The transient steps induced by calcium binding to sarcoplasmic reticulum vesicles were concomitantly studied by stopped flow fluorometry and chemical quenching methods. The rate constant \( k_{\text{obs}} \) for the transition between the forms of ATPase with low and high intrinsic fluorescence ("on" rate) was found to be \( 1.3 \pm 0.4 \text{ s}^{-1} \) at \( \text{pCa} 4.2 \), pH 6, 20 °C, in the absence of KCl. A complete \( k_{\text{obs}} \) versus \( \text{pCa} \) curve was also obtained; magnesium concentrations from 0 to 20 mM had little effect on these \( k_{\text{obs}} \) values. We designed a separate experimental setup allowing the study of chemical quenching methods of the transition between the two unphosphorylated forms of ATPase capable of reacting with P, and ATP, respectively. In the absence of any possible activation by nucleotides, the observed rate constant for this transition was found to be 1.5 s\(^{-1}\), a value consistent with the one derived from fluorometry.

Addition of calcium to enzyme phosphorylated with P, reduced the rate constant for sarcoplasmic reticulum ATPase transformation and subsequent phosphorylation by ATP \( (k_{\text{obs}} \approx 0.8 \text{ s}^{-1} \text{ at } 5 \text{ mM Mg}^{2+} \text{ and } 20 \text{ mM phosphate}) \). Under these conditions, the rate constant for fluorescence changes was also reduced. When the phosphoenzyme level rose under the initial conditions, the observed rate constant fell. In addition, detailed examination of the traces recorded indicated the presence of a second faster exponential of small amplitude. Different initial experimental conditions, in which calcium and phosphate were added simultaneously to the \( \text{Ca}^{2+}\)-deprived enzyme, made this biphasic behavior more clearly evident.

All these results are discussed in terms of the calcium binding mechanism, the acceleration by ATP of the \( \text{Ca}^{2+} \)-binding-induced transition, and the competition between the calcium-induced transition and the calcium-independent phosphorylation by P.

Calcium is transported across sarcoplasmic reticulum membranes by a calcium-dependent ATPase. The hydrolytic cycle can be described by the following minimal scheme:

\[
\text{Ca}_{\text{obs}} \xrightarrow{k_{\text{obs}}} E \xrightarrow{k_{\text{on}}} \text{ATP} \xrightarrow{k_{\text{off}}} E \xrightarrow{k_{\text{on}}} \text{ATP} \xrightarrow{k_{\text{off}}} \text{Pi} \xrightarrow{k_{\text{on}}} \text{Pi} \xrightarrow{k_{\text{off}}} \text{Pi} \]

\[ \text{Scheme I} \]

In this scheme, *E and E are the P- and ATP-reactive conformations of the unphosphorylated enzyme, and \( *E-P \) and \( E \sim P \) are the corresponding phosphorylated forms. Although the detailed events associated with calcium binding are not known, it has been suggested that under certain experimental conditions, the calcium binding-induced transition \( (*E \rightarrow E) \) may become the rate-limiting step in the \( \text{Ca}^{2+}\)-pump reaction sequence (for review, see de Meis and Vianna, 1979).

This transition was first studied by rapid quenching techniques by measuring the amount of phosphoenzyme appearing after the addition of both \( \text{Ca}^{2+} \) and ATP to a \( \text{Ca}^{2+}\)-deprived enzyme (Takisawa and Tomonura, 1978; Rauch et al., 1978; Sumida et al., 1978; Vieyra et al., 1979; Scofano et al., 1979). However, the available rapid quenching data only allowed estimation of the \( *E \rightarrow \text{ATP} \) transition rate in the presence of ATP which, unfortunately, is supposed to activate this transition. As regards P-deriv phosphoenzyme \( (*E-P) \), its slow disappearance after addition of calcium provided a much lower estimation of the rate constant of the \( *E \rightarrow \text{ATP} \) reaction (de Meis and Tume, 1977; Rauch et al., 1978; Chaloub et al., 1979).

It recently was shown that both the \( *E \Rightarrow E \) and the \( *E \Rightarrow *E-P \) transitions could be monitored separately by stopped flow fluorometry (Dupont and Leigh, 1978; Guillain et al., 1980; Lacapère et al., 1981). Due to different experimental conditions, the fluorescence-derived rate constant for the "on" transition could not, however, be directly compared to that available from chemical quenching experiments (Guillain et al., 1980). Furthermore, the significance of fluorescence measurements was questioned (Nakamura et al., 1979).

For the purposes of the present work, we therefore designed an experimental setup allowing the study by chemical quenching methods of the \( *E \rightarrow E \) transition in the absence of ATP, and compared the results with direct fluorescence measurement.
ments performed under exactly the same conditions. Both methods gave the same rate constant for the transition, namely \(1.3 \pm 0.4 \, \text{s}^{-1}\) at pH 6 and in the absence of KCl (20 °C), and the results allowed examination of the calcium binding mechanism. We also performed both stopped flow and rapid quenching experiments in the presence of phosphate, and related our findings to previous measurements of the rate of transition between the \(P_i\)-derived phosphoenzyme (*E-P) and the \(E\) form. Under certain conditions, competition between the calcium-induced transition and the calcium-independent phosphorylation by \(P_i\) in fact resulted in a complex situation in which fluorescence proved a valuable tool to monitor biphasic evolutions.

**Experimental Procedures**

Fragmented sarcoplasmic reticulum was prepared and tested as already described (Champney et al., 1978). Fluorescence equilibrium and stopped flow measurements were performed as described by Guillain et al. (1980), except that the excitation wavelength for stopped flow measurements was 275 nm, because when excited at 290 nm, the Corning 0.54 cutoff filter itself emitted some light at 330 nm, Inorganic phosphate was added as sodium phosphate salt.

For calculation of the free Mg\(^{2+}\) and \(P_i\) concentrations in our media, the following apparent association constants (pH 6) were used: [Mg\(^{2+}\)]/[Mg\(^{2+}\)\(\cdot\)P\(_i\)] = 12.4 \, \text{M}^{-1} and [Mg\(_{\text{EGTA}}\)]/[Mg\(_{\text{EGTA}}\)\(\cdot\)EGTA] = 3.2 \, \text{M}^{-1}, where free \(P_i\) was the sum of the phosphate species not complexed with Mg\(^{2+}\), i.e. essentially \(\text{H}_2\text{PO}_4^-\) (84%) and \(\text{HPO}_4^{2-}\) (16%) (see Fabiato and Fabiato, 1979; Guillain et al., 1980). Unless otherwise specified, the medium contained 150 mM Mes-Tris, pH 6 (20 °C), and no KCl.

Rapid mixing and quenching experiments were performed at 20 °C with a Durrum O-133 multimixing device, allowing electronically controlled aging procedures up to 10 s. Due to the rather long single reaction coil used, the minimum reaction time was 150 ms. Accuracy of time calibration was tested by monitoring alkaline hydrolysis of 2,4-dinitrophenyl acetate at a final NaOH concentration of 0.5 M (Barman and Gutfreund, 1979). ESR spectra were measured at 20 °C, and the excitation wavelength for rapid mixing and quenching experiments was 290 nm, because when excited at 290 nm, the Corning 0.54 cutoff filter itself emitted some light at 330 nm, Inorganic phosphate was added as sodium phosphate salt.

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values obtained here in the absence of KCl were systematically lower than those previously found in the presence of KCl (Gullain et al., 1980). Magnesium concentrations between 0 and 20 mM had little effect on the \( h_{obs} \) values measured. In agreement with our previous results at pH 6, the main feature of the curve in Fig. 2b is the continuous decline in \( h_{obs} \) as calcium concentrations increased from pCa 8 to pCa 5, i.e. for concentrations well below the pCa_{1/2} deduced from equilibrium measurements. For higher calcium concentrations, \( h_{obs} \) either remained constant or rose slightly. Starting from a Ca\(^{2+}\)-saturated enzyme, transition to the \( \cdot E \) form took place with a \( k_{obs} \) reaching as much as 4 s\(^{-1}\) ("off" rate), whereas the reverse calcium binding-induced transition for final pCa 4-2 occurred with a \( k_{obs} \) around 1.3 s\(^{-1}\) (on rate).

**Multimixing Experiments**—Initial studies by rapid quenching techniques of the \( \cdot E \rightarrow E \) on rate were based on measurements of the phosphorylation time course of SR ATPase after the addition of ATP (Takishawa and Tomomura, 1978; Rauch et al., 1978; Sumida et al., 1978). When the enzyme is in its Ca\(^{2+}\)-saturated (\( E \) form), phosphorylation is very rapid; when the enzyme is initially in its Ca\(^{2+}\)-deprived state (\( \cdot E \) form), phosphorylation after addition of both Ca\(^{2+}\) and ATP proceeds at a much more slower rate, presumably due to the limiting \( \cdot E \rightarrow E \) transition rate. Under our conditions, the mixing of SR ATPase in its \( E \) form with both Ca\(^{2+}\) and ATP at final concentrations of 100 and 5 \( \mu \)M, respectively, led to phosphorylation with a \( k_{obs} \) value around 7 s\(^{-1}\) (Fig. 3).

Besides phosphorylation of the \( E \) form, however, ATP is believed to accelerate the \( \cdot E \rightarrow E \) transition (Vieyra et al., 1978; Scofano et al., 1979). Consequently, for an enzyme initially in either the \( \cdot E \) or the \( E \) state, comparison of the phosphorylation time courses at a given ATP concentration does not reflect the true \( \cdot E \rightarrow E \) transition rate. In order to measure this rate, we designed an original experimental setup derived from the one previously used to measure the rate of the off reaction (Sumida et al., 1978; Rauch et al., 1978).

\[ \text{SR, EGTA} \]

\[
\begin{align*}
+\text{Ca} & \\
+\text{ATP} & \\
\text{Quenching} & \\
 t & \\
 t + \theta & \\
\text{Inset} &
\end{align*}
\]

The rationale for such a setup is the following: since only \( E \) and not \( \cdot E \) can be phosphorylated by ATP, the initial rate of phosphorylation at any time \( t \) during the \( \cdot E \rightarrow E \) transition depends solely on the amount of \( E \) present at the time when ATP is added.

\[
\left( \frac{dE}{dt} \right)_{(t=0)} = k_{ATP}[E].
\]

This initial phosphorylation rate and the appearance of \( E \) therefore have the same time course as a function of \( t \). In practice, we can only measure the phosphoenzyme level at a finite time interval \( (\theta) \) after ATP addition, i.e. we only measure \( E \sim P_{(t=\theta)} \). Fortunately however, it can be shown mathematically that the finite level \( E \sim P_{(t=\theta)} \) varies in the same way as the initial phosphorylation rate \( \left[ (dE/dt)_{(t=0)} \right. \). \( E \sim P_{(t=\theta)} \) as a function of \( t \) is therefore an index of the nonaccelerated \( \cdot E \rightarrow E \) transition.

The Durrum multimixer was used in a somewhat unconventional mode. As depicted in the Fig. 4 inset, SR vesicles were suspended in an EGTA medium and mixed with a calcium-containing solution (final pCa = 4). After various reaction times (from 150 ms to 10 s), ATP was added to a final concentration of 5 \( \mu \)M (pCa 4). Then 35 ms later, acid quenching took place within the collect syringe, whose entrance was narrowed to achieve good mixing, and which already contained 240 mM perchloric acid 15 mM P, and 0.5 mM ATP. Phosphoenzyme was measured by the filtration technique. For the minimum reaction time (150 ms, see "Experimental Procedures"), interaction between SR and calcium took place as the solution flowed continuously through the reaction zone between the first mixing jet and the second, i.e. between points A and B in Fig. 4 inset. For longer times of reaction between SR and calcium, the solutions in the reaction zone were allowed to age further for an electronically controlled time interval, before being mixed with the contents of

![Fig. 3. Phosphorylation time course of SR ATPase after addition of both ATP and calcium to the \( \cdot E \) form.](image)

![Fig. 4. Time course of the appearance of the ATP-reactive ATPase form (\( E \)) after addition of calcium.](image)
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The phosphoenzyme level for zero reaction time was obtained separately by mixing SR + EGTA (syringe 1) with EGTA (syringe 2), so that no reaction occurred after the first mixing jet (A); the Ca⁺⁺ concentration in the ATP-containing syringe 3 was such that the final pCa during phosphorylation was the same as before (pCa 4). Under all conditions, the phosphorylation reaction took place between mixing jet B and the entrance to the collect syringe (C), i.e. within a time (θ) which was separately deduced from parallel acid quench measurements of the di- and tetraethylammonium hydrolysis at a final concentration of 0.2 mM NaOH. This time was found to be 35 ms under our conditions. Note that the measured phosphoenzyme level for zero time in Fig. 4, i.e. after 35 ms incubation with ATP and Ca⁺⁺, fits reasonably well with the data in Fig. 3, which shows the phosphorylation time course for an enzyme initially in the *E state (see Δ in Fig. 3 at 35 ms).

The upper curve in Fig. 4 shows that after addition of calcium, SR ATPase becomes available for phosphorylation by ATP according to an exponential with kobs = 1.5 s⁻¹. Incidentally, Figs. 3 and 4 show that after 35 ms, phosphorylation of the E form is virtually complete (see E → P level at t = 10 s in Fig. 4, and maximum E → P level in Fig. 3). Compared to kobs = 1.5 s⁻¹ in Fig. 4, the phosphorylation time course in Fig. 3 (kobs ≈ 7 s⁻¹) shows that 5 μM ATP already slightly activates the E → P transition, a result which is not obvious from Scofano’s paper (1979). Our Fig. 3 is at variance with Scofano’s Fig. 3, both from the point of view of the initial phosphorylation rate and of phosphoenzyme stability (our phosphoenzyme level is only slightly reduced after 10 s of incubation; see Fig. 3 inset). We do not know the reason for these differences.

We already mentioned that the same kind of experimental setup with three mixing jets had been previously used to estimate the rate constant of the off transition induced by calcium removal. In addition to our original on measurements, we repeated these off experiments (not shown). Results were consistent with both Rauch’s data, obtained under conditions similar to ours, and with our own fluorescence results (kobs = 4 s⁻¹).

**E-P → E Transition**

The lower trace of Fig. 4 shows a chemical quenching experiment performed in the presence of 20 mM phosphate, i.e. with an enzyme initially in equilibrium between E → P (the P-derived gradient-independent phosphoenzyme species) and *E. After calcium addition under those conditions, the final level of enzyme phosphorylated within 35 ms was the same irrespective of the presence or absence of phosphate, whereas the initial level (time zero in Fig. 4) was lower in the presence of phosphate. In addition, the presence of phosphate also markedly slowed down the rate at which the ATP-reactive conformation appeared after the addition of calcium (kobs ≈ 0.8 s⁻¹).

In parallel stopped flow fluorescence measurements, SR vesicles (syringe 1) were suspended in 0.5 mM EGTA, 10 mM Mg⁺⁺, and 10 mM PO₄, i.e. under conditions where a significant proportion of the enzyme is phosphorylated by inorganic phosphate. Syringe 2 contained various amounts of calcium plus 10 mM EGTA and 10 mM PO₄. The fluorescence changes after mixing were found to be significantly slower than in the absence of phosphate and were analyzed as single exponentials. Resulting kobs values are plotted in Fig. 5 for final calcium concentrations between pCa 4 and pCa 2, giving kobs = 0.6-0.7 s⁻¹ (t₁/₂ ≈ 1 s). Incidentally, Fig. 5 shows that kobs determinations within a given set of experiments are much more precise than would be expected from Fig. 2b, in which we compiled data collected from different experiments and preparations.

The *E-P → E transition in the presence of phosphate was further investigated as a function of the Pᵢ concentration. Large amounts of calcium (final pCa = 4 or 3) were added at time zero to SR vesicles suspended in a medium containing 0.5 mM EGTA, 10 mM free magnesium, and various amounts of phosphate, in addition to our standard 150 mM Mes KCl-free medium (pH 6, 20 °C). When the intrinsic fluorescence rise was first analyzed as a single exponential, its rate constant was found to be a function of the phosphate concentration with an apparent affinity around 2 mM (Fig. 6). In the absence of phosphate, a kobs of 1.5-1.6 s⁻¹ was found, which under the present conditions is characteristic of the *E → E transition rate (see above).

The same rate constant (1.5 s⁻¹) was observed irrespective of the Pᵢ concentration when the experiment shown in Fig. 6 was repeated in the absence of magnesium, i.e. when calcium was added to an enzyme suspended, for instance, in a medium containing 40 mM Pᵢ, but no magnesium (not shown). These features, namely, a phosphate affinity of about 2 mM and magnesium dependence, suggest that the calcium-independent phosphoenzyme *E-P level at the beginning of the experiment is the crucial parameter for determining the observed rate constant. Competition between the Ca⁺⁺-induced transi-

**FIG. 5. Effect of phosphate on the observed rate constant of the calcium-induced fluorescence changes.** Various amounts of calcium were added to SR vesicles suspended in 10 mM MgCl₂ and 0.5 mM EGTA, either in the absence of phosphate (C) *E-P → E as in Fig. 2) or with 10 mM PO₄ present in both syringes (Δ, *E-P → E).

**FIG. 6. Phosphate dependence of the observed rate constant for fluorescence changes after calcium addition to SR ATPase.** Syringe 1, SR vesicles were suspended in a medium containing 0.5 mM EGTA, 10 mM free magnesium, and various amounts of phosphate. In addition to magnesium and phosphate, syringe 2 contained calcium, so that its free concentration after mixing was 10⁻⁴ M (▲) or 10⁻² M (●). The time course of the fluorescence rise was fitted to a single exponential whose rate constant (kobs) was plotted as a function of the free phosphate concentration.
tion and Ca\(^{2+}\)-independent phosphorylation therefore manifests itself by slowing down the rate of appearance of the E form.

Biphasic Nature of the *E-P* \(\rightarrow*E\) Transition—Fig. 7 shows the typical fluorescence records from which the Fig. 6 results were in fact obtained (pCa 3). The trace in Fig. 7a corresponds to 10 mM P\(_o\), and the fit to a single exponential is quite correct. The trace in Fig. 7b is the one for 2 mM P\(_o\); in this case, the fit to a single exponential clearly does not match the first experimental points, although the last ones are correctly simulated. Under these conditions, therefore, competition between the calcium-induced transition and calcium-independent phosphorylation also manifests itself by biphasic behavior, as shown in Fig. 7b.

We worked out experimental conditions emphasizing this biphasic behavior. Following our interpretation of this phenomenon (see "Discussion"), Mg and P\(_o\) concentrations were chosen so that the two anticipated resulting rate constants were in a convenient ratio, and the initial conditions were chosen so that the amplitudes of the resulting exponentials were similar. This led us to the experiment described in Fig. 8. Both Ca\(^{2+}\) and P\(_o\), at final free concentrations of 3 \(\times\) 10\(^{-4}\) M and 20 mM, respectively, were added to ATPase in its *E form, i.e. suspended in a medium containing 0.5 mM EGTA and 2.5 mM free magnesium. Obviously the signal rise could not be fitted to a single exponential. When the data for times longer than 500 ms were fitted to a single exponential, extrapolation to shorter times (Fig. 8, dashed line) clearly did not match the first experimental points. On the other hand, a correct fit (solid line) was obtained with two exponentials, with respective time constants and amplitudes of 1.1 s\(^{-1}\) and 80 nV, and of 8.6 s\(^{-1}\) and 50 mV. A more precise estimation of the initial phase was obtained by monitoring the fluorescence rise during the first 500 ms, which gave a rate constant of 6 s\(^{-1}\). With the same final Mg and P\(_o\) concentrations as those used in the experiment described in Fig. 8, an experiment similar to those reported in Fig. 7 was performed (not shown). For this purpose, calcium only was added to an enzyme in equilibrium between *E-P* and *E*, i.e. suspended in EGTA + Mg + Pi. Under these initial conditions, the resulting rate constants (0.96 s\(^{-1}\) and 5.2 s\(^{-1}\)) were found to be very close to those of the Fig. 8 experiment; whereas the slow component, as in Fig. 7, dominated the fluorescence evolution (respective amplitudes of 70 and 10 mV).

Ca\(^{2+}\)-induced Conformational Changes Differed from Spin Label Studies—As the cooperativity of the calcium-induced events will be discussed at the end of this paper, we wish to show here the results obtained in separate experiments using the spin label technique. Selective labeling of SR ATPase with a paramagnetic iodoacetamide analogue has been shown by Prof. Inesi's group to provide a method for detection of conformational changes in the calcium pump, mainly in the presence of substrate (e.g. ATP, ADP, or ATP analogues). We showed that even in the absence of substrate, calcium binding to the ATPase high affinity sites induced a small but definite spectral change in the protein-bound label (Champeil et al., 1976). However, measurements were not precise enough to allow any comment upon the possible cooperativity of the Ca\(^{2+}\)-induced effect. In later work, Inesi et al. only tested the Ca\(^{2+}\) concentration dependence of label spectrum in the presence of a substrate. These studies showed evidence for cooperativity (see for example Inesi et al., 1980, Figs. 4 and 5).

Measurement of the Ca\(^{2+}\)-induced spectral changes in the absence of substrate can, however, be precise enough to allow similar studies. This is shown in Fig. 9 (compare to Fig. 2 in Champeil et al., 1976), in which the label spectrum outer splitting 2T\(_1\) was plotted as a function of pCa for a spin labeled preparation suspended at 2 or 22 °C (pH 6). The calcium effect is more easily visible at the lower temperature. Like the fluorescence data in Fig. 1, the relatively steep curves in Fig. 9 suggest positive cooperativity of the calcium effect.

---

**Fig. 7.** Typical fluorescence recordings from which the results in Fig. 6 were obtained. a, P\(_o\) = 10 mM; b, P\(_o\) = 2 mM (pCa 3). Recorded signals were analyzed as a single exponential curve after correction for the photolysis drift. This drift was estimated from the last points, which were registered at a slower rate. The dashed line is the extrapolating to short times of the exponential. Compared to the 150-mV amplitude of the *E* \(\rightarrow*E\) transition (see Fig. 2a), the *E-P* \(\rightarrow*E\) transition in a and b displayed much smaller amplitudes (about 100 and 50 mV). This is of course because the *E-P* conformation itself has a higher level of fluorescence than the *E* form (Lacapere et al., 1981). Therefore, when, under the initial conditions, the phosphoenzyme level rises, the amplitude of the resulting transition falls.

**Fig. 8.** Typical stopped flow recording of the fluorescence changes after simultaneous addition of calcium and phosphate to Ca\(^{2+}\)-deprived SR vesicles. In syringe 1, SR vesicles were suspended in a medium containing 0.5 mM EGTA and 2.5 mM Mg. In addition to magnesium, syringe 2 contained calcium and phosphate, so that final calcium and phosphate concentrations after mixing were 3 \(\times\) 10\(^{-4}\) M and 20 mM, respectively. The dashed line is the extrapolation to short times of the best exponential fit to the last points.
transformation has been suggested to be the rate-limiting step in overall hydrolysis (de Meis and Vianna, 1979). It is worth mentioning, however, that under the conditions of this present work (20 °C, pH 6, no KCl) the dephosphorylation step (*E-P \rightarrow *E) was found to occur with a _k_{obs} around 1.5-2 s^{-1} (Lacapère et al., 1981), a value not so different from the *E \rightarrow E rate constant of 1.3 ± 0.4 s^{-1} determined here.

Competition between *E \equiv E and *E \equiv *E-P—Regarding the results obtained in the presence of phosphate, we found basically that its presence induced both an apparent slowing down of the rate of appearance of the E form and a biphasic system evolution.

Phosphorylation of the enzyme by P, has been shown to proceed through several random binding steps (Punzengruber et al., 1978; Lacapère et al., 1981).

\[ \text{Scheme IIa} \]

For given magnesium and phosphate concentrations, Scheme IIa can be reduced to a simple fictive scheme *E \equiv *E-P (Scheme IIb), where *E is the sum of all the noncovalent complexes. The rate constant of the *E \equiv *E-P transition is the observed constant measurable from the fluorescence evolution at the Mg and P, concentrations used in the experiment (Lacapère et al., 1981).

In the present work, we measured the rate of transition toward the E form, starting from *E, Mg, *E, or *E-P (respectively, without magnesium or phosphate, with magnesium but without phosphate, and with phosphate but without magnesium; see "Results"). The _k_{obs} values were all found to be around 1.3 ± 0.4 s^{-1}. Furthermore, we previously showed that under equilibrium conditions, the ternary complex was almost totally transformed into the covalent species (Lacapère et al., 1981). This leads us to assume that, at a first approximation, all the noncovalent species in Scheme IIa were transformed at the same rate into the E form after the addition of calcium. In Scheme IIb the rate constant for the transformation of *E into the E form is therefore the same.

Consequently, competition experiments like the ones in Figs. 6 and 7 can be described by the following reduced scheme, which simply includes the two transitions just described.

\[ \text{Scheme IIc} \]

Compared to the *E \rightarrow E transition, which is relatively slow (1.3 ± 0.4 s^{-1}), phosphorylation of the enzyme by P, is believed to be fast (Rauh et al., 1977; Chaloub et al., 1975; Lacapère et al., 1981). At a first approximation, the *E-P and *E forms can therefore be considered in quasi-equilibrium. This would make the evolution of such a system virtually monoexponential, with an observed rate constant that is a direct function of the *E/*E + *E-P ratio. This is consistent with the P, affinity observed in Fig. 6.

In Scheme IIc, calcium is shown to bind to the *E form and

\[ 1.3 ± 0.4 \text{ s}^{-1} \]
also hypothetically to the covalent phosphoenzyme. Starting from an enzyme mainly in its $E-P$ form (high phosphate and magnesium concentrations), the rate of appearance of the $E$ form after calcium addition should approach either zero or the hypothetical $h$ rate constant. Fig. 6 data do not allow clear discrimination between these two possibilities, but imply that if present, the $h$ transition would be much slower than the $*E \rightarrow E$ one.

Compared to Fig. 7a, the biphasic behavior found in Fig. 7b can be easily understood from Scheme IIc. For high phosphate concentrations, phosphorylation is fast enough to justify the quasi-equilibrium approximation in this scheme. For lower phosphate concentrations, on the other hand, the $*E \rightarrow E$ transition is only a little faster than the $*E \rightarrow E$ transition (Lacapere et al., 1981) and gives rise to a faster phase in the system's evolution. On the basis of this reasoning and of the phosphorylation data of Lacapere et al. (1981), we designed experimental conditions allowing good separation of these two phases (Fig. 8). The assumption that the fast phase is in fact correlated with the $*E \rightarrow E$ transition is supported by a recent very similar experiment by Guimarães-Motta and de Meis (1980). They showed that transient phosphorylation by $P_i$, as determined from radioactive measurements, can be observed when the enzyme is incubated in the absence of calcium and then added to a medium containing $P_i$ and an excess of calcium.

**Calcium Binding Mechanism**—The possible mechanism for calcium binding can be discussed on the basis of the dependence of both equilibrium and kinetic fluorescence on $pCa$ (Figs. 1 and 2b). At a first approximation, the often postulated Scheme IIIa (de Meis and Vianna, 1979) might explain the essentially decreasing $k_{obs}$ curve in Fig. 2b.

**Scheme IIIa**

According to this scheme, calcium binding only takes place after slow pump isomerization, allowing the transfer of calcium binding sites from a low affinity inward orientation to a high affinity outward orientation. However, such a simple scheme would not account for both the moderate ratio of $k_6$ to $k_{10}$ (4 s$^{-1}$/1.3 s$^{-1}$) and the large difference observed between the apparent affinities derived from the equilibrium ($pCa_{1/2} \approx 5.15$) and $k_{obs}$ ($pCa_{1/2} \approx 6.5$) data (Guillain et al., 1980). This discrepancy therefore leads us to assume that calcium binding takes place either before or after pump isomerization.

In our preceding paper (Guillain et al., 1980) fluorescence data obtained in the presence of KCl were in fact correctly fitted by a two-route mechanism for Ca$^{2+}$ binding.

\[
\begin{align*}
E + Ca & \xrightarrow{k^+} ECa \\
E & \xrightarrow{k^-} E + Ca \\
E & \xrightarrow{k} E + Ca
\end{align*}
\]

**Scheme IIIb**

However, a major problem in Scheme IIIb is that the equilibrium between the $P_i$-reactive and the ATP-reactive conformations would never be totally displaced toward one of these forms. For instance at high Ca, Scheme IIIb is reduced to the simpler one, $*ECa \xrightarrow{k^+} ECa$; if the data in Fig. 2b are fitted to Scheme IIIb, $k^+$ and $k^-$ are found to be, respectively, 0.9 s$^{-1}$ and 0.4 s$^{-1}$ ($k_{10} = k^+ + k^- = 1.3$ s$^{-1}$). With those constants, therefore, $0.4/(0.9 + 0.4) \approx 30\%$ of the enzyme would be $P_i$-reactive even at high calcium concentrations, which is obviously wrong. Similarly, at zero calcium, Scheme IIIb is reduced to the simpler one, $*E \xrightarrow{k^+} E$, and a definite amount of ATP-reactive enzyme would be present even with excess EGTA, which is also wrong.

In addition, Scheme IIIb does not take into account any cooperativity in the Ca$^{2+}$ binding-induced events, even though such positive cooperativity has often been pointed out (Inesi et al., 1980) and is also evident from Figs. 1 and 9. Our data were therefore tentatively fitted to the following scheme:

\[
\begin{align*}
K_i & \rightarrow E + Ca \rightarrow *ECa \rightarrow ECa \\
K_i & \rightarrow E + Ca \rightarrow ECa
\end{align*}
\]

$K_i > K_f$

**Scheme IIIc**

Like Scheme IIIb, this scheme comprises calcium binding steps located before and after slow isomerization. For this reason it also accounts for both the descending and the ascending portions of our $k_{obs}$ curves (see for instance Fig. 2b).

Scheme IIIc is identical with the one of Inesi et al. (1980). Some further comments can be made, however. (i) For the sake of simplicity we assumed that binding and dissociation steps were fast, compared to isomerization rates, which of course might be an oversimplification. Note, however, that we always found monoeXponential evolutions after either addition or removal of calcium in the absence of phosphate. (ii) We also assumed that $ECa$ and $ECa^2$ had the same fluorescence level. In any case, $ECa$ is not a dominant species at equilibrium in this scheme. (iii) Exactly like the Scheme IIIb proposed in our preceding paper, Scheme IIIc seems to imply that the $*E$ conformation of the ATPase does possess an outward facing binding site with a rather high affinity for calcium, in apparent contradiction with previous suggestions (see de Meis and Vianna, 1979). The molecular significance of the $*E$ specie remains, therefore, to be discussed. Such a molecular description has recently been attempted by Y. Dupont.

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