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OCCURRENCE AND CHARACTERISTICS OF A RAPID EXCHANGE OF PHOSPHATE OXYGENS CATALYZED BY SARCOPLASMIC RETICULUM VESICLES*

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ABSTRACT

Sarcoplasmic reticulum vesicles prepared from rabbit skeletal muscle catalyze a rapid $P_i \rightleftarrows$ HOH exchange in the presence of Mg^{++} and absence of ATP and Ca^{++}. The capacity for oxygen exchange is about fourteen times the potential capacity for ATP cleavage in the presence of Ca^{++}. No detectable exchange is found without added $MgCl_2$. The exchange is unaffected by oligomycin, 2,4-dinitrophenol or ouabain, but strongly inhibited by low concentrations of Ca^{++} in the medium. The Ca^{++} ion concentration giving a half-maximum inhibition is 2.0 μM in the presence of 5 mM $MgCl_2$. A Hill plot of the Ca^{++} inhibition gives a straight line with a Hill coefficient of 1.8. The Ca^{++} inhibition is competitively overcome by additional Mg^{++}. The $P_i \rightleftarrows$ HOH exchange is almost completely inhibited by the detergent Triton X-100 at low concentrations where the ATPase activity is not disturbed. Sarcoplasmic reticulum vesicles are phosphorylated by P_i in the presence of Mg^{++} and absence of Ca^{++} under conditions similar to those for the $P_i \rightleftarrows$ HOH exchange. The phosphorylation requires Mg^{++} and is strongly inhibited by low concentrations of Ca^{++}. The response of the phosphorylation to Ca^{++} is quite similar to that of the $P_i \rightleftarrows$ HOH exchange; the Ca^{++} ion concentration giving a half-maximum inhibition is 2.0 μM in the presence of 5 mM $MgCl_2$ and the Hill coefficient is about 2.0. The various properties of the exchange give strong support to the probability that it results from reversal of steps in the overall process associated with Ca^{++} transport driven by ATP cleavage.

RUNNING TITLE: Oxygen Exchanges of Sarcoplasmic Reticulum

INTRODUCTION

Sarcoplasmic reticulum vesicles isolated from skeletal muscle actively take up Ca^{++} from the medium in the presence of Mg^{++} and ATP (1-3). This transport is coupled to ATP hydrolysis catalyzed by membrane-bound Ca^{++}, Mg^{++}-ATPase which is activated by concurrent presence of Ca^{++} and Mg^{++} (2-6). Considerable informations have accumulated that give insight into the ATPase and its coupling to the calcium transport. The hydrolysis of ATP by this enzyme occurs through a phosphorylated intermediate (7-11). Formation and decomposition of the intermediate show vectorial requirements for Ca^{++} and Mg^{++} (12,13), suggesting an intimate involvement of the intermediate in the transport process. ATP synthesis from P_i and ADP coupled to outflow of Ca^{++} from sarcoplasmic reticulum vesicles has recently been demonstrated (14,15). This indicates the reversibility of the entire process of calcium transport in sarcoplasmic reticulum vesicles.

In the present article, it has been demonstrated that sarcoplasmic reticulum vesicles catalyze a rapid $P_i \rightleftarrows HOH$ exchange, which can be reasonably attributed to the reversal of steps in the process associated with calcium transport driven by ATP hydrolysis. The characteristics of the $P_i \rightleftarrows HOH$ exchange recently found in Na^+, K^+-ATPase of cell membranes (16) are quite similar to those of sarcoplasmic reticulum vesicles. This suggests mechanistic similarities between the two transport systems.

EXPERIMENTAL PROCEDURE

Materials. Water of approximately 1.4 atom % excess ^{18}O was obtained from Yeda Research and Development Company, Rehovoth, Israel. KH_2PO_4-^{18}O with about 1.7 atom % excess ^{18}O was prepared as described by Cohn and Drysdale (17) and appropriately diluted with carrier P_i before use. Disodium salts of ATP and ADP were purchased from Boehringer and Mannheim. γ-^{32}P-labelled ATP was prepared as described by Glynn and Chappell (18). Acetylphosphate was obtained as the lithium salt from Sigma. Ouabain was purchased from Calbiochem and oligomycin from Sigma. All the other chemicals were of reagent grade.

Preparation of sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle by the method of Weber et al. (3) with slight modifications. About 300 g of white dorsal and hind leg muscles were homogenized with 900 ml of chilled 0.1 M NaCl-5 mM Tris-maleate buffer, pH 7.0, in a Waring blender for 2 min. The homogenate was centrifuged at 4,000 x g for 20 min to remove myofibrils, nuclei and debrils. The supernatant was filtered through four layers of gauze, and the filtrate was centrifuged at 10,000 x g for 30 min to remove mitochondria. The resulting supernatant was centrifuged at 54,000 x g for 60 min. The pellet was suspended in 190 ml of 0.6 M KCl-5 mM Tris-maleate buffer, pH 6.5, and centrifuged at 64,000 x g for 80 min to remove contaminating actomyosin and other proteins. The precipitate was suspended in 190 ml of 0.1 M KCl-5 mM Tris-maleate buffer, pH 6.5, and centrifuged at 8,000 x g for 20 min. The resulting supernatant was centrifuged at 54,000 x g for 60 min. The precipitate was washed once with 90 ml of 0.1 M KCl-5 mM Tris-maleate buffer, pH 6.5. The preparation of sarcoplasmic reticulum vesicles thus obtained was suspended in 0.1 M KCl-5 mM Tris-maleate buffer, pH 6.5, stored at 0° on ice and used within 7 days.

<u>Determination of $P_i \rightleftarrows$ HOH Exchange</u>. The $P_i \rightleftarrows$ HOH exchange catalyzed by sarcoplasmic reticulum vesicles was determined by two different methods. In the first method, sarcoplasmic reticulum vesicles were incubated with P_i in $H^{18}OH$ with approximately 0.8 to 1.2 atom % excess ^{18}O under conditions described under "Results." Reactions were quenched by addition of perchloric acid, and the denatured vesicles and precipitate of potassium perchlorate were removed by centrifugation. P_i in the supernatant was isolated as KH_2PO_4, and ^{18}O incorporated from $H^{18}OH$ into P_i was determined as described below. Total amount of oxygen atoms exchanged was calculated, as described by Boyer and Bryan (19), from the equation,

$$\mu\text{atoms oxygen exchanged} = 4 \times p_o \times \ln \frac{1}{1-F}$$

where p_o = mM P_i in the reaction medium and $F = \dfrac{\text{atom \% excess } ^{18}O \text{ in } P_i}{\text{atom \% excess } ^{18}O \text{ in HOH}}$

The second method was utilized to measure the extent of $P_i \rightleftarrows$ HOH exchange during cleavage of ATP or acetylphosphate. Sarcoplasmic reticulum vesicles were incubated with P_i-^{18}O of approximately 0.43 atom % excess ^{18}O under conditions described under "Results." Reactions were quenched by adding perchloric acid at zero time and 40 minutes after the start of the incubation. Subsequent procedures for isolation of P_i and determination of ^{18}O in the P_i were the same as described for the first method with the exception that in the experiment where ATP was present in the medium isolation of P_i was performed at 4°, while in the experiment where acetylphosphate was present the samples were allowed to stand for 1 hour at room temperature after addition of ammonium molybdate in the process of P_i isolation described below to allow decomposition of acetylphosphate. The amount of oxygen exchanged during

ATP cleavage was calculated as described previously (20) by the following equation.

$$\mu\text{atoms oxygen exchanged} = \left(\ln \frac{p_o\,{}^{18}O_1}{(p_o+p)\,{}^{18}O_2}\right)\left(\frac{4k(p_o+a)}{\ln \frac{1+p/p_o}{1-p/a}}\right) \times t$$

where a = initial mM ATP, p = mM P_i released from ATP, p_o = initial mM P_i, k = rate constant (sec^{-1}) for ATP hydrolysis governed by $\frac{dp}{dt} = k(a-p)$, t = seconds of incubation, and ${}^{18}O_1$ and ${}^{18}O_2$ = initial and final atom % excess ${}^{18}O$ in P_i. The amount of oxygen exchanged in the absence of ATP or acetylphosphate was calculated by the equation,

$$\mu\text{atoms oxygen exchanged} = 4p_o \ln \frac{{}^{18}O_1}{{}^{18}O_2}$$

The amount of oxygen exchanged in the presence of acetylphosphate was also calculated using the above equation because no significant cleavage of acetylphosphate occurred during incubation.

P_i isolation and ${}^{18}O$ analysis. The procedure of Boyer and Bryan (19) was used for P_i isolation and ${}^{18}O$ analysis, with some modifications. After denatured vesicles were removed by centrifugation, concentrated HCl was added to the sample to a final concentration of 1.1 N. The sample was washed once with about 2 ml of isobutanol-benzene (1:1), then 50 mM aqueous ammonium molybdate was added to a final concentration of 12 mM. The resulting phosphomolybdate complex was removed by two extractions with isobutanol-benzene. The isobutanol-benzene mixture was washed with 2 ml of 1 N HCl, then P_i was extracted into 1.5 ml of 7.5 N NH_4OH. After discarding the isobutanol-benzene layer, 1.5 ml of cold magnesia mixture was added to the extract, and the sample was refrigerated overnight for precipitation of $MgNH_4PO_4$. The precipitate was

washed with 1 ml of cold 5 N NH_4OH and dissolved by addition of 0.1 ml of 6 N HCl. When completely dissolved, 1.1 ml of 7.5 N NH_4OH was added to reprecipitate the phosphate. After overnight refrigeration, the precipitate was washed again with 1 ml of 5 N NH_4OH and then lyophilized. 5 µl of 0.1% aqueous bromocresol green was added to the dry precipitate, and the sample was titrated with 0.1 N HCl to a pH slightly higher than 4 (yellow end point). After addition of water to a final volume of 0.3 ml, the sample was passed through a Dowex-50-K^+ column to convert the phosphate to the KH_2PO_4 form. The eluent was lyophilized and ^{18}O determined by the guanidine-HCl pyrolysis procedure (21).

Determination of P_i liberated and phosphorylated protein formed by the ATPase reaction. ATPase reactions catalyzed by sarcoplasmic reticulum were performed using $AT^{32}P$ as substrate as described under "Results," and $^{32}P_i$ liberated and the phosphorylated protein formed from $AT^{32}P$ were measured as described by Kanazawa et al. (13) using Millipore filtration (1.2 µ pore size). Radioactivity of ^{32}P was measured using Bray's solution (22) with a Nuclear Chicago liquid scintillation system, Model 725.

Determination of phosphorylated protein formed from P_i and sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles were incubated with $^{32}P_i$ for 30 sec at 15° and pH 7.0 in 1.0 ml of a medium as described under "Results." The reaction was quenched by adding 10 ml of 4% perchloric acid. The denatured vesicles were washed 7 times by centrifugation procedure with 10 ml of 4% perchloric acid containing 20 mM carrier P_i and then suspended in 2 ml of 0.01 N NaOH containing 0.1 mM carrier P_i. After heating at 100° for 5 min, the suspension was centrifuged and ^{32}P in an aliquot of the supernatant was measured as described above.

Adjustment of free Ca^{++} concentration in the medium. Free Ca^{++} concentrations in the reaction medium were adjusted using a Ca-EGTA buffer, where the concentration of $CaCl_2$ added to the medium was fixed at 0.5 mM and EGTA concentrations in the medium were varied. Calculations of free Ca^{++} concentrations were carried out assuming that apparent stability constant of Ca-EGTA complex at pH 7.0, association constant of $CaHPO_4$ from Ca^{++} and $HPO_4^{=}$ and pK_2 for P_i at 15° were, respectively, $1.3 \times 10^6\ M^{-1}$ (23), 50 M^{-1} (24) and 7.23 (25).

Protein concentrations were determined by the method of Lowry et al. (26) with bovine serum albumin as a standard. Acetylphosphate was measured by the method of Lipmann and Tuttle (27). Contaminant P_i in acetylphosphate was measured as described by Lowry and Lopez (28).

RESULTS

The $P_i \rightleftarrows HOH$ exchange catalyzed by sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles prepared from rabbit skeletal muscle were incubated with 40 mM P_i at pH 7.0, 37°, in the presence and absence of 14.5 μM free Ca^{++} in the medium containing $H^{18}OH$ and other reagents as indicated in Fig. 1. As shown in Fig. 1, the vesicles were found to catalyze a rapid incorporation of water oxygen atoms into P_i when any free Ca^{++} ion was almost completely chelated by addition of 5 mM EGTA, a potent chelating reagent for Ca^{++}. The amount of water oxygen atoms incorporated into P_i molecule increased with time and readily approached isotopic equilibrium when higher concentrations of sarcoplasmic reticulum vesicles and longer incubation periods than those shown in Fig. 1 were used. The rate of $P_i \rightleftarrows HOH$ exchange calculated as described under "Experimental Procedure" was 520 gram atoms oxygen exchanged/sec$\cdot 10^6$ gm protein. This rate was approximately fourteen-fold higher

than the rate of Ca^{++}-dependent ATP hydrolysis, which was 37 moles P_i liberated/ sec·10^6 gm protein, as measured in the presence of 5 mM ATP, 0.5 mM $CaCl_2$ and 0.5 mM EGTA without added P_i under otherwise the same conditions. Without addition of $MgCl_2$ to the medium, no detectable $P_i \rightleftarrows$ HOH exchange was found. In contrast, the $P_i \rightleftarrows$ HOH exchange was strikingly inhibited by the presence of 14.5 μM free Ca^{++} in the medium.

Absence of effects of oligomycin, 2,4-dinitrophenol, and ouabain on the $P_i \rightleftarrows$ HOH exchange. As tests of the possibility that the observed $P_i \rightleftarrows$ HOH exchange might be due to mitochondria or Na^+,K^+-ATPase present as contaminants in the sarcoplasmic reticulum preparation, effects of oligomycin, 2,4-dinitrophenol and ouabain on the $P_i \rightleftarrows$ HOH exchange were investigated. As seen in Fig. 1, the $P_i \rightleftarrows$ HOH exchange observed in the absence of free Ca^{++} was quite unaffected by concentrations of oligomycin, 2,4-dinitrophenol, or ouabain that sharply inhibit exchanges of mitochondria or Na^+,K^+-ATPase. Similarly, the slight $P_i \rightleftarrows$ HOH exchange activity remaining in the presence of 14.5 μM free Ca^{++} also was not affected by these reagents.

Time course of the $P_i \rightleftarrows$ HOH exchange. Data of Fig. 1 indicates that the exchange proceeded approximately linearly with time at 37° when the reaction was initiated by addition of P_i. Additional check of linearity with time was felt desirable, in particular in view of the interesting lag in onset of exchange noted with the actomyosin system (29,30). As a further assessment, experiments reported in Fig. 2 were conducted with initiation of exchange by addition of the sarcoplasmic reticulum vesicles, and at 15° instead of 37° to increase the possibility of detecting a lag phase. As seen in the figure, upon addition of the sarcoplasmic reticulum vesicles, the $P_i \rightleftarrows$ HOH exchange proceeded linearly with time without a detectable lag phase. The exchange

rate was 28.3 gram atoms oxygen exchanged/sec$\cdot 10^6$ gm protein. As noted in Fig. 2, when sarcoplasmic reticulum vesicles were added to the medium containing 14.5 μM free Ca^{++}, the $P_i \rightleftarrows HOH$ exchange was almost completely inhibited.

Effect of Ca^{++} ion concentration on inhibition of the $P_i \rightleftarrows HOH$ exchange. In the experiment shown in Fig. 3, the rates of $P_i \rightleftarrows HOH$ exchange were measured at varying concentrations of free Ca^{++} ion in the medium under otherwise the same conditions as those for Fig. 2. Concentrations of free Ca^{++} in the medium were adjusted using Ca-EGTA buffer as described under "Experimental Procedure." As shown in the figure, the $P_i \rightleftarrows HOH$ exchange was progressively inhibited as the free Ca^{++} ion concentration in the medium was increased more than 0.1 μM and almost completely disappeared above 34 μM Ca^{++} ion. Fig. 4 shows the Hill plot taken from the data of Fig. 3. This plot gives a straight line with a Hill coefficient of 1.8; the Ca^{++} ion concentration giving a half-maximum inhibition was found to be 2.0 μM.

Effect of Ca^{++} on the Mg^{++} ion dependence of the $P_i \rightleftarrows HOH$ exchange. The rate of $P_i \rightleftarrows HOH$ exchange at varying Mg^{++} ion concentrations was measured in the absence and presence of 3.6 μM free Ca^{++} to examine Ca^{++} ion effect on the Mg^{++} ion dependence of the $P_i \rightleftarrows HOH$ exchange. Fig. 5 shows the double reciprocal plots of the rates of $P_i \rightleftarrows HOH$ exchange against concentration of $MgCl_2$ added. The plots give straight lines both in the absence and presence of free Ca^{++} over the $MgCl_2$ concentration range tested. The apparent K_m for $MgCl_2$ calculated from these plots was markedly increased by the presence of 3.6 μM Ca^{++}, being 4.9 and 77 mM, respectively, in the absence and presence of 3.6 μM Ca^{++}. On the other hand, the maximum rate obtained by extrapolating the $MgCl_2$ concentration to infinity remained unaffected, being equivalent to 59 gram atoms oxygen exchanged/sec$\cdot 10^6$ gm protein. This indicates that the

observed calcium inhibition can be competitively overcome by additional $MgCl_2$.

Ca^{++} ion effects on phosphorylated protein and ATPase. In the experiment reported in Fig. 6, the rates of P_i liberation and amounts of phosphorylated protein present during the steady state of the ATPase reaction catalyzed by sarcoplasmic reticulum vesicles were measured at varying concentrations of free Ca^{++} in the medium. The reactions were performed under the same conditions as those for the $P_i \rightleftarrows$ HOH exchange measurement shown in Fig. 3 except that 5 μM AT^{32}P at a final concentration was used in place of 40 mM P_i. As seen in the figure, the steady state rate of P_i liberation was increased with increase in free Ca^{++} concentration in the medium and reached a maximum at 19 μM free Ca^{++}, followed by decrease with further increase in the free Ca^{++} concentration. The amount of phosphorylated protein showed a quite similar response, but was increased in proportion to the increase in the rate with less increase above 8 μM Ca^{++}. Thus, the ratio of the rate of P_i liberation to amount of phosphorylated protein in the steady state remained approximately constant (1.0 sec^{-1}), particularly in the samples up to 8 μM Ca^{++}. Fig. 7 shows the Hill plot of the rates of P_i liberation given in Fig. 6. The Hill coefficient was 1.05 and the free Ca^{++} concentration giving half-maximum activation was 2.6 μM.

Effects of ATP, ADP and acetylphosphate on the $P_i \rightleftarrows$ HOH exchange. Table I shows the effect of ATP on the $P_i \rightleftarrows$ HOH exchanges as measured in the absence and presence of 60 μM free Ca^{++} in the medium. The $P_i \rightleftarrows$ HOH exchange in the absence of both free Ca^{++} and ATP showed a high value of 23 to 24 gram atoms oxygen exchanged/sec·10^6 gm protein, in good agreement with that obtained from ^{18}O incorporation from H^{18}OH into P_i under the same conditions (Figs. 2 and 3). The important point of Table I is that the $P_i \rightleftarrows$ HOH exchange in the

absence of free Ca^{++} in the medium was pronouncedly inhibited by presence of 4 mM ATP. The ATP added was hydrolyzed by 28% in the absence of free Ca^{++} during the 40 minutes incubation.

As anticipated from the previous experiments, the $P_i \rightleftarrows HOH$ exchange was completely inhibited in the presence of 60 µM free Ca^{++} in the medium. The inhibition of $P_i \rightleftarrows HOH$ exchange by the Ca^{++} was unaffected by addition of 4 mM ATP, while ATP hydrolysis was activated by the Ca^{++} and 62% of the added ATP was hydrolyzed in the presence of 60 µM Ca^{++} during the 40 minutes incubation.

Similar measurements were performed to measure the effect of acetylphosphate on the $P_i \rightleftarrows HOH$ exchange in the absence and presence of 60 µM Ca^{++}. As shown in Table II, addition of 3.3 mM acetylphosphate showed no appreciable or a slightly inhibitory effect on the $P_i \rightleftarrows HOH$ exchange in the absence of free Ca^{++} in the medium, while the $P_i \rightleftarrows HOH$ exchange inhibited by 60 µM Ca^{++} appeared to be somewhat reactivated by the addition of acetylphosphate. No hydrolysis of acetylphosphate was detected either in the absence of or in the presence of Ca^{++}.

Fig. 8 shows the effect of ADP on the $P_i \rightleftarrows HOH$ exchange catalyzed by sarcoplasmic reticulum vesicles in the absence of free Ca^{++} in the medium. A rather high concentration (20 mM) of $MgCl_2$ was used to minimize change in the free Mg^{++} concentration due to addition of ADP. The rate of $P_i \rightleftarrows HOH$ exchange without added ADP, equivalent to 37.6 gram atoms oxygen exchanged/ sec$\cdot 10^6$ gm protein, was considerably higher than that measured at 5 to 7 mM $MgCl_2$ under otherwise the same conditions (Figs. 2,3 and 9, Tables I and II). As seen in the figure, the rate of $P_i \rightleftarrows HOH$ exchange was markedly decreased with increase in concentration of ADP added. Addition of 0.5 and 3.0 mM ADP caused, respectively, 67 and 87% inhibition. However, the inhibition was not

appreciably enhanced by further increase in added ADP up to 4 mM. To estimate the adenylate kinase activity which might obscure the experimental results, decrease in ADP concentration in the 20 minutes incubation was measured under the same conditions as those for Fig. 8 using the method of Reynard et al. (31). The result showed that decreases in ADP concentration were 26, 8 and 4% in the 20 minutes incubation when ADP was added to the medium to give, respectively, 0.5, 2 and 4 mM. Thus, the adenylate kinase activity was not so high as to disturb accurate measurements of $P_i \rightleftarrows HOH$ exchange.

Effect of Triton X-100 on the $P_i \rightleftarrows HOH$ exchange. Fig. 9 shows the effect of Triton X-100 on the $P_i \rightleftarrows HOH$ exchange and ATPase reactions catalyzed by sarcoplasmic reticulum vesicles. In this experiment sarcoplasmic reticulum vesicles were incubated with Triton X-100 of various concentrations in the presence of 0.625 mM EGTA and other reagents as indicated in the figure without added $CaCl_2$. The $P_i \rightleftarrows HOH$ exchange was started by adding P_i to the preincubation mixture, while the ATPase reaction was initiated by addition of $CaCl_2$ and $AT^{32}P$. As seen in the figure, the rate of $P_i \rightleftarrows HOH$ exchange was progressively decreased with increase in the Triton X-100 concentration; 92% of the exchange activity was lost when Triton X-100 was added to a final concentration of 0.4 to 0.6 µl/ml. In contrast, the rate of P_i liberation and the amount of phosphorylated protein formed in the ATPase reaction remained on the same level as that of control even when 0.6 µl/ml Triton X-100 was present in the medium.

Phosphorylation of sarcoplasmic reticulum vesicles by P_i. The occurrence of the $P_i \rightleftarrows HOH$ exchange suggested that the phosphorylated enzyme is formed from P_i and sarcoplasmic reticulum vesicles with elimination of water under conditions where the $P_i \rightleftarrows HOH$ exchange takes place. In the experiment reported in Table III, sarcoplasmic reticulum vesicles were incubated with 5 mM $^{32}P_i$

for 30 sec under conditions similar to those for the $P_i \rightleftarrows HOH$ exchange measurement, and ^{32}P incorporated into the protein was measured. As seen in the table, a quite small but significant fraction of the enzyme was phosphorylated by P_i when 5 mM $MgCl_2$ was present in the medium and any free Ca^{++} was removed by addition of EGTA. The phosphorylation disappeared almost completely when 0.1 mM $CaCl_2$ was added without EGTA or when no $MgCl_2$ was added and free Mg^{++} ion contaminating the medium was chelated by EDTA. Fig. 10 shows a double reciprocal plot of the amount of phosphorylated protein formed from P_i against P_i concentration in the presence of 20 mM $MgCl_2$ and absence of free Ca^{++} ion at pH 7.0 and 15°. The maximum amount of the phosphorylation was equivalent to 1.25 moles of phosphoryl group per 10^7 gm protein. The apparent K_m for P_i was 4.1 mM.

<u>Effect of Ca^{++} ion concentration on inhibition of the formation of phosphorylated protein from P_i.</u> In the experiment shown in Fig. 11, the formations of phosphorylated protein from P_i were measured at varying concentrations of free Ca^{++} ion in the medium. The conditions for the measurement were the same as those for the $P_i \rightleftarrows HOH$ exchange reaction given in Fig. 3 except that sarcoplasmic reticulum vesicles (3 mg of protein per ml) were incubated with 5 mM $^{32}P_i$ for 30 sec. As is clear from comparison with Fig. 3, the inhibitory effect of Ca^{++} ion on the formation of phosphorylated protein from P_i showed quite similar features to the inhibition of the $P_i \rightleftarrows HOH$ exchange. Fig. 12 shows the Hill plot taken from the data of Fig. 11. A Hill coefficient obtained from this plot was about 2.0, and the Ca^{++} ion concentration giving a half-maximum inhibition was 2.0 μM. These values were in good agreement with those obtained from the Ca^{++} inhibition of $P_i \rightleftarrows HOH$ exchange given in Figs. 3 and 4.

DISCUSSION

The results clearly demonstrate that the sarcoplasmic reticulum preparation has the capacity to catalyze a rapid $P_i \rightleftarrows HOH$ exchange. The various properties of the exchange give strong support to the probability that it results from reversal of steps in the overall process associated with calcium transport driven by ATP cleavage. The implications and possible nature of the exchange reaction may be conveniently discussed in relation to the scheme presented in Fig. 13 that gives steps in the transport process consistent with the present and earlier findings (13,32).

The oxygen exchange could quite logically result from the dynamic reversal of steps 7 and 8 of this sequence, namely the formation of an enzyme$\cdot P_i$ complex in presence of Mg^{++}, and the formation of a covalent enzyme-phosphate with elimination of water. A striking feature is the unusual facility of this reaction. As noted in Table III, only a small fraction of the enzyme is phosphorylated by P_i in presence of Mg^{++} and absence of ATP and Ca^{++}. Yet the capacity for oxygen exchange is some ten to fifteen times the potential capacity for ATP cleavage in the presence of calcium. An estimate is that the small fraction of E-P present must be turning approximately 300 times per second at 15° to account for the exchange.

The data showing the lack of effect of inhibitors of mitochondrial exchange processes (Fig. 1 and text), the pronounced inhibition of the exchange by Ca^{++} (Figs. 1 and 2) and the similar concentration dependencies of the exchange (Fig. 3) and the ATP hydrolysis (Fig. 6) on Ca^{++}, as well as the sharp decrease caused by Ca^{++} in the capacity for phosphoryl enzyme formation from P_i (Table III and Fig. 11) all suggest that the oxygen exchange is a property of the transport ATPase system. The similar characteristics of

the Ca^{++} inhibition of phosphoryl enzyme formation from P_i and of the $P_i \rightleftarrows HOH$ exchange give additional evidence that the observed oxygen exchange is a property of the transport ATPase system. On the other hand, although the ATP hydrolysis is activated in nearly the same range of Ca^{++} ion concentration as the $P_i \rightleftarrows HOH$ exchange is inhibited, the concentration dependencies are somewhat different as seen from the observed difference in Hill coefficients (1.05 in the ATPase reaction and 1.8 in the $P_i \rightleftarrows HOH$ exchange).

The Ca^{++} inhibition of the exchange, in terms of the scheme given in Fig. 13, can be explained if the Ca^{++} can combine with the free enzyme as a partial reaction of step 2 to give a form of a complex unable to catalyze the exchange. Such a mechanism can explain the observed cooperativity of Ca^{++} assuming that in step 1 occurs a conformational change which results in simultaneous formation of two Ca^{++}-binding sites.

The Ca^{++} responses as well as the lack of effect of ouabain, a potent inhibitor of the exchange catalyzed by Na^+,K^+-ATPase (16), effectively eliminate the Na^+,K^+-ATPase as being responsible for the exchange.

Similarly, the observed $P_i \rightleftarrows HOH$ exchange is quite different in the effects of divalent cations and nucleotides from what has been demonstrated with a contractile protein. In the $P_i \rightleftarrows HOH$ exchange catalyzed by myosin, Ca^{++} has been shown to be an activator much more effective than Mg^{++} (33), whereas Ca^{++} was a strong inhibitor in the $P_i \rightleftarrows HOH$ exchange with the preparation of sarcoplasmic reticulum vesicles. The $P_i \rightleftarrows HOH$ exchanges with myosin and actomyosin are stimulated by nucleotides such as ATP and ADP (20,33). In contrast, the $P_i \rightleftarrows HOH$ exchanges with the preparation of sarcoplasmic reticulum vesicles were markedly inhibited by addition of ATP or ADP.

It is characteristic of the Ca^{++}-transport system, including the

Ca^{++},Mg^{++}-ATPase, of sarcoplasmic reticulum that the system is activated by very low Ca^{++} concentration. The present results showed that a half-maximum inhibition of the $P_i \rightleftarrows HOH$ exchange was caused by 2 μM Ca^{++} which was found to be nearly equivalent to that giving a half-maximum activation of the Ca^{++},Mg^{++}-ATPase measured under similar conditions to those for the $P_i \rightleftarrows HOH$ exchange measurement. Such results add to the probability that the observed $P_i \rightleftarrows HOH$ exchange is catalyzed by the Ca^{++},Mg^{++}-ATPase of sarcoplasmic reticulum.

Implications of the time course of the exchange warrant comment. When sarcoplasmic reticulum vesicles were added to the reaction medium containing P_i and excess EGTA, the $P_i \rightleftarrows HOH$ exchange occurred immediately without showing any appreciable lag phase and proceeded linearly with time (Fig. 2), while the exchange was prevented from the start of the incubation when sarcoplasmic reticulum vesicles were added to the medium containing P_i and free Ca^{++}. These observations indicate that the inhibition of the $P_i \rightleftarrows HOH$ exchange is caused by external Ca^{++} located outside membranes, but not by internal Ca^{++}. Vesicles usually contain about 20 nmoles or more of endogenous Ca^{++} per mg protein (34,35) and this Ca^{++} does not diffuse out in a few minutes at neutral pH when medium Ca^{++} is chelated by excess EGTA (15,36). EGTA added to the medium cannot diffuse into the vesicles (3).

As mentioned earlier, the fraction of the enzyme that is phosphorylated in the presence of P_i and Mg^{++} is small. It amounts to only about 1/50 of that present during the steady-state hydrolysis of ATP in presence of Ca^{++} and Mg^{++}, and suggests a particularly rapid turnover for participation of this phosphoryl enzyme in the oxygen exchange. Whether the same phosphoryl enzyme participates in the synthesis of ATP from ADP and P_i upon outward flux

of Ca^{++} reported by Makinose and Hasselbach (14) is uncertain. In our experiments, addition of external Ca^{++} inhibited formation of the phosphoryl enzyme, but it could form from P_i in presence of internal Ca^{++} when ATP synthesis accompanies Ca^{++} efflux. Indeed Hasselbach _et al_. (37) have quite recently reported that $^{32}P_i$ was incorporated into the protein of sarcoplasmic reticulum vesicles preloaded with Ca^{++}. The amount of ^{32}P incorporated (2 moles/10^6 gm protein) was much larger than that obtained by us (0.125 moles/10^6 gm protein) using Ca^{++}-unloaded sarcoplasmic reticulum vesicles. Such apparent contrast in behavior can be explained by shifts of steps 4 and 5 toward the right due to binding of preloaded Ca^{++} with free E-P at step 4.

The cooperativity observed in the Ca^{++} inhibition of the $P_i \rightleftarrows$ HOH exchange suggests that an allosteric transition could be induced by Ca^{++} in the Ca^{++}-transport system of sarcoplasmic reticulum. A possibility previously suggested (13), based on the dependence of the breakdown of the phosphorylated intermediate on internal Mg^{++}, is that Mg^{++} is transported by sarcoplasmic reticulum as counter ion to Ca^{++}. The cooperative inhibition of the $P_i \rightleftarrows$ HOH exchange may reflect a Ca^{++}-induced transition of the transport system from one form (Mg^{++}-carrier), which has a binding site with high affinity for Mg^{++} and catalyzes translocation of Mg^{++} across membranes coupled with Mg^{++}-dependent $P_i \rightleftarrows$ HOH exchange, to the other form (Ca^{++}-carrier) which has two binding sites with high affinity for Ca^{++} and catalyzes translocation of Ca^{++}. This explanation is consistent with the fact that the breakdown of the phosphorylated intermediate was unaffected by external Ca^{++} as shown in Fig. 6 in spite of almost complete inhibition of the $P_i \rightleftarrows$ HOH exchange by external Ca^{++}.

The inhibition of the $P_i \rightleftarrows$ HOH exchange by nucleotides such as ATP and ADP could be explained by assuming that Mg^{++}-carrier form of the enzyme is

converted to Ca^{++}-carrier form by these nucleotides even in the absence of Ca^{++}.

This assumption is compatible with the present observation that the Ca^{++} activation of the ATPase measured in the presence of ATP shows no cooperativity. It is also compatible with de Meis and Hasselbach's recent observation (38,39) that the Ca^{++} uptake by sarcoplasmic reticulum vesicles shows a hyperbolic Ca^{++} ion concentration dependence with high Ca^{++} ion affinity when ATP is used as an energy source, but a sigmoid dependence with low Ca^{++} ion affinity when acetylphosphate is used in place of ATP. However, this assumption seems not to be in harmony with the fact that Ca^{++} activation of the ATPase occurred in the concentration range similar to that for Ca^{++} inhibition of the $P_i \rightleftarrows HOH$ exchange (Figs. 3 and 6) and with the cooperative activation of phosphorylated intermediate formation from ATP by Ca^{++} (13). Although at present these difficulties cannot be readily resolved, it should be noted that the activation of phosphorylated intermediate formation has been measured at low temperature (0°) which might be unfavorable to nucleotide-induction of the transition.

A need for membrane integrity for the $P_i \rightleftarrows HOH$ exchange is implied by the almost complete inhibition by low concentration of Triton X-100. Such low detergent concentrations did not disturb the ATPase activity. Transport capacity is likewise destroyed by detergents, again indicating that the exchange is intimately related to the transport mechanism. A similar inhibition by a detergent, deoxycholate, has been found in the $P_i \rightleftarrows HOH$ exchange catalyzed by Na^+, K^+-ATPase (16).

It has recently been found by Dahms and Boyer (41) that Na^+,K^+-ATPase also catalyzes $P_i \rightleftarrows HOH$ exchange which is activated by K^+ and inhibited by Na^+. Thus, the dependencies of the $P_i \rightleftarrows HOH$ exchanges on cations transported across

membranes are similar between the Ca^{++}, Mg^{++}-ATPase of sarcoplasmic reticulum and the Na^{+}, K^{+}-ATPase of cell membranes. The $P_i \rightleftarrows HOH$ exchanges catalyzed by these two transport-ATPases may reflect mechanistic similarities between the two systems. Similarly, the occurrence of the exchanges suggests possible mechanistic similarities to the membrane activation accompanying oxidative phosphorylation (42) and active transport of sugars and amino acids (43).

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FOOTNOTES

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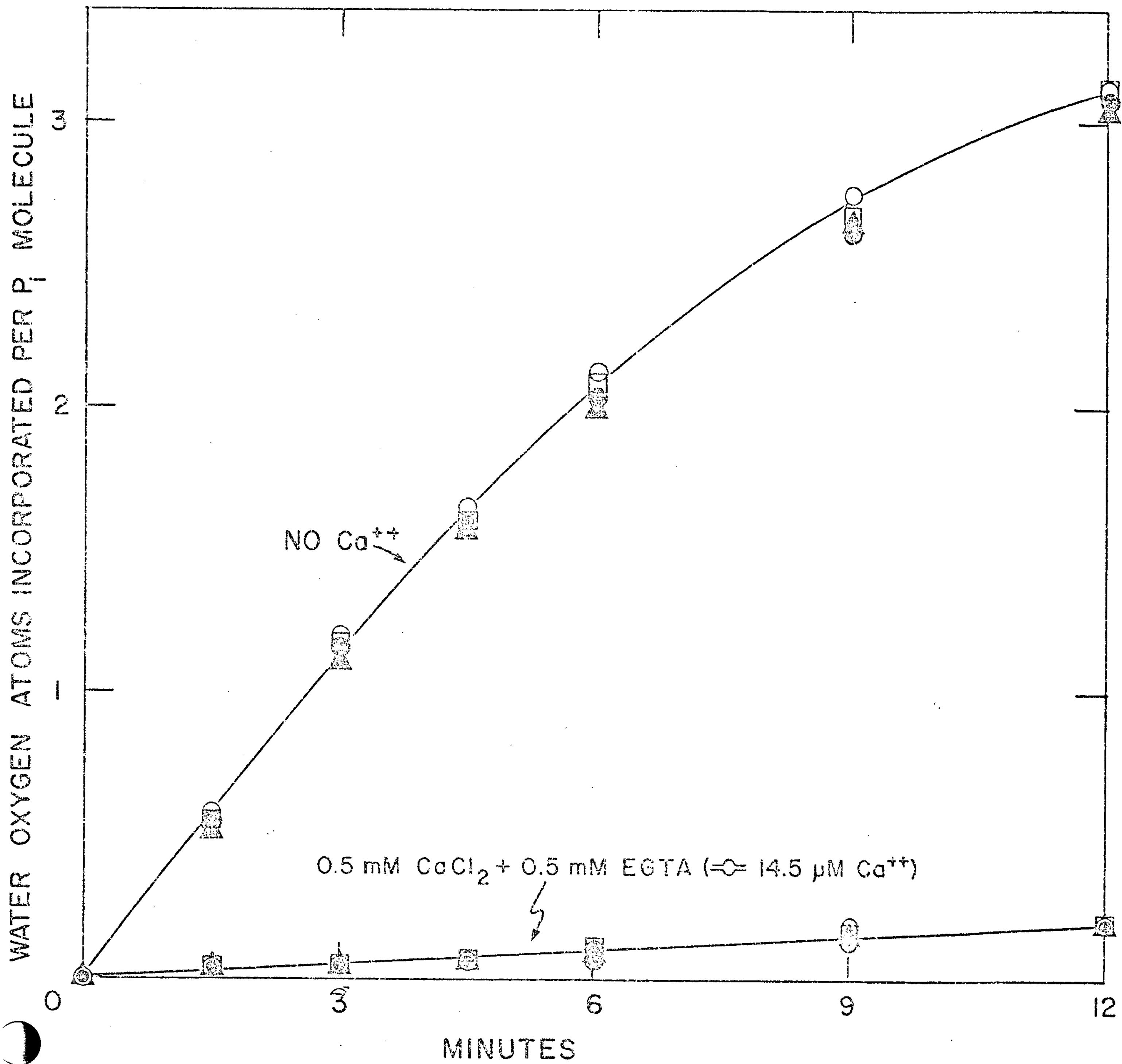
† On leave from the Department of Biology, Faculty of Science, Osaka University, Japan.

LEGEND FOR FIGURE 1

SOME CHARACTERISTICS OF THE $P_i \rightleftarrows$ HOH EXCHANGE

CATALYZED BY SARCOPLASMIC RETICULUM VESICLES

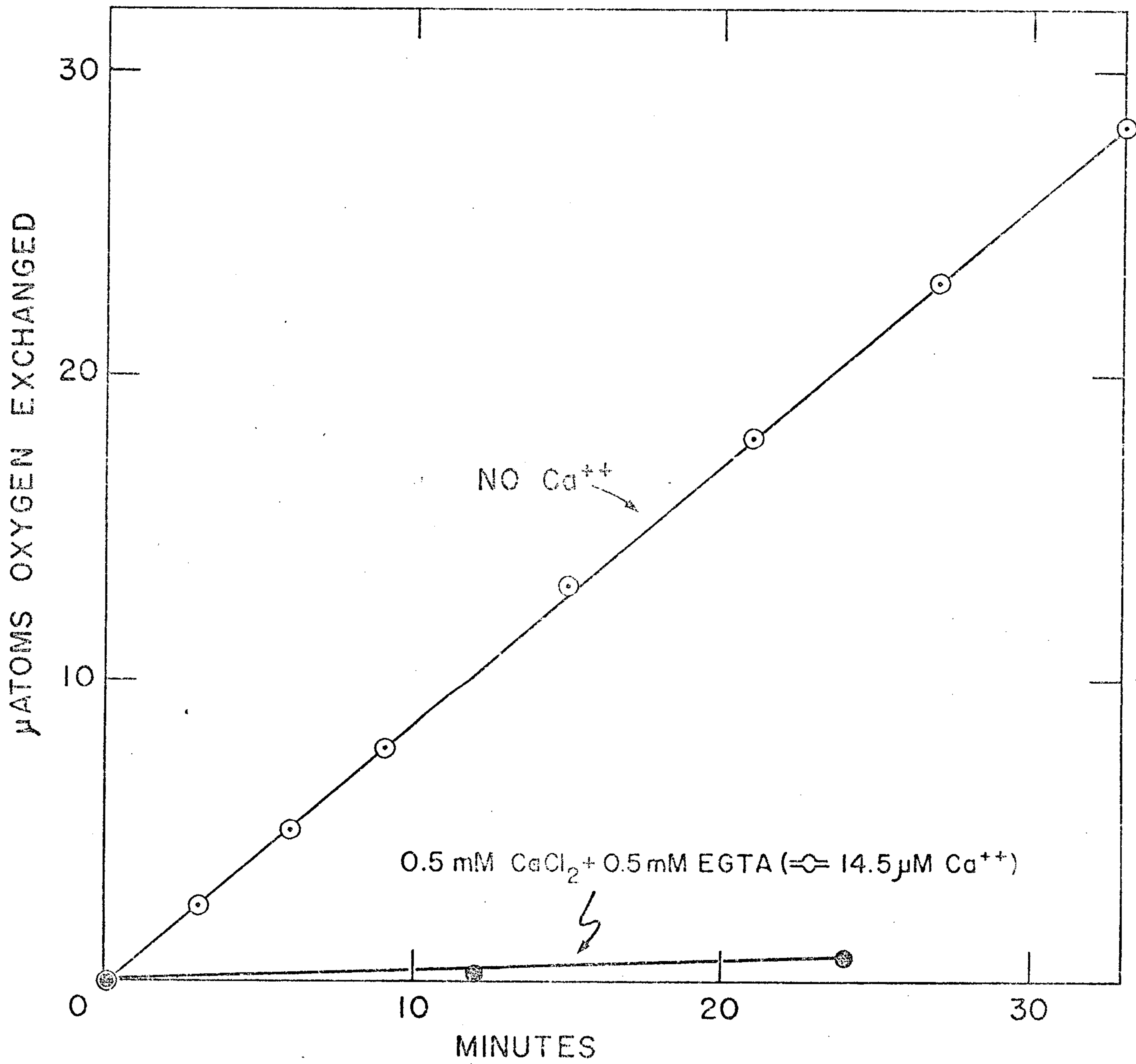
Sarcoplasmic reticulum vesicles, equivalent to 0.5 mg of protein, were incubated for 3 min at 37° and pH 7.0 without potential inhibitors or with 5.56 μg/ml oligomycin, 0.222 mM 2,4-dinitrophenol or 1.11 mM ouabain in 0.9 ml of $H^{18}OH$ (0.88 atom % excess ^{18}O) containing 5.56 mM EGTA, 5.56 mM $MgCl_2$, 111 mM KCl and 111 mM Tris-HCl. A second incubation series had the same composition as above except for 0.556 mM EGTA and 0.556 mM $CaCl_2$. $P_i \rightleftarrows$ HOH exchange reactions were initiated by adding 0.1 ml of 400 mM potassium phosphate at pH 7.0 to give final concentrations of 40 mM P_i and 5 mM EGTA or 40 mM P_i, 0.5 mM $CaCl_2$ and 0.5 mM EGTA; 5 mM EGTA without added $CaCl_2$ almost completely removed free Ca^{++} ions, while in the presence of 40 mM P_i and 0.5 mM $CaCl_2$, 0.5 mM EGTA left 14.5 μM free Ca^{++} ions. The reactions were quenched at the times indicated by adding 1 ml of 8% perchloric acid and determinations of ^{18}O incorporated into P_i were made as described under "Experimental Procedure." In a control the procedure was the same as above except that the preincubation mixture contained none of oligomycin, 2,4-dinitrophenol and ouabain. ○ —— ○, no further additions; ◎ —— ◎, 5 μg/ml oligomycin; ▲ —— ▲, 0.2 mM 2,4-dinitrophenol; □ —— □, 1 mM ouabain.


WATER OXYGEN ATOMS INCORPORATED PER P_i MOLECULE
3
2
1
0
3
6
9
12
MINUTES
NO Ca^{++}
0.5 mM $CaCl_2$ + 0.5 mM EGTA (=○= 14.5 μM Ca^{++})

LEGEND FOR FIGURE 2

TIME COURSE OF THE $P_i \rightleftarrows$ HOH EXCHANGE

$P_i \rightleftarrows$ HOH exchange reactions were initiated by adding 0.05 ml of sarcoplasmic reticulum vesicles (10 mg of protein per ml) suspended in 100 mM KCl-5 mM Tris-maleate at pH 6.5, to 0.95 ml of $H^{18}OH$ (1.24 atom % excess ^{18}O) containing 5.26 mM EGTA, 42.1 mM potassium phosphate, 5.26 mM $MgCl_2$, 105 mM KCl and 105 mM Tris-HCl at pH 7.0, or to 0.95 ml of $H^{18}OH$ with the same composition as above except for 0.526 mM $CaCl_2$ and 0.526 mM EGTA, at 15°. The reactions were quenched at the time indicated by adding 1 ml of 8% perchloric acid and determinations of ^{18}O incorporated into P_i and calculations of the amount of oxygen atoms exchanged were made as described under "Experimental Procedure." ○——○, 5 mM EGTA (final concentration) without added $CaCl_2$; ◎——◎, 0.5 mM $CaCl_2$ + 0.5 mM EGTA (final concentration).



μATOMS OXYGEN EXCHANGED
30
20
10
0
10
20
30
MINUTES
NO Ca++
0.5 mM CaCl2 + 0.5 mM EGTA (= 14.5 μM Ca++)

LEGEND FOR FIGURE 3

INHIBITION OF THE $P_i \rightleftarrows$ HOH EXCHANGE BY Ca^{++} IN THE MEDIUM

$P_i \rightleftarrows$ HOH exchange reactions were carried out for 30 min as described in Fig. 2 except that free Ca^{++} concentrations in the reaction medium were varied using a Ca-EGTA buffer, where final concentration of $CaCl_2$ in the reaction medium was fixed at 0.5 mM and final concentration of EGTA was changed in the range from 0.3 to 5.0 mM. Removal of nearly all free Ca^{++} ion from the medium was achieved by adding EGTA to give a final concentration of 5 mM without added $CaCl_2$. Determinations of ^{18}O incorporated into P_i and calculations of the $P_i \rightleftarrows$ HOH exchange rates and free Ca^{++} concentrations in the medium were made as described under "Experimental Procedure."

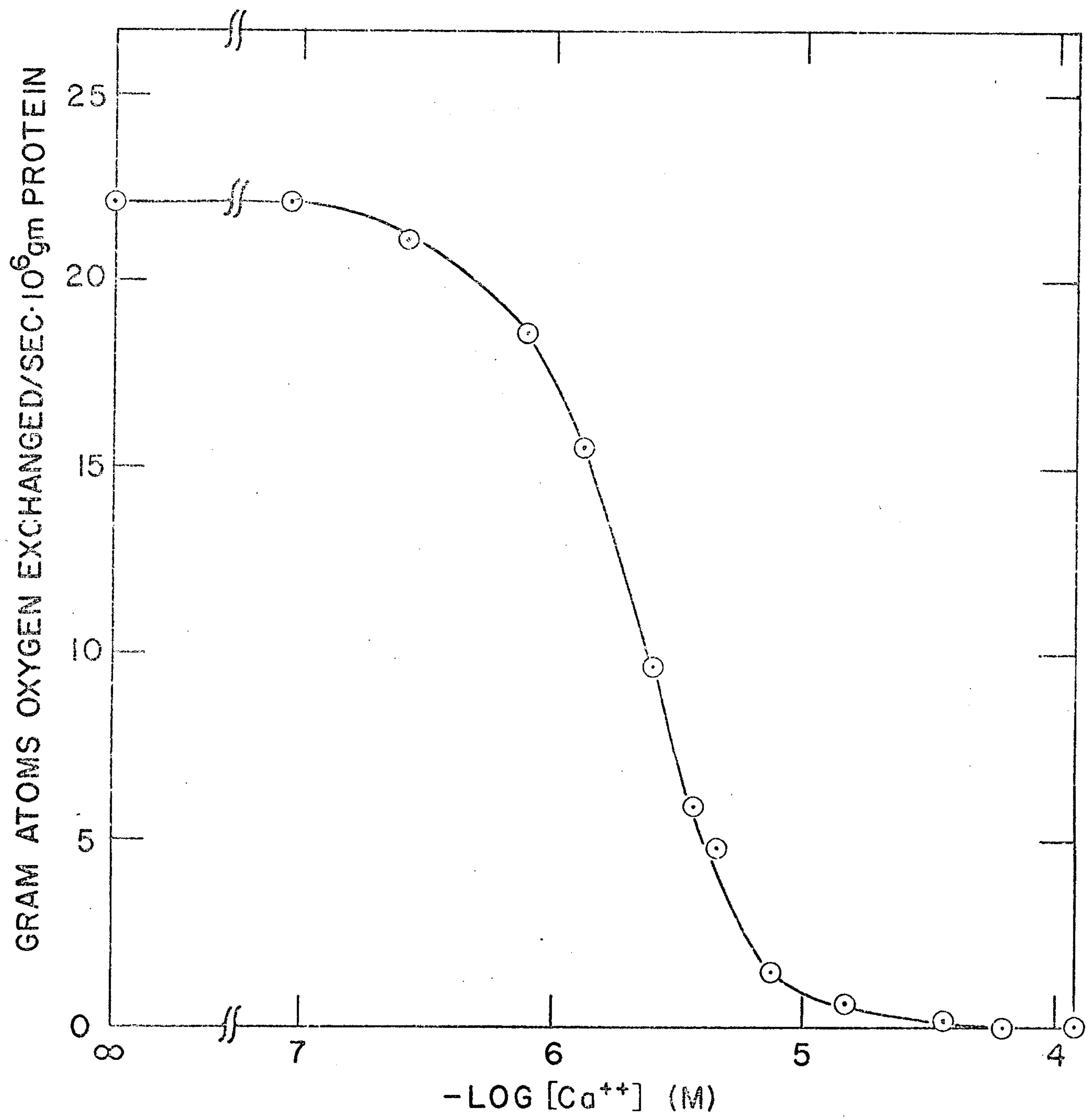
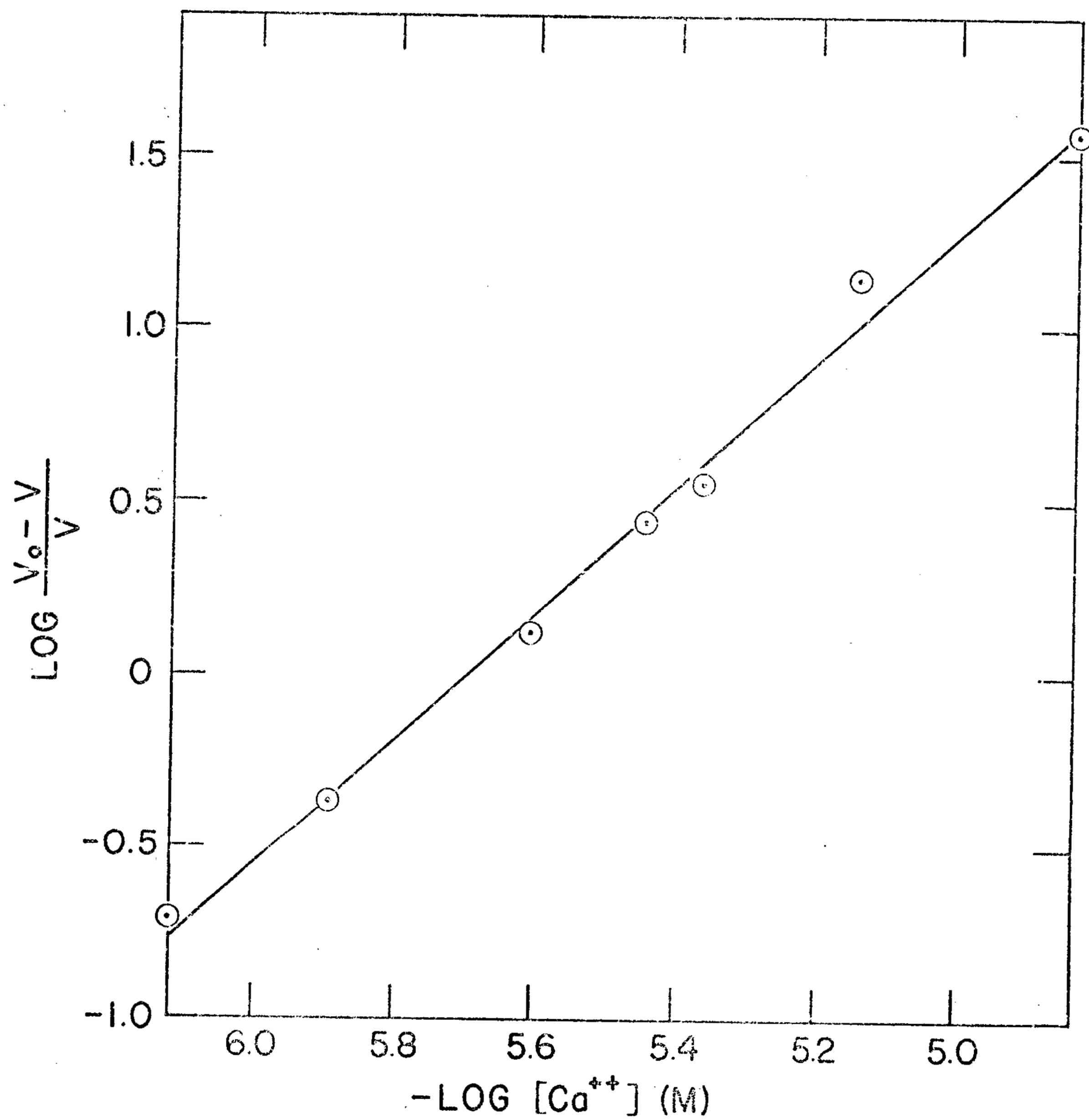


Fig 3

LEGEND FOR FIGURE 4

HILL PLOT OF Ca^{++} INHIBITION OF THE $P_i \rightleftarrows$ HOH EXCHANGE

The rates of $P_i \rightleftarrows$ HOH exchange (V) are taken from those given in Fig. 3. V_o represents the rate of $P_i \rightleftarrows$ HOH exchange in the absence of free Ca^{++}, i.e. in the presence of 5 mM EGTA without added $CaCl_2$.

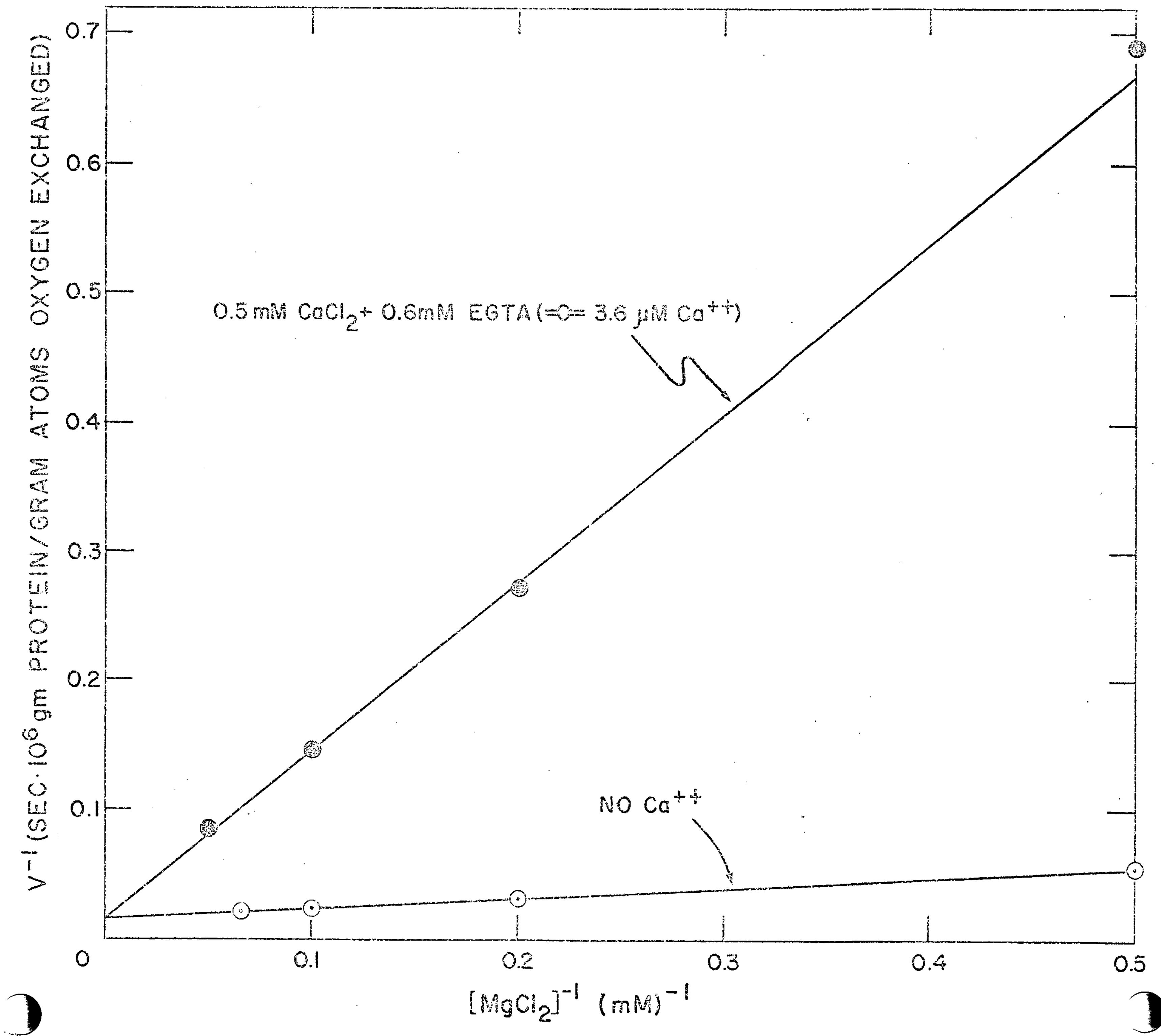


1.5
1.0
0.5
0
-0.5
-1.0
LOG $\frac{V_o - V}{V}$
6.0
5.8
5.6
5.4
5.2
5.0
-LOG [Ca^{++}] (M)

LEGEND FOR FIGURE 5

EFFECT OF Ca^{++} ION ON THE Mg^{++}-DEPENDENCE OF THE $P_i \rightleftarrows$ HOH EXCHANGE

$P_i \rightleftarrows$ HOH exchange reactions were initiated by adding 0.05 ml of sarcoplasmic reticulum vesicles (20 mg of protein per ml) to 0.95 ml of $H^{18}OH$ containing all other reagents at pH 7.0 and 15°. The reaction mixture had a final composition of $H^{18}OH$ (1.19 atom % excess ^{18}O), sarcoplasmic reticulum vesicles (1.0 mg protein per ml), 40 mM potassium phosphate, 100 mM KCl, 100 mM Tris-HCl, $MgCl_2$ as indicated and 1 mM EGTA, or had the same composition as above except for 0.5 mM $CaCl_2$ and 0.6 mM EGTA. 1 mM EGTA without added $CaCl_2$ almost completely removed free Ca^{++} ions, while in the presence of 40 mM P_i and 0.5 mM $CaCl_2$, 0.6 mM EGTA left 3.6 μM free Ca^{++} ions. The rate of the $P_i \rightleftarrows$ HOH exchange (V) was determined as described under "Experimental Procedure." ○ —— ○, 1 mM EGTA without added $CaCl_2$, 30 min reaction; ● —— ●, 0.5 mM $CaCl_2$ + 0.6 mM EGTA, 60 min reaction.

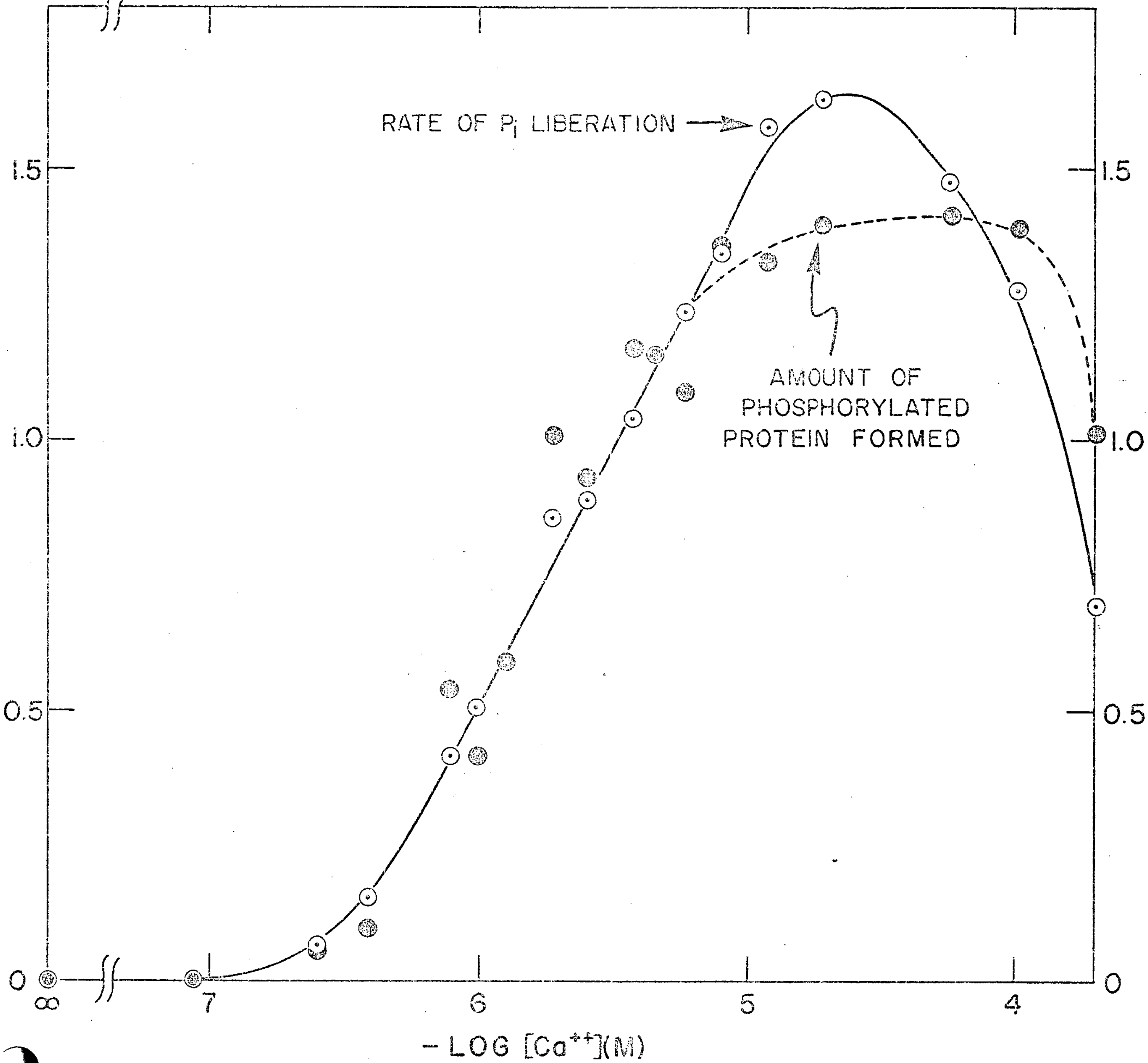


V^{-1} (SEC·10^{6}gm PROTEIN/GRAM ATOMS OXYGEN EXCHANGED)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.5 mM CaCl$_2$+ 0.6mM EGTA (=C= 3.6 μM Ca^{++})
NO Ca^{++}
0.1
0.2
0.3
0.4
0.5
[MgCl$_2$]$^{-1}$ (mM)$^{-1}$

LEGEND FOR FIGURE 6

DEPENDENCE OF THE RATE OF P_i LIBERATION AND AMOUNT OF PHOSPHORYLATED PROTEIN FORMED IN THE STEADY STATE ON THE Ca^{++} ION CONCENTRATION

ATPase reactions were initiated by adding 0.1 ml of 50 μM $AT^{32}P$ to 0.9 ml of reaction mixtures containing sarcoplasmic reticulum vesicles (0.011 mg protein), 5.56 mM $MgCl_2$, 111 mM KCl, 111 mM Tris-HCl, 0.556 mM $CaCl_2$ and 0.3 to 5.0 mM EGTA at pH 7.0 or to 0.9 ml of a reaction mixture with the same composition as above except for 5.56 mM EGTA without added $CaCl_2$, at 15°. Reactions were quenched by adding 1 ml of a mixture of 8% perchloric acid containing 1 mM carrier ATP and 0.04 mM carrier P_i 10 and 60 sec after the start of reactions. $^{32}P_i$ liberated and amounts of phosphorylated protein formed were measured as described under "Experimental Procedure." The amounts of phosphorylated protein were measured only with the samples of 60 sec reactions, while the rates of P_i liberation were calculated from the difference in the amount of P_i liberated between 10 and 60 sec after the start of reactions. These are presented in the figure after subtracting the rate of P_i liberation and amount of ^{32}P incorporated into protein in the presence of 5 mM EGTA without added $CaCl_2$, respectively, from those with added $CaCl_2$. Free Ca^{++} ion concentrations were calculated as described under "Experimental Procedure."

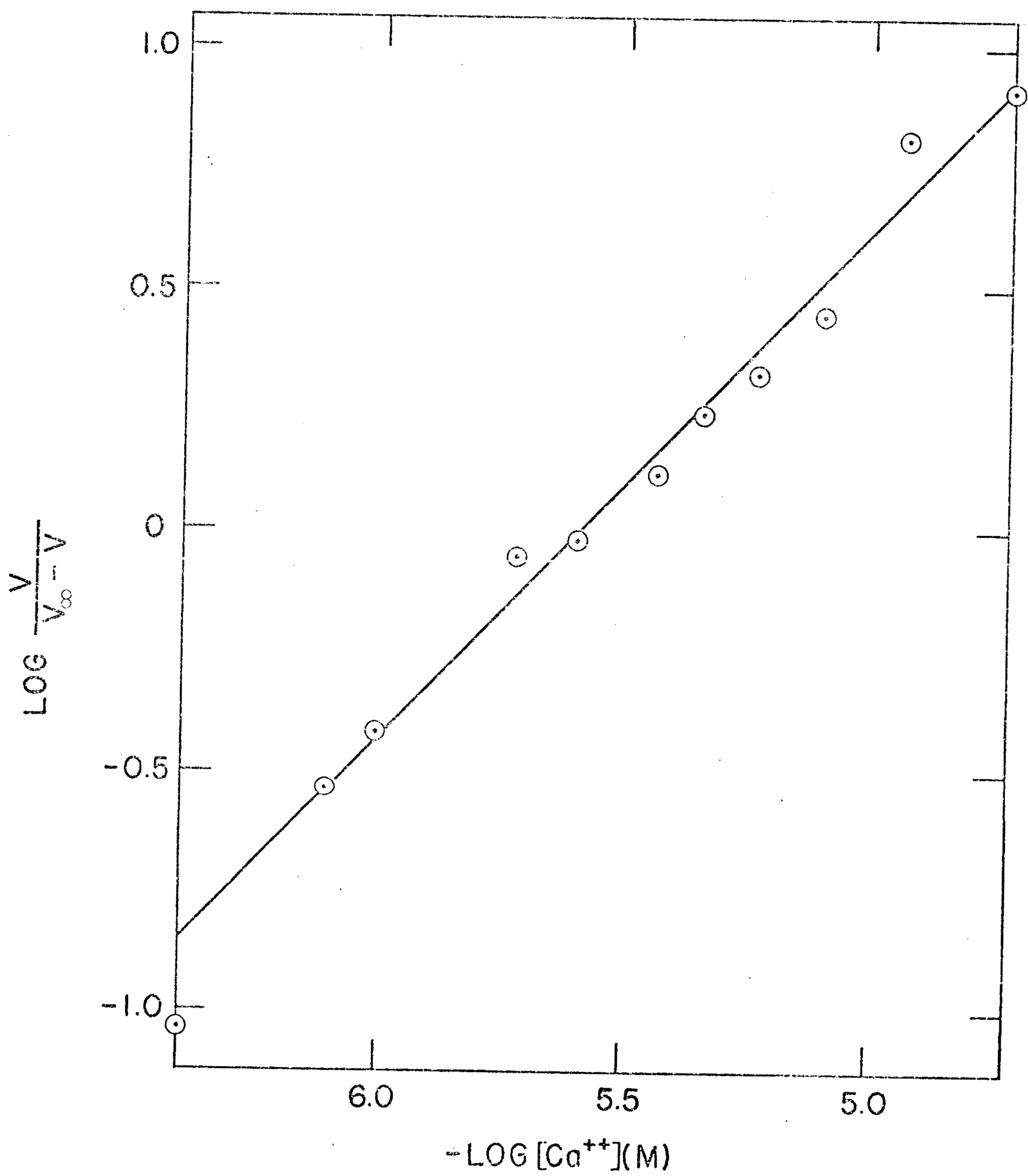


RATE OF P_i LIBERATION
AMOUNT OF PHOSPHORYLATED PROTEIN FORMED
MOLES P_i LIBERATED/SEC·10^6gm PROTEIN
MOLES PHOSPHORYLATED PROTEIN FORMED/10^6gm PROTEIN
0
0.5
1.0
1.5
∞
7
6
5
4
− LOG [Ca^{++}](M)

LEGEND FOR FIGURE 7

HILL PLOT OF Ca^{++} ACTIVATION OF THE ATP HYDROLYSIS

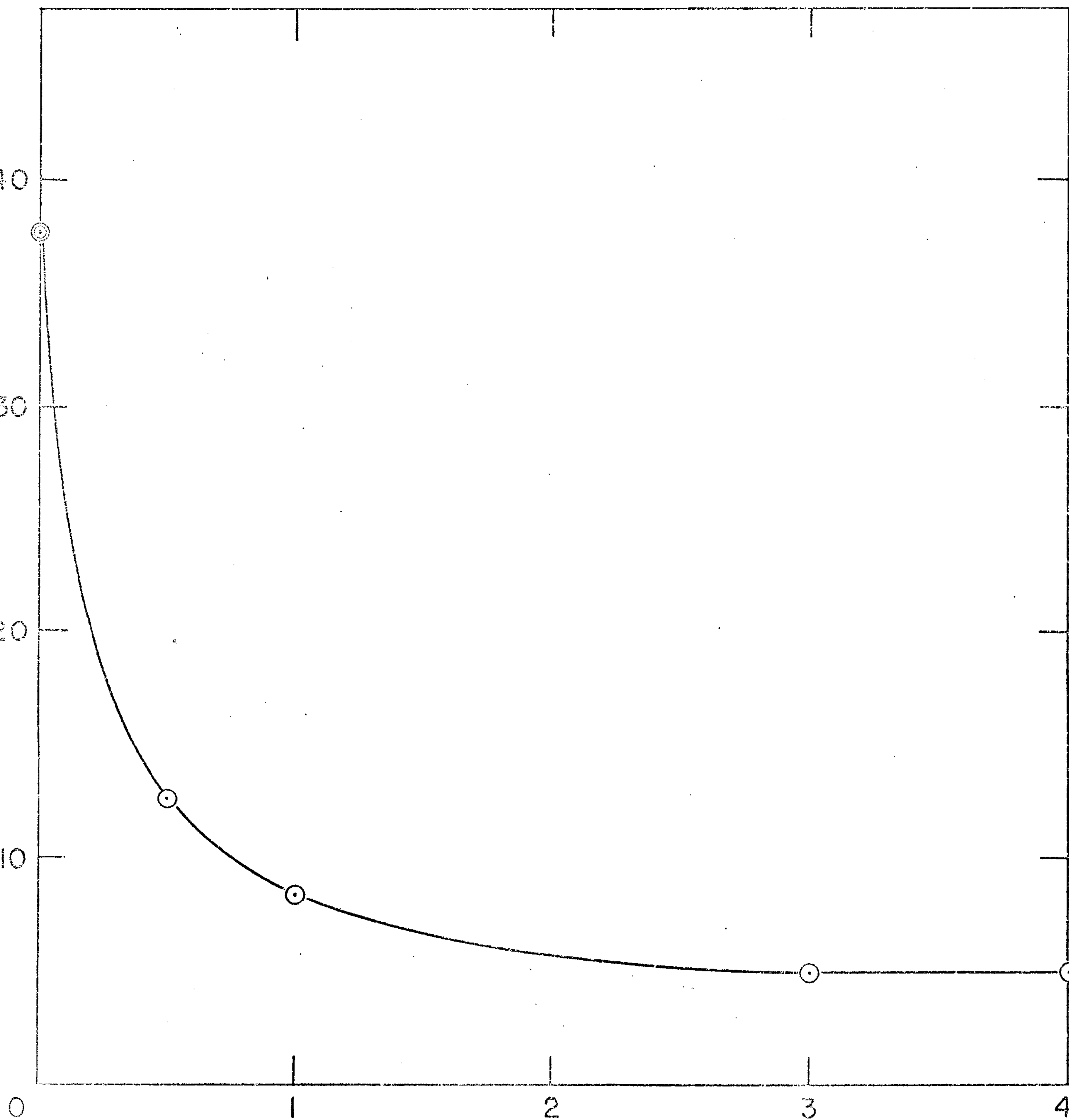
The rates of ATP hydrolysis (V) are taken from those given in Fig. 6. V_{∞} represents the rate of ATP hydrolysis at an infinite concentration of Ca^{++} obtained by extrapolating the double reciprocal plot of the rate of ATP hydrolysis against Ca^{++} ion concentration.


1.0
0.5
0
-0.5
-1.0
LOG $\frac{V}{V_{\infty} - V}$
6.0
5.5
5.0
-LOG [Ca^{++}](M)

LEGEND FOR FIGURE 8

INHIBITION OF THE $P_i \rightleftarrows$ HOH EXCHANGE BY ADDITION OF ADP

$P_i \rightleftarrows$ HOH exchange reactions were initiated as described in Fig. 2 by adding sarcoplasmic reticulum vesicles to give a final composition of $H^{18}OH$ (1.19 atom % excess ^{18}O), sarcoplasmic reticulum vesicles (0.5 mg protein per ml), 40 mM potassium phosphate, 5 mM EGTA, 20 mM $MgCl_2$, 100 mM KCl, 100 mM Tris-HCl and ADP as indicated at pH 7.0, 15°. Reactions were quenched by adding 1 ml of 8% perchloric acid 20 min after addition of the vesicles, and determinations of ^{18}O incorporated into P_i and calculations of the $P_i \rightleftarrows$ HOH exchange rates were made as described under "Experimental Procedure."

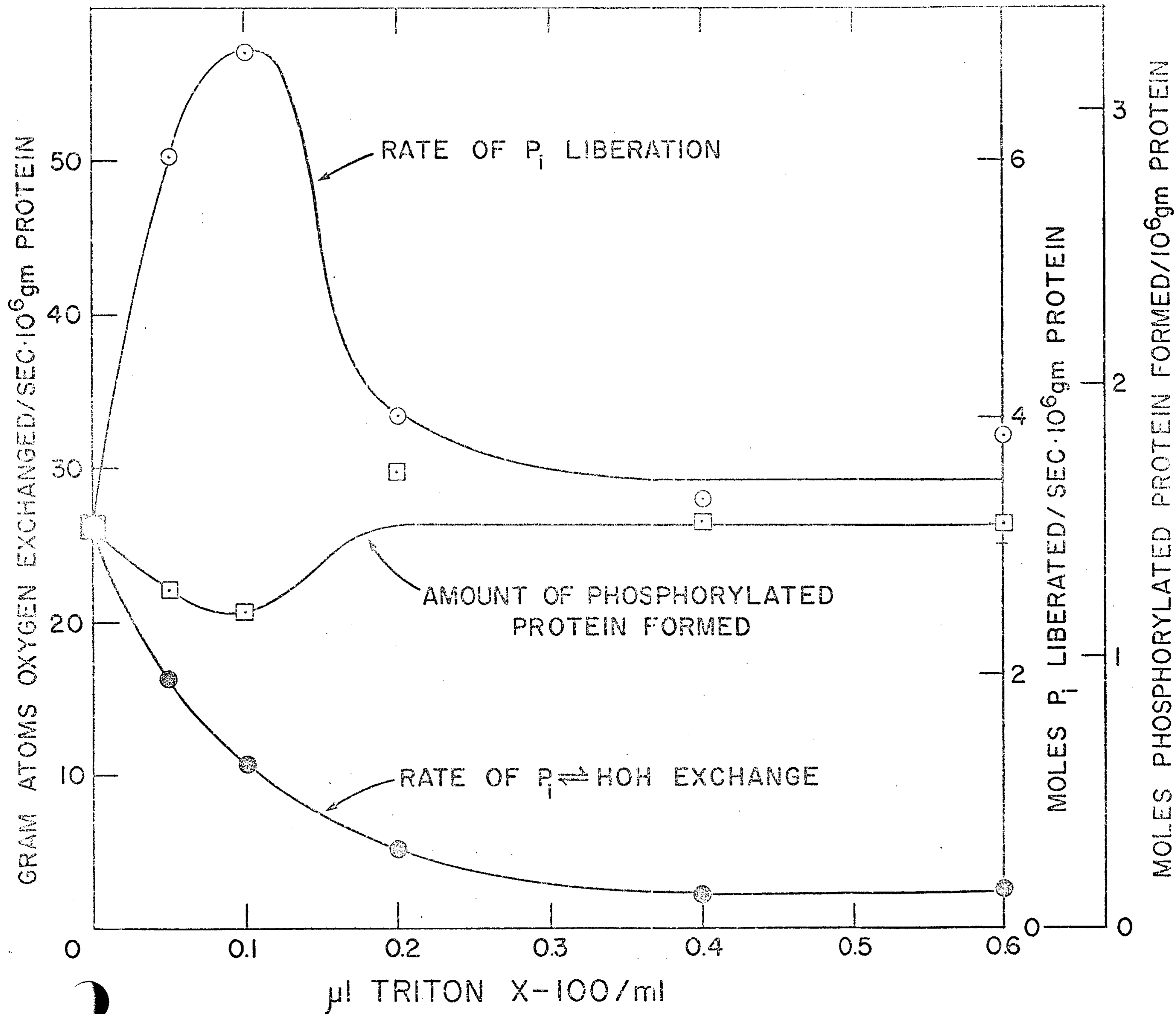


GRAM ATOMS OXYGEN EXCHANGED / SEC · 10⁶ gm PROTEIN
40
30
20
10
0
1
2
3
4
ADP CONCENTRATION ADDED (mM)

LEGEND FOR FIGURE 9

EFFECT OF TRITON X-100 ON THE $P_i \rightleftarrows$ HOH EXCHANGE AND ATPase REACTIONS

For the $P_i \rightleftarrows$ HOH exchange measurements, sarcoplasmic reticulum vesicles (0.5 mg of protein) were incubated in 0.8 ml of $H^{18}OH$ (0.96 atom % excess ^{18}O) containing Triton X-100 at varying concentrations, 0.625 mM EGTA, 6.25 mM $MgCl_2$, 125 mM KCl and 125 mM Tris-HCl at pH 7.0, 15°, for 20 to 30 min, and then $P_i \rightleftarrows$ HOH exchange reactions were initiated by adding 0.2 ml of 200 mM potassium phosphate at pH 7.0 to the incubation mixture at 15°. Reactions were quenched by adding 1 ml of 8% perchloric acid 40 min after the addition of P_i, and determinations of ^{18}O incorporated into P_i and calculations of the $P_i \rightleftarrows$ HOH exchange rates were made as described under "Experimental Procedure." For the ATPase measurements, sarcoplasmic reticulum vesicles (0.5 mg of protein) were incubated in 0.775 ml of a medium containing Triton X-100 at varying concentrations, 0.645 mM EGTA, 6.45 mM $MgCl_2$, 129 mM KCl and 129 mM Tris-HCl at pH 7.0, 15°, for 30 to 50 min, and then 0.025 ml of 20 mM $CaCl_2$ or water alone was added. ATPase reactions were initiated by adding 0.2 ml of 181 µM $AT^{32}P$ to the incubation mixture at pH 7.0, 15°, 30 sec after addition of $CaCl_2$ or water. Reactions were quenched by adding 4 ml of 5% perchloric acid containing 5 mM ATP 10 sec after the addition of $AT^{32}P$. $^{32}P_i$ liberated and amounts of phosphorylated protein formed were measured as described under "Experimental Procedure." The rates of P_i-liberation and amounts of phosphorylated protein presented in the figure are corrected for the rate of P_i liberation and amount of ^{32}P incorporated into protein, respectively, in samples with added $CaCl_2$ at each concentration of Triton X-100. The abscissa indicates the concentration of Triton X-100 in the reaction medium after addition of P_i or $AT^{32}P$.

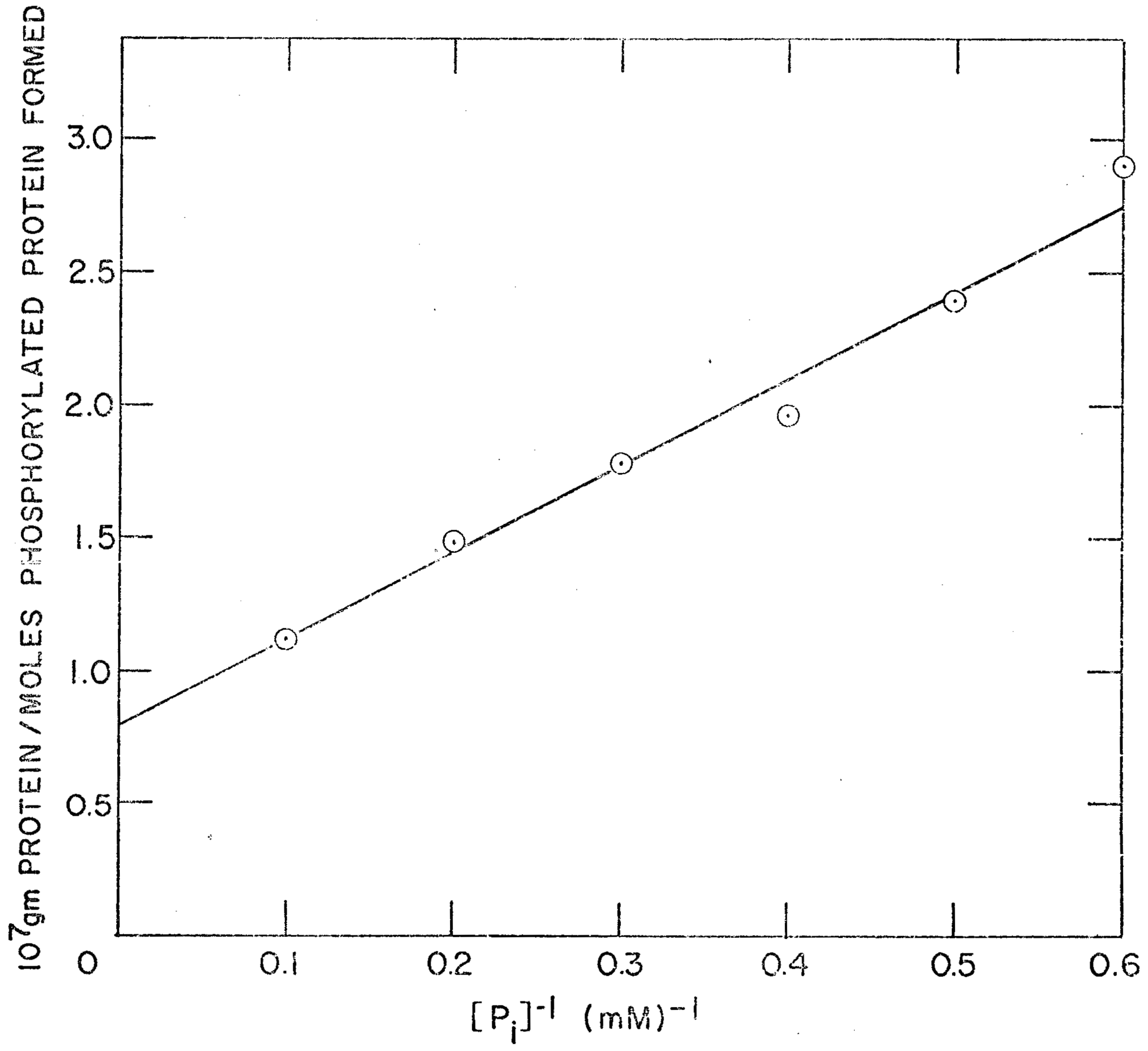


RATE OF P_i LIBERATION
AMOUNT OF PHOSPHORYLATED PROTEIN FORMED
RATE OF $P_i \rightleftharpoons$ HOH EXCHANGE
GRAM ATOMS OXYGEN EXCHANGED/SEC·10^6gm PROTEIN
MOLES P_i LIBERATED/SEC·10^6gm PROTEIN
MOLES PHOSPHORYLATED PROTEIN FORMED/10^6gm PROTEIN
μl TRITON X-100/ml
0
0.1
0.2
0.3
0.4
0.5
0.6
10
20
30
40
50
2
4
6
1
3

LEGEND FOR FIGURE 10

DOUBLE RECIPROCAL PLOT OF THE AMOUNT OF PHOSPHORYLATED PROTEIN FORMED FROM P_i AGAINST P_i CONCENTRATION

Sarcoplasmic reticulum vesicles, equivalent to 3 mg of protein, were incubated for 30 sec at pH 7.0 and 15° in 1.0 ml of a medium containing 1.67-10 mM $^{32}P_i$, 20 mM $MgCl_2$, 100 mM KCl, 100 mM Tris-HCl and 5 mM EGTA. The amount of ^{32}P incorporated into the protein was measured as described under "Experimental Procedure." The amount of ^{32}P-incorporation independent of Mg^{++} was measured at each $^{32}P_i$ concentration under the same conditions as above except for 5 mM EDTA without added $MgCl_2$ and was subtracted from the above value obtained at corresponding $^{32}P_i$ concentration before plotting.



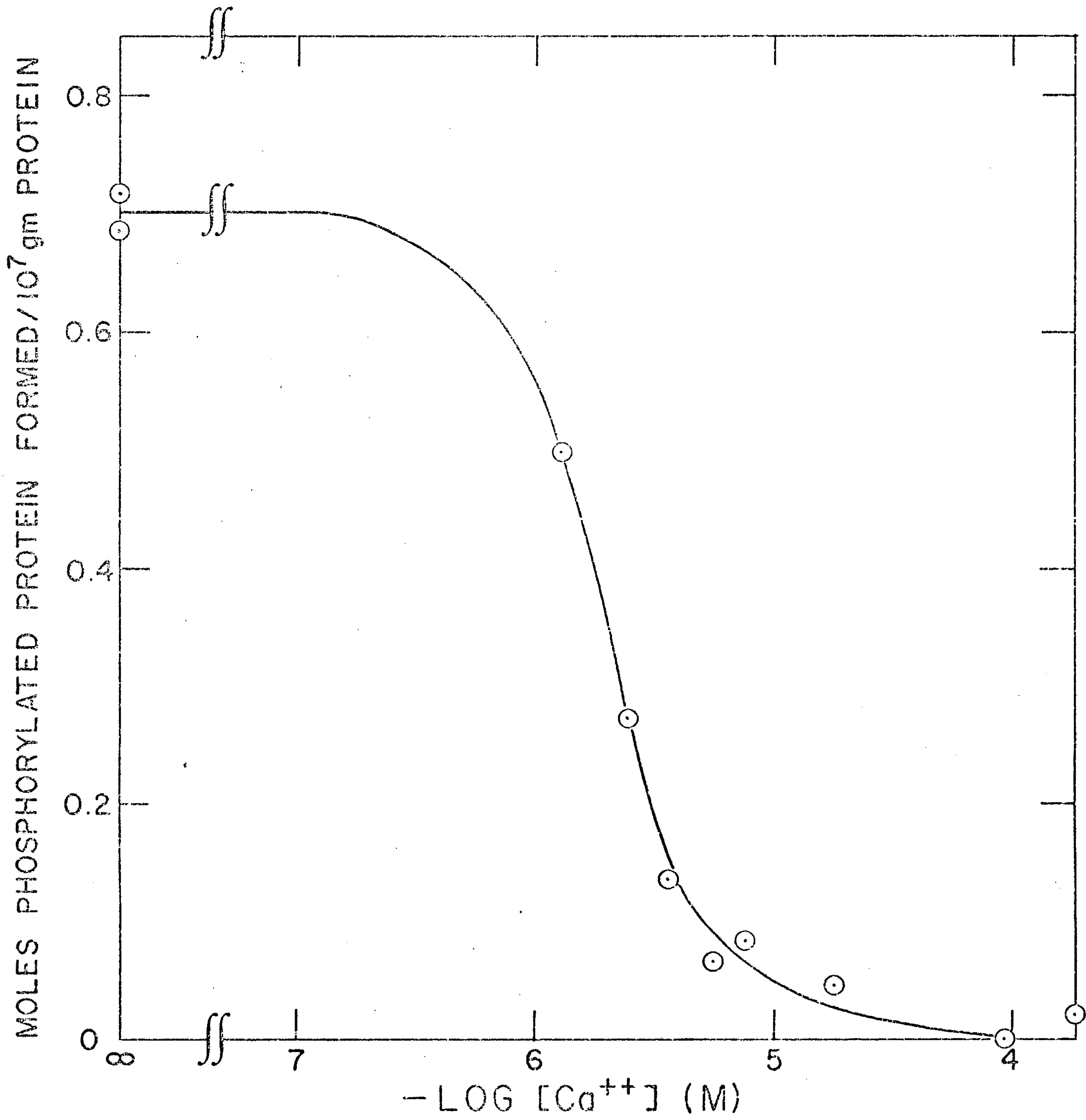
10^7gm PROTEIN / MOLES PHOSPHORYLATED PROTEIN FORMED
3.0
2.5
2.0
1.5
1.0
0.5
0
0.1
0.2
0.3
0.4
0.5
0.6
$[P_i]^{-1}$ $(mM)^{-1}$

LEGEND FOR FIGURE 11

INHIBITION OF THE FORMATION OF PHOSPHORYLATED

PROTEIN FROM P_i BY Ca^{++} IN THE MEDIUM

Sarcoplasmic reticulum vesicles, equivalent to 3 mg of protein, were incubated for 30 sec at pH 7.0 and 15° in 1.0 ml of a medium containing 5 mM $^{32}P_i$, 5 mM $MgCl_2$, 100 mM KCl, 100 mM Tris-HCl, 0 or 0.5 mM $CaCl_2$, and 0.3-5.0 mM EGTA. Free Ca^{++} concentrations in the incubation medium were varied using a Ca-EGTA buffer, where $CaCl_2$ concentration in the medium was fixed at 0.5 mM and EGTA concentration was changed in the range from 0.3 to 2.0 mM. Removal of nearly all free Ca^{++} ions from the medium was achieved by adding EGTA to give a final concentration of 5 mM without added $CaCl_2$. The amount of ^{32}P incorporated into the protein was measured as described under "Experimental Procedure" and was plotted after subtracting the amount of ^{32}P incorporated under the same conditions as above except for 5 mM EDTA without added $MgCl_2$. Free Ca^{++} ion concentrations in the medium were calculated as described under "Experimental Procedure" except that formation of $MgHPO_4$ from Mg^{++} and $HPO_4^{=}$ with association constant of 74 M^{-1} (24) was taken account of for this calculation.

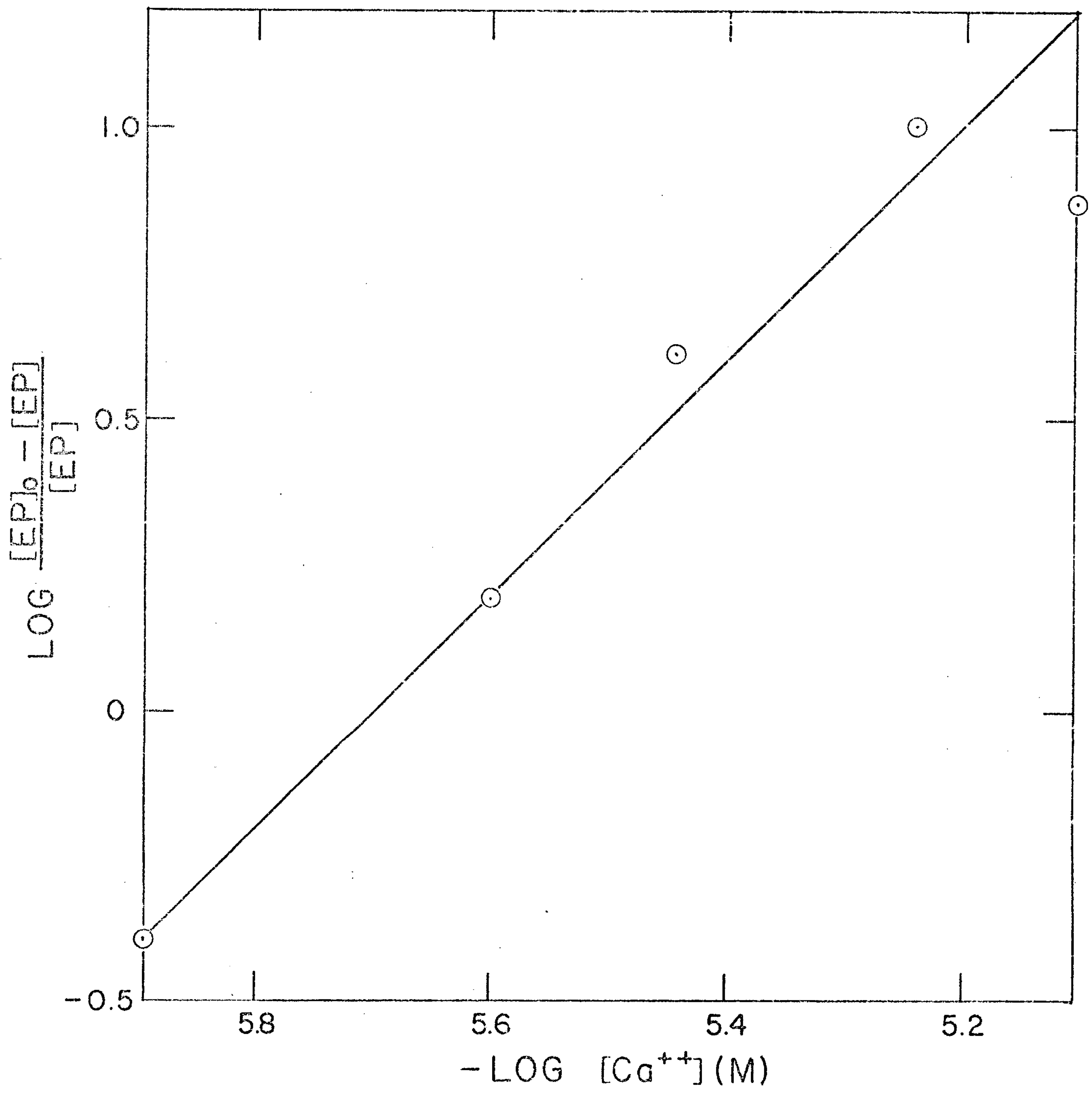

MOLES PHOSPHORYLATED PROTEIN FORMED/10^7 gm PROTEIN
0.8
0.6
0.4
0.2
0
∞
7
6
5
4
−LOG [Ca^{++}] (M)

LEGEND FOR FIGURE 12

HILL PLOT OF Ca^{++} INHIBITION OF THE FORMATION

OF PHOSPHORYLATED PROTEIN FROM P_i

The amount of phosphorylated protein ([EP]) is taken from those given in Fig. 11. $[EP]_o$ represents the amount of phosphorylated protein in the absence of free Ca^{++}, i.e. in the presence of 5 mM EGTA without added $CaCl_2$.



1.0
0.5
0
−0.5
LOG $\frac{[EP]_o - [EP]}{[EP]}$
5.8
5.6
5.4
5.2
−LOG [Ca^{++}] (M)

LEGEND FOR FIGURE 13

SCHEME FOR CATION TRANSPORT MEDIATED BY Ca^{++}, Mg^{++}-ATPase
IN SARCOPLASMIC RETICULUM MEMBRANES

E and EP represent, respectively, nonphosphorylated and phosphorylated enzymes which have two binding sites with high affinity for Ca^{++} but not for Mg^{++} on each molecule and catalyze translocation of Ca^{++} across membranes (Ca^{++}-carriers). E* and E*P indicate, respectively, nonphosphorylated and phosphorylated enzymes which have one binding site with an affinity for Mg^{++} but not for Ca^{++} on each molecule and catalyze translocation of Mg^{++} across membranes (Mg^{++}-carriers).

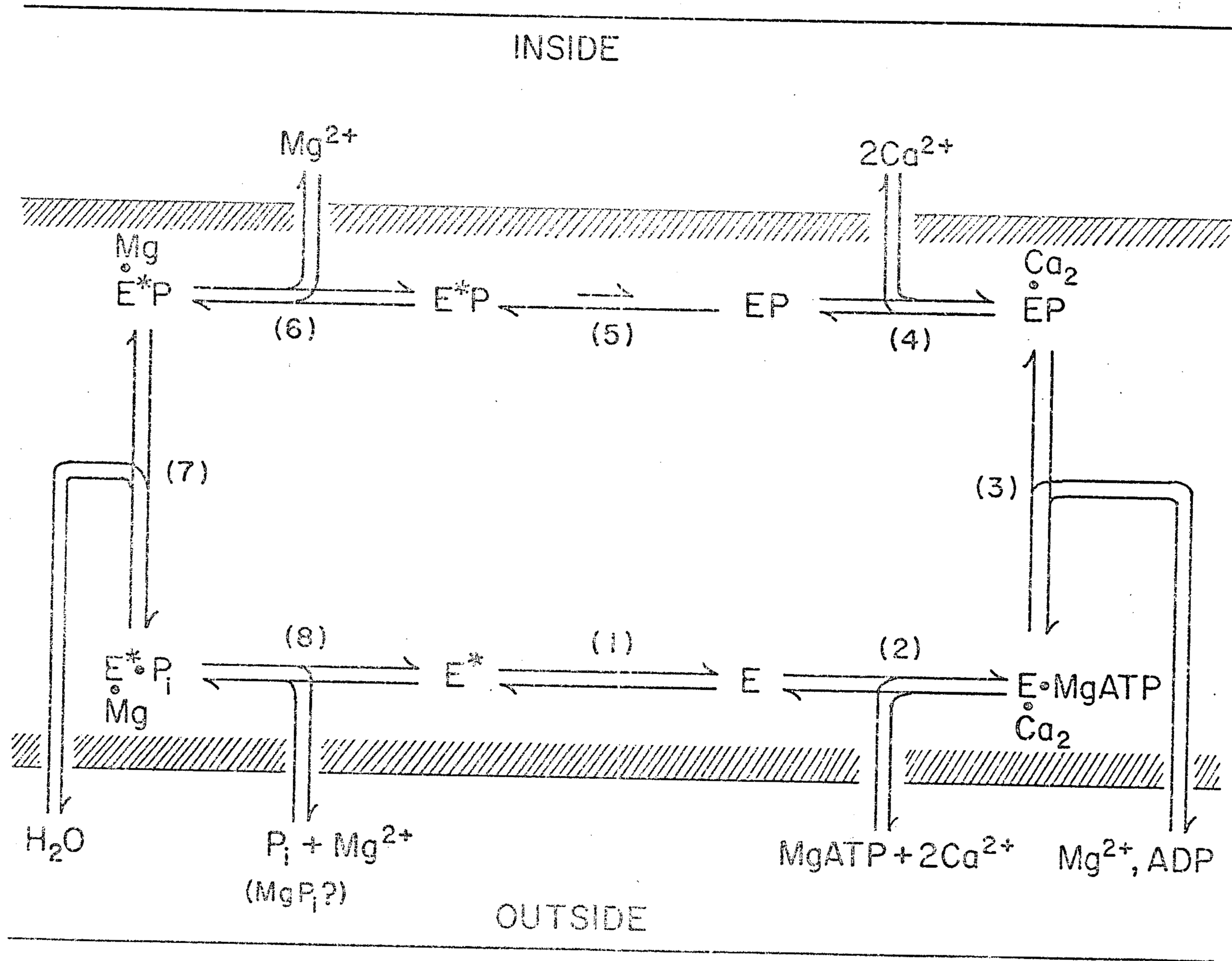

INSIDE
Mg^{2+}
$2Ca^{2+}$
Mg
E*P
(6)
E*P
(5)
EP
(4)
Ca_2
EP
(7)
(3)
(8)
(1)
(2)
$E^{*} \cdot P_i$
Mg
E^{*}
E
E·MgATP
Ca_2
H_2O
$P_i + Mg^{2+}$
$(MgP_i?)$
$MgATP + 2Ca^{2+}$
Mg^{2+}, ADP
OUTSIDE

TABLE I

EFFECT OF ATP ON THE $P_i \rightleftarrows$ HOH EXCHANGE

Reactions were initiated by adding 0.05 ml of sarcoplasmic reticulum vesicles to 0.45 ml of a reaction medium containing all other constituents. Reaction mixtures contained, at the start of reactions, sarcoplasmic reticulum vesicles (0.5 mg protein per ml), 40 mM potassium phosphate-^{18}O with 0.432 atom % excess ^{18}O, 7 mM $MgCl_2$, 100 mM KCl and 100 mM Tris-HCl at pH 7.0, 15°, as well as 0 or 0.5 mM $CaCl_2$, 0.4 or 5.0 mM EGTA and 0 or 4.0 mM $AT^{32}P$ as indicated. 0.5 mM $CaCl_2$, 0.4 mM EGTA and 40 mM P_i gave 60.4 μM free Ca^{++} to the reaction medium. 1.5 ml of 5.3% perchloric acid was added to the reaction mixture before (as zero time) or 40 min after addition of sarcoplasmic reticulum vesicles. Loss of ^{18}O from P_i-^{18}O and hydrolysis of $AT^{32}P$ in the 40 minute reactions were measured and the rates of $P_i \rightleftarrows$ HOH exchange were calculated as described under "Experimental Procedure."

$CaCl_2$ added	EGTA added	ATP concentration		Rate of $P_i \rightleftarrows$ HOH exchange
		Initial	Final	
mM	mM	mM		gram atoms oxygen exchanged/ sec·10^6 gm protein
0	5.0	0	0	22.5
0	5.0	0	0	24.3
0	5.0	4.00	2.90	1.1
0	5.0	4.00	2.89	0.1
0.5	0.4	0	0	0
0.5	0.4	0	0	0
0.5	0.4	4.00	1.52	0
0.5	0.4	4.00	1.52	0

TABLE II

EFFECT OF ACETYLPHOSPHATE ON THE $P_i \rightleftarrows$ HOH EXCHANGE

$P_i \rightleftarrows$ HOH exchange reactions were performed at pH 7.0, 15°, for 40 min as described in Table I except that acetylphosphate was added in place of $AT^{32}P$ to the medium to give 3.3 mM at the start of reactions. The addition of acetylphosphate resulted in an increase in P_i concentration by 0.65 mM at the start of reactions since acetylphosphate used was contaminated with P_i. Determination of loss of ^{18}O from P_i-^{18}O in the 40 minute reactions and calculation of the rate of $P_i \rightleftarrows$ HOH exchange were made as described under "Experimental Procedure."

$CaCl_2$ added	EGTA added	Acetylphosphate added	Rate of $P_i \rightleftarrows$ HOH exchange
mM	mM	mM	gram atoms oxygen exchanged/sec·10^6 gm protein
0	5.0	0	23.3
0	5.0	0	28.8
0	5.0	3.3	20.6
0	5.0	3.3	22.5
0.5	0.4	0	0
0.5	0.4	0	0
0.5	0.4	3.3	7.0
0.5	0.4	3.3	3.6

TABLE III

PHOSPHORYLATION OF THE SARCOPLASMIC RETICULUM PROTEIN BY P_i

Sarcoplasmic reticulum vesicles, equivalent to 3 mg of protein, were incubated for 30 sec at pH 7.0 and 15° in 1.0 ml of a medium containing 5 mM $^{32}P_i$, 100 mM KCl, 100 mM Tris-HCl and other reagents as indicated. The reaction was quenched by adding 10 ml of 4% perchloric acid and ^{32}P incorporated into the protein was measured as described under "Experimental Procedure."

Addition				^{32}P incorporated
$MgCl_2$	$CaCl_2$	EDTA	EGTA	into the protein
mM	mM	mM	mM	mole/10^7 gm protein
5	0	0	5	0.94
5	0.1	0	0	0.05
0	0	1	5	0.03