical specificities for reaction of the unphosphorylated enzyme in the presence of strontium (Scheme 7) are not consistent with the E1-E2 model. According to the E1-E2 model, the E1 conformation binds calcium on the cytoplasmic side of the membrane and reacts with ATP but not with inorganic phosphate, whereas the E2 conformation binds calcium on the luminal side of the membrane and reacts with inorganic phosphate but not with ATP. However, there are three conformations of the enzyme in the presence of strontium, as shown in Fig. 5: the area to the left of both curves represents free enzyme, which reacts with inorganic phosphate but not with ATP; the area to the right of both curves represents enzyme with two strontium ions bound, which reacts with ATP but not with inorganic phosphate; the area between the two curves represents enzyme with one strontium ion bound, which cannot react with either ATP or inorganic phosphate. The value of 28 \( \mu M \) Sr\(^{2+}\) for 50% inhibition of phosphorylation by inorganic phosphate is much lower than the value of 83 \( \mu M \) Sr\(^{2+}\) for 50% activation of phosphorylation by ATP (Fig. 5). The E1-E2 model predicts that the half-maximal concentrations of strontium for these two processes should be identical because there are only two conformations, E1 and E2. The changes in chemical reactivities that are caused by strontium are not consistent with the E1-E2 model and require the presence of a third species of unphosphorylated enzyme, which is not able to be phosphorylated by either ATP or inorganic phosphate. This species is formed when one strontium ion is bound to the cytoplasmic transport sites. Therefore, the binding of strontium to the cytoplasmic transport sites changes the chemical specificities of the enzyme in a stepwise mechanism, which agrees with the proposal that a conformational transition may be associated with nearly every step in the catalytic cycle (48). Several other predictions of the E1-E2 model are not consistent with experimental results (18, 27, 48, 49).

**Calcium and Strontium Bind to the Cytoplasmic Transport Sites with Different Degrees of Cooperativity**—Two calcium ions bind to the cytoplasmic transport sites with positive cooperativity: Hill slopes of \( n_H = 1.6-2.0 \) have been measured for the binding of calcium to the cytoplasmic transport sites at equilibrium using a variety of methods including direct binding assays, spectroscopy, and changes in chemical specificities (6, 7, 9, 19). The Hill slopes measured for the binding of strontium to the cytoplasmic transport sites at equilibrium are lower than those for the binding of calcium. Values of \( n_H = 1.0-1.3 \) for the change in chemical specificities caused by strontium (Figs. 3B and 4B) agree with the value of \( n_H = 0.93 \) for the change in the fluorescence of ANS\(^*\) bound to the enzyme in the presence of strontium (16). A value of \( n_H = 1.64 \) was reported for the change in the intrinsic fluorescence of the enzyme caused by strontium, which is slightly lower than the value of \( n_H = 1.83 \) observed with calcium (13). However, the Hill plot with strontium was curved, and a value of \( n_H = 0.8 \) was estimated at high concentrations of strontium, \( \log[AF/(\Delta F_0 - \Delta F)] > 0.2 \), whereas less curvature was observed with calcium. This type of downward curvature in Hill plots is predicted by Equation A.13 (Appendix) for the sequential binding of two ions if \( K_i \approx K_e \). Values of \( K_{o1} = 55 \mu M \) and \( K_{o2} = 55 \mu M \) were calculated from the values of \( K_{o1} = 28 \mu M \) Sr\(^{2+}\) for inhibition of phosphorylase formation from inorganic phosphate and \( K_{o2} = 83 \mu M \) for activation of phosphorylase formation from ATP and indicate that two strontium ions bind with negative cooperativity.

Although negative cooperativity is observed with strontium, the properties of the outer cytoplasmic transport sites appear to be identical for both \( 'E-Sr \) and \( 'E-Ca \): the association constants calculated from Table I for the binding of Sr\(^{2+}\) to \( 'E-Sr \) and \( 'E-Ca \) are not significantly different, 1.8 \( \times 10^4 \) M\(^{-1}\) and 2.2 \( \times 10^4 \) M\(^{-1}\), respectively. The conformational change caused by the binding of one calcium ion is known to be critical for the positive cooperativity that is observed for the binding of two calcium ions to the cytoplasmic transport sites (6, 7, 19, 27, 50, 51). Therefore, the E to \( 'E \) conformational change, which can be induced by the binding of either one calcium ion or one strontium ion, specifically increases the affinity of the enzyme for calcium but has little effect on the affinity of the enzyme for strontium.

The negative cooperativity that is observed with strontium cannot easily be explained by electrostatic repulsion. The unfavorable electrostatic interaction between two ions bound to the cytoplasmic transport sites is expected to be similar with strontium and with calcium: they are both divalent cations, and the distance between the two binding sites has been estimated to be \(-10\) \( \AA \), which is much larger than the radius of either ion (52-56). Therefore, any electrostatic repulsion that is produced upon the binding of two strontium ions to the cytoplasmic transport sites should also be observed in the binding of two calcium ions. The negative cooperativity that is observed with strontium is more likely to arise from either the steric requirements of the outer transport sites or the ability of calcium, which has a higher charge density, to interact more strongly with ligands at this site.

**Strontium and Calcium Ions Bind Sequentially to the Cytoplasmic Transport Sites and Form Two Different Mixed Complexes, \( 'E-Sr.Ca \) and \( 'E-Ca-Sr \):** The mixed species, \( 'E-Sr.Ca \), has strontium bound to the inner site and calcium bound to the outer site and is formed by the addition of Ca\(^{2+}\) to \( 'E-Sr \). The strontium ion bound to the outer site is rapidly replaced by external Ca\(^{2+}\), whereas the strontium ion bound to the inner site dissociates much more slowly in the presence of 190 \( \mu M \) Ca\(^{2+}\). Strontium bound to the inner site cannot dissociate if calcium is bound to the outer site. The other mixed species, \( 'E-Ca-Sr \), represents enzyme with calcium bound to the inner site and strontium bound to the outer site and was formed by the addition of Sr\(^{2+}\) to \( 'E-Ca \). The calcium ion bound to the outer site is rapidly replaced by strontium, whereas the calcium ion bound to the inner site dissociates much more slowly in the presence of 300 \( \mu M \) Sr\(^{2+}\). Calcium bound to the inner site cannot dissociate if strontium is bound to the outer site. Therefore, strontium can bind to both the inner and outer transport sites. The sequential binding of one strontium ion and one calcium ion to the cytoplasmic transport sites results in the formation of two different mixed species, \( 'E-Sr.Ca \) and \( 'E-Ca-Sr \); these forms of enzyme react rapidly with ATP.

The trivalent cations of the lanthanide series have also been used to probe the cytoplasmic transport sites of the calcium ATPase. Recently, Inesi and co-workers observed biphasic dissociation of calcium from \( 'E-Ca \), in the presence of \( Pr^{3+} \), with rapid dissociation of 50% of the bound calcium, followed by slow dissociation of the remaining calcium; they suggested that the lanthanide ion was binding to the outer transport site of \( 'E-Ca \) to form the mixed complex, \( 'E-Ca-Pr \) (57). However, Shigekawa and co-workers (58) observed only a small burst, \(-10-20\%\), of calcium dissociation in the presence of \( Gd^{3+} \), with the majority of the calcium dissociating with monophase kinetics; they concluded that lanthanides do not inhibit the dissociation of calcium by binding to the
outer transport site of \( ^{3}E\cdot Ca \) under these conditions (58).

Instead, they concluded that lanthanide ions inhibit the dissociation of both the inner and the outer calcium ions of \( ^{3}E\cdot Ca \) by binding to a site that is distinct from the cytoplasmic transport sites. The biphasic dissociation of calcium observed in the presence of contaminating calcium in the micromolar range, which would inhibit the dissociation of only the inner calcium ion (9, 19–23). Therefore, the mixed species of enzyme, \( ^{3}E\cdot Ca\cdot Pr \), may not involve the binding of \( Pr^{3+} \) to a transport site.

It has also been reported recently that the binding of lanthanum activates the enzyme for phosphorylation by ATP (57). Inesi and co-workers observed that incubation of the enzyme with \( 40 \mu M LaCl_{3}, 20 \mu M CaCl_{2}, 10 \mu M MgCl_{2}, 80 \mu M KCl \), and \( 50 \mu M [\gamma-^{32}P]ATP \) at pH 6.8 for several seconds results in phosphorylation of the enzyme. They observed that this concentration of lanthanum inhibits the binding of \( Ca^{2+} \) to the enzyme at equilibrium under these conditions. These data suggested that the binding of lanthanum to the cytoplasmic transport sites activates the enzyme for phosphorylation by ATP. However, in the presence of ATP, the binding of calcium to the transport sites is not at equilibrium because ATP increases the concentration of bound calcium by phosphorylating the enzyme. In fact, LaATP is particularly good for trapping calcium at the transport sites because the binding of lanthanum to the catalytic site for phosphoryl transfer greatly decreases the rate of calcium dissociation from both the cytoplasmic transport sites of the unphosphorylated enzyme and the luminal transport sites of the phosphorylated enzyme (29). The effectiveness of lanthanum as a competitor of calcium for binding to the cytoplasmic transport sites is different in the presence and absence of ATP. The results observed by Inesi and co-workers are consistent with the conclusion that the binding of lanthanide ions to the cytoplasmic transport sites inhibits the formation of phosophoenzyme (28, 58). Therefore, although lanthanide ions can be used to probe the specificity of the cytoplasmic transport sites for binding, the inability of lanthanide ions to change the chemical specificity of the enzyme for phosphorylation by ATP (28, 58) limits their usefulness as probes of the cytoplasmic transport sites for activation of catalysis.

In contrast, strontium may be used to determine the specificity of the cytoplasmic transport sites both for binding and for activation of catalysis. Strontium binds to both the inner and outer cytoplasmic transport sites, and the binding of strontium and calcium to these sites is sequential. The binding of two strontium ions or two calcium ions causes the same changes in chemical reactivity. Strontium does not bind with positive cooperativity and was used to determine the chemical specificity of the enzyme with one ion bound to the cytoplasmic transport sites. The enzyme species with one bound strontium ion can be formed at equilibrium and has a novel chemical specificity: \( ^{3}E\cdot Sr \) does not react with either ATP or inorganic phosphate.

**APPENDIX: DERIVATION OF EQUATIONS**

The Dependence of \( ^{3}E\cdot Ca \) Concentration on \( [Ca^{2+}] \)—Two calcium ions bind to the cytoplasmic transport sites in a sequential mechanism as shown in Scheme A (9, 19, 21). Two calcium ions must be bound to the transport sites to activate the enzyme for phosphorylation by ATP in the presence of 100 mM KCl and 40 mM MOPS, pH 7.0, at 25 °C (9). The fraction of enzyme that is activated for phosphorylation by ATP is described as \( \alpha \). Equation A.3 describes the dependence of \( \alpha \) on \( [Ca^{2+}] \) and is derived below. The equations below are also valid for the formation of \( ^{3}E\cdot Ca_{4} \) from \( E \), which includes a conformational change that increases the affinity of the enzyme for calcium.

\[
K_{1} = \frac{[Ca][E]}{[Ca][E]_{Ca}} \quad (A.1)
\]

\[
K_{2} = \frac{[Ca][E]}{[Ca][E]_{Ca}} \quad (A.2)
\]

\[
\alpha = \frac{[E]_{Ca}}{[E]_{total}} = \frac{[E]_{Ca}}{[E] + [E]_{Ca} + [E]_{Ca}} = \frac{1}{1 + \frac{[Ca]}{K_{Ca}} + \frac{[Ca]}{K_{Ca}} + \frac{[Ca]}{K_{Ca}} + \frac{[Ca]}{K_{Ca}}} \quad (A.3)
\]

Substitute \( K_{Ca}/[Ca] = \frac{1}{2} = \frac{K_{Ca}}{[Ca]} + \frac{K_{Ca}}{[Ca]} + \frac{K_{Ca}}{[Ca]} + \frac{K_{Ca}}{[Ca]} \)

\[
\alpha = \frac{1}{2} \frac{(K_{Ca})^{2}}{K_{Ca} + K_{Ca} + K_{Ca} + (K_{Ca})^{2}} \quad (A.4)
\]

Solve for \( K_{Ca} \) by using the quadratic equation.

\[
K_{Ca} = \frac{K_{Ca} + \sqrt{(K_{Ca})^{2} + 4 K_{Ca} K_{Ca}}}{2} \quad (A.5)
\]

In the case of infinite positive cooperativity (\( K_{1} \gg K_{2} \)) Equation A.5 simplifies to the following.

\[
K_{Ca} = K_{Ca} \quad (A.6)
\]

In the case of infinite negative cooperativity (\( K_{2} \gg K_{1} \)) Equation A.5 simplifies to the following.

\[
K_{Ca} = K_{Ca} \quad (A.7)
\]

Petithory and Jencks (9, 27) calculated a value of \( K_{1} = 9 \mu M \) from Equation A.8 and the observed values of \( K_{Ca} = 3.4 \mu M Ca^{2+} \) for the formation of \( ^{3}E\cdot Ca_{4} \) and \( K_{2} = 1.4 \mu M \) for the dissociation of the outer calcium ion.

\[
K_{1} = \frac{(K_{Ca})^{2}}{K_{Ca}} \quad (A.8)
\]

Equation A.8 is an algebraic rearrangement of Equation A.6 and is only valid in the limiting case of infinite positive cooperativity, \( K_{1} \gg K_{2} \). The general solution for \( K_{1} \) is obtained by rearranging Equation A.5.

\[
K_{1} = \frac{(K_{Ca})^{2}}{K_{Ca}} - K_{Ca} \quad (A.9)
\]

By using the general solution for \( K_{1} \), Equation A.9, and the values of \( K_{Ca} = 3.4 \mu M \) and \( K_{Ca} = 1.4 \mu M \) (9, 27), a smaller value of \( K_{1} = 4.9 \mu M \) was calculated.
Binding of Strontium to the SR Calcium-ATPase

$K_{a3} = 3.4 \, \mu M$, reported by Petithory and Jencks (29, 36). A value of $K_0 = 0.3 \, \mu M$ was calculated from $K_0 = 1.4 \, \mu M$ and the apparent dissociation constant of $3.9 \times 10^{-5} \, M$ (27, 32). A value of $K_i = 3.0 \, \mu M$ was calculated from Equation A.9 for $K_{a0} = 2.0 \, \mu M$ and $K_0 = 0.8 \, \mu M$.

The Hill slope, $n_H$, is calculated from Equation A.10.

$$n_H = \frac{d \log \left( \frac{[E_{Ca}]}{[E_{Ca}] - [E_{Ca}]} \right)}{d \log [Ca]} = \frac{d \log \left( \frac{1}{1 - \alpha} \right)}{d \log [Ca]} \quad (A.10)$$

Substitute from Equation A.3.

$$n_H = 2 - \frac{[Ca]}{[Ca] + K_i} \quad (A.13)$$

Substitute from Equation A.1.

$$n_H = 2 - \frac{[E_{Ca}]}{[E_{Ca}] + K_i} \quad (A.14)$$

The Hill slope varies between values of $2 \leq n_H \leq 1$. At low concentrations of calcium, when $[Ca^{2+}] << K_0$, the Hill slope is $n_H \sim 2.0$. At high concentrations of calcium, when $[Ca^{2+}] >> K_0$, the Hill slope is $n_H \sim 1.0$. This variation in the value of the Hill slope results from the change in the ratio of $[E]$ and $[E_{Ca}]$. These two forms of enzyme, $E$ and $E_{Ca}$, do not react with ATP. If the concentration of $E$ is much greater than the concentration of $E_{Ca}$, then most of the enzyme that is unreactive toward ATP must bind two calcium ions to become reactive to ATP. The Hill slope of $n_H \sim 2.0$ is an indication that two calcium ions are binding. If the concentration of $E_{Ca}$ is much greater than the concentration of $E$, then most of the enzyme that is unreactive toward ATP must bind one calcium ion to become reactive to ATP. The Hill slope of $n_H \sim 1.0$ is then an indication that only one calcium ion must bind to the predominant species of enzyme that is unreactive to ATP.

A value of $n_H = 1.6$ for the formation of $E_{Ca}$ at the half-maximal concentration of $K_{a0} = 2.0 \, \mu M$ was calculated from the corrected value of $K_i = 3 \, \mu M$. This value is close to the value of $n_H = 1.9$ that was reported by Petithory and Jencks (9).

Inhibition of EP Formation from Inorganic Phosphate by Strontium—Scheme 4 under “Results” shows a model in which the binding of strontium is competitive with the binding of inorganic phosphate. The strontium binds in an ordered sequential mechanism.

$$K_{int} = \frac{[EP]}{[E \cdot P]} \quad (A.15)$$

The value of $Y$ is defined as the amount of phosphoenzyme formed divided by the maximal amount of phosphoenzyme, $EP/EP_{max}$ or $\beta/\beta_{max}$.

$$Y = \frac{\beta}{\beta_{max}} \quad (A.22)$$

The concentration of strontium that causes half-maximal inhibition of phosphoenzyme formation from inorganic phosphate is calculated by substituting $Y = 1/2$ and $[Sr] = K_{S0}$.

$$K_{S0} = \frac{1}{2} \left( \frac{1}{\frac{K_{int}}{K_o} + \frac{[P]}{K_o} + 1} \right) \quad (A.23)$$

Solve for $K_{S0}$ by rearranging and the quadratic equation.

$$K_{S0} = \frac{K_{int}}{2} \left( \sqrt{1 + \frac{4}{K_{S0}} \frac{K_{int} [P]}{K_p} + \frac{[P]}{K_o} + 1} - 1 \right) \quad (A.24)$$

The Hill slope, $n_H$, is calculated from Equations A.25 and A.26.

$$n_H = \frac{d \log \left( \frac{[EP_{max}] - [EP]}{[EP]} \right)}{d \log \left( \frac{\beta_{max} - \beta}{\beta} \right)} \quad (A.25)$$

$$\frac{\beta_{max} - \beta}{\beta} = \left( \frac{K_p}{K_{int} [P] + [P] + K_p} \frac{[Sr]}{K_{S0}} + 1 \right) \quad (A.26)$$
Substitute from Equation A.26 into Equation A.25 and differentiate.

\[ n_{H} = 1 + \frac{[\text{Sr}]}{[\text{Sr}]} + K_{S2} \]  
\[ (A.27) \]

Substitute \([E_{S1}]/[E_{S2}]\) for \(K_{S2}/[\text{Sr}]\) and rearrange.

\[ n_{H} = 1 + \frac{[E_{S1}]}{[E_{S1}] + [E_{S2}]} \]  
\[ (A.28) \]

Activation of Phosphoenzyme Formation from ATP by Strontium in the Presence of Inorganic Phosphate.—Scheme 6 under “Results” shows a model in which the binding of two strontium ions activates the enzyme for reaction with ATP; this binding occurs by a sequential mechanism and is competitive with the binding of inorganic phosphate. The fraction of enzyme that has two strontium ions bound, \(E_{S2}/E_{total}\), is defined as \(\alpha\). The value of \(\alpha\) is equivalent to the fraction of the maximal amount of enzyme that is phosphorylated by ATP in the experiment shown in Fig. 4, A and B.

\[ \alpha = \frac{[E_{S1}]}{[E_{S1}] + [E_{S2}]} \]  
\[ (A.29) \]

\[ \frac{1}{\alpha} = \frac{[E_{S2}] + [E_{S1}]}{[E_{S1}] + [E_{S2}]} \]  
\[ (A.30) \]

Substitute values from A.15.

\[ \frac{1}{\alpha} = \frac{K'_{S1}[P]K_{S2}K_{S2}}{K_{p}[S]} + \frac{K'_{S1}[P]K_{S2}K_{S2}}{K_{p}[S]} + \frac{K_{S2}K_{S2}}{[S]} + \frac{K_{S2}K_{S2}}{[S]} + \frac{K_{S2}K_{S2}}{[S]} + \frac{K_{S2}K_{S2}}{[S]} + 1 \]  
\[ (A.31) \]

To calculate the concentration of strontium at which 50% of the enzyme is \(E_{S2}\), substitute \(\alpha = 1/2\) and \([S] = K_{S2}v.\)

\[ \frac{1}{2} = \frac{(K_{S2})^{2}}{[S]^{2}} + 2K_{S2}[S] + 2K_{S2}[S] + \frac{K'_{S1}[P]K_{S2}K_{S2}}{K_{p}[S]} + \frac{K'_{S1}[P][P]}{K_{p}^{2}} + 1 \]  
\[ (A.32) \]

Solve for \(K_{S2}\) by rearranging and the quadratic equation.

\[ K_{S2} = \frac{1}{2} \left( 1 + \sqrt{1 + 4 \frac{K_{S2}}{K_{p}} + \frac{K'_{S1}[P][P]}{K_{p}^{2}} + 1} \right) \]  
\[ (A.34) \]

The Hill slope, \(n_{H}\), for the formation of \(E_{S2}\) is calculated from the following.

\[ n_{H} = \frac{d \log ([E_{S1}] - [E_{S2}])}{d \log [Sr]} = \frac{d \log \left( \frac{1}{1 - \alpha} \right)}{d \log [Sr]} \]  
\[ (A.35) \]

\[ \frac{1}{1 - \alpha} = \frac{[Sr]}{K_{S}} + \frac{K_{S2}K_{S2}}{K_{p}} + \frac{K'_{S1}[P][P]}{K_{p}^{2}} + 1 \]  
\[ (A.36) \]