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Membrane Adenosine Triphosphatase of *Escherichia coli*: Activation by Calcium Ion and Inhibition by Monovalent Cations[1]

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Membrane ghost preparations of *Escherichia coli* K-12 obtained by osmotic lysis of lysozyme-induced spheroplasts were found to possess both Mg^{++}- and Ca^{++}-activated adenosine 5'-triphosphatase (ATPase, EC 3.6.1.3) activities. Maximal activities of 1.0 to 1.5 μmoles of orthophosphate released per min per mg of protein were obtained at *p*H 9.0 with a molar Mg^{++} to adenosine 5'-triphosphate (ATP) ratio of 2:5 and at *p*H 9.9 with a molar Ca^{++} to ATP ratio of 1:5. These ATPase activities were not altered by ouabain, fluoride, *N*-ethylmaleimide, 2,4-dinitrophenol, cyanide, or dithionite, but were inhibited by low concentrations of azide, *p*-chloromercuribenzoate, and pentachlorophenol. Mg^{++} ATPase was more susceptible to inhibition by azide than was Ca^{++} ATPase. Fifty per cent inactivation of both activities was observed when membrane ghost preparations were preincubated at 66 C for 10 min. The Mg^{++} and Ca^{++} ATPase activities of these preparations were not additive, but did respond independently to inhibition by monovalent cations. Ca^{++} ATPase was found to be very sensitive to inhibition by K^+, Na^+, Li^+, Rb^+, and Cs^+; Mg^{++} ATPase was relatively insensitive to these ions. One possible interpretation of the results presented in this paper is that the membrane of *E. coli* possesses an ATPase which is activated by either Mg^{++} or Ca^{++} and that activation by Ca^{++} increases the susceptibility of this enzyme to inhibition by monovalent cations. Increased susceptibility of *E. coli* membrane ATPase to inhibition by monovalent cations such as Na^+ and K^+ as a consequence of Ca^{++} activation could represent a regulatory mechanism.

Adenosine 5'-triphosphatase (ATPase) activities have been demonstrated in a large variety of gram-positive and gram-negative bacteria. These include members of the genera *Streptococcus* (4), *Staphylococcus* (11), *Escherichia* (27), *Vibrio* (14), *Bacillus* (28), *Micrococcus* (15), *Lactobacillus* (7), and *Pseudomonas* (8). There is ample evidence that most, if not all, of these bacteria possess ATPase activities which are bound to or otherwise intimately associated with the cell membrane structure. Although a few bacterial ATPases exhibit some degree of stimulation by Na^+ or K^+, or by both (11, 13, 14), the membrane-associated Mg^{++} ATPases of bacteria clearly do not conform to the well-accepted definition of an ion transport ATPase (ouabain-sensitive, $Na^+ + K^+$-stimulated, Mg^{++}-activated ATPase) put forth by Skou (24, 25) and others (4, 16, 21, 29). Many investigators have pointed out properties of bacterial membrane-associated ATPases which are similar to those of mitochondrial ATPase (1, 7, 10, 11, 15, 20). Nevertheless, the function or functions of bacterial membrane-associated Mg^{++} ATPases and their significance can only be speculated upon at this time.

This laboratory has chosen to investigate the membrane-associated, Mg^{++}-activated ATPase of *E. coli* on the premise that further characterization of this particular enzyme might provide the basis for a more direct approach to the question of the functional significance of this enzymatic activity.

E. coli ATPase activity was first described by Solomon (26), in 1962, and by Voelz (27), in 1964. In 1966, Gunther and Dorn (12) compared the Mg^{++} ATPase activity of *E. coli* strain B-163 and that of its K^+-transport mutant, strain

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B-525 and found no significant differences. Bragg and Hou (5) investigated ATPase activities found in a number of cell fractions of *E. coli*. Hafkenscheid and Bonting (13) reported the occurrence of a ($Na^+ + K^+$)-activated ATPase in freeze-dried *E. coli* cells homogenized in 1.5 M urea. This activity could be differentiated from the much more prominent Mg^{++} ATPase found in the same preparations.

In the present investigation, enzyme preparations consisted of washed membrane ghosts obtained by lysis of lysozyme-induced spheroplasts. For comparative purposes, parallel studies were carried out with the well-characterized (1–4) membrane-associated Mg^{++} ATPase of *S. faecalis* (ATCC 9790).

Although previous investigators (5, 12) reported only slight activation of *E. coli* ATPase by Ca^{++}, reports of Ca^{++} ATPase activities in *B. megaterium* (10, 28) and in *M. lysodeikticus* (15, 19) prompted an investigation into the effects of Ca^{++} on *E. coli* membrane ATPase activity. Significant levels of Ca^{++} ATPase activity were found. This report describes the *p*H dependence and some other properties of this activity, including its relationship to membrane Mg^{++} ATPase activity.

MATERIALS AND METHODS

Preparation of intact cells. *E. coli* K-12 (ATCC 10798) was grown aerobically in Difco Tryptose Phosphate Broth supplemented with 0.5% NaCl and 0.5% yeast extract. *S. faecalis* (ATCC 9790) was grown under the same conditions, except that additional supplements of 0.8% glucose and 1.0% K_2HPO_4 were employed to increase cell yields. Cells were harvested by centrifugation during the mid-exponential phase of growth and were washed three times with distilled water (*S. faecalis*) or 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.5 (*E. coli*).

Preparation of membranes. Washed cells of *S. faecalis* were suspended in 0.15 M Tris-hydrochloride buffer, *p*H 6.4, containing 0.6 M sucrose and 180 μg of lysozyme per ml. Cells of *E. coli* were suspended in the same buffer-sucrose solution containing 20 μg of lysozyme per ml and 1.0 mM ethylenediaminetetraacetate (EDTA). Final volumes were approximately one-fourth that of the original culture. After incubation for 120 min (*S. faecalis*) or 75 min (*E. coli*) at 37 C, the protoplasts or spheroplasts were harvested by centrifugation at 12,000 × *g* for 20 min and were washed once with cold buffer-sucrose solution, *p*H 6.4, by centrifugation under the same conditions. The resultant pellets were suspended in approximately two-thirds the original (culture) volume of cold 0.02 M Tris-hydrochloride, *p*H 7.5. Vigorous stirring resulted in rapid and essentially complete lysis. The resulting membrane "ghosts" were harvested by centrifugation at 35,000 × *g* for 20 min and washed thoroughly three times with 0.02 M Tris-hydrochloride, *p*H 7.5, containing 1.0 mM $MgCl_2$, by repeated resuspension and centrifugation under the same conditions. The final pellets of membranous material were homogeneously suspended in a minimal volume of the Mg^{++}-containing Tris-hydrochloride buffer. Small portions were immediately frozen at −55 C in vials. Only freshly thawed material was employed in the work reported here.

Chemical reagents and substrates. Egg white lysozyme, Na_2 and K_2 adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), ouabain, and sodium *p*-chloromercuribenzoate were obtained from Calbiochem, Los Angeles, Calif. Pentachlorophenol, 2,4-dinitrophenol, sodium dithionite, N-ethylmaleimide, sodium azide, Tris (THAM), and purified LiCl, CsCl, and RbCl were obtained from Fisher Scientific Co., Pittsburgh, Pa. Reagent-grade NaCl, KCl, $MgCl_2$, and $CaCl_2$ were employed. All reagents were prepared in glass-distilled water.

Substrates (ATP, ADP, and AMP) were titrated to *p*H 7.0 with 1.0 M Tris base, adjusted to a final concentration of 0.05 M, divided into small portions, and kept frozen.

Assay methods. In the ATPase assay, reaction mixtures always contained a final concentration of 5.0 mM substrate, prepared as above, and 0.1 M Tris-hydrochloride buffer of the appropriate *p*H. In addition to enzyme (membrane preparation), salts, and other test compounds, distilled water was added to adjust the mixtures to a final volume of 1.0 ml. All test compounds were adjusted to the appropriate *p*H with Tris base or HCl prior to use. The ATPase reaction was initiated by the addition of enzyme and was terminated, after 30 min at 37 C in a water bath, by the addition of 1.0 ml of 5% perchloric acid. After 5 min in an ice bath, precipitated material was removed by centrifugation for 5 min in a Clay-Adams Serofuge. Inorganic phosphate was determined by the method of Fiske and SubbaRow (9). Controls were employed to account for extraneous inorganic phosphate present in the reaction mixtures and for nonenzymatic hydrolysis of substrate. All assays were done in triplicate, and the data presented here are average values. Protein was determined by the biuret (17) method with the use of a bovine albumin standard.

Standard ATPase assay conditions. *S. faecalis* Mg^{++} ATPase activity was assayed at *p*H 7.5 in the presence of 3.0 mM $MgCl_2$. *E. coli* Mg^{++} ATPase activity and Ca^{++} ATPase activity were assayed at *p*H 9.0 in the presence of 2.0 mM $MgCl_2$ and 1.0 mM $CaCl_2$, respectively, unless otherwise specified. Choice of these values was based upon preliminary results (*see* Results).

S. faecalis and *E. coli* ATPase activities were linear with respect to time during the 30-min assay and also with respect to protein concentration within the range employed in this work. With increasing protein concentration, the *E. coli* Mg^{++} ATPase to Ca^{++} ATPase activity ratio increased slightly. This potential variable was avoided by

employing a standardized protein concentration for each individual set of experiments.

Preincubation and heating experiments. Two methods were employed for pretreatment of membrane ghost preparations by heating. In the first method, a 15-liter water bath, equipped with a Porta-Temp (Precision Scientific Co.) portable circulating and heating element, was heated from room temperature to 80 C at approximately 2.4 degrees per min. All test samples were placed in the bath when the temperature reached 37 C, and one tube was removed every 3 min thereafter. The temperature was monitored by a thermometer placed in a "blank" tube; reproducibility between experiments was excellent. Upon removal, each sample was immediately cooled in a melting-ice bath and remained so until the standard ATPase assays were performed.

In the second method, the same water bath-heating system was employed. Samples were heated for 10 min at the desired temperatures, during which time the temperature varied no more than ±0.2 C. Upon removal, the samples were treated as above. In all experiments, control samples were immediately placed in the ice bath.

RESULTS

Effect of Mg^{++}, Mn^{++}, and Ca^{++} on the ATPase activity of membrane preparations. As reported by previous investigators (4, 5, 12), the ATPase activities of *S. faecalis* and *E. coli* were maximal in the presence of added Mg^{++} at *p*H 7.5. At this *p*H, substitution of equivalent amounts of Mn^{++} produced only partial activation, and Ca^{++} had an insignificant effect on these activities. However, significant ATPase activity was observed with *E. coli* membrane ghost preparations when Ca^{++} was substituted for Mg^{++} at low concentrations and the assays were performed at *p*H 9.0. The effects of various concentrations of Mg^{++} and Ca^{++} on the liberation of orthophosphate from a constant amount of ATP by an *E. coli* membrane ghost preparation can be seen in Fig. 1. *E. coli* ATPase activity was maximal at a molar Ca^{++} to ATP ratio of approximately 0.2:1.0 and a molar Mg^{++} to ATP ratio of approximately 0.4:1.0. Ca^{++} and Mg^{++} caused a considerable decrease in activity when added in excess of these values.

Influence of pH on ATPase activity of E. coli membrane ghost preparations. The effect of *p*H on the Ca^{++} ATPase and Mg^{++} ATPase activities of *E. coli* was investigated by employing a range of reaction *p*H values between 5.95 and 9.90 and otherwise standard assay conditions (*see* Materials and Methods). As can be seen in Fig. 2, Mg^{++} ATPase responded to a rather broad range of *p*H values, with an optimum at approximately *p*H 9.0. This is in good agreement with previous reports concerning this point (12, 13). The Ca^{++} ATPase activity increased sharply above *p*H 7.5

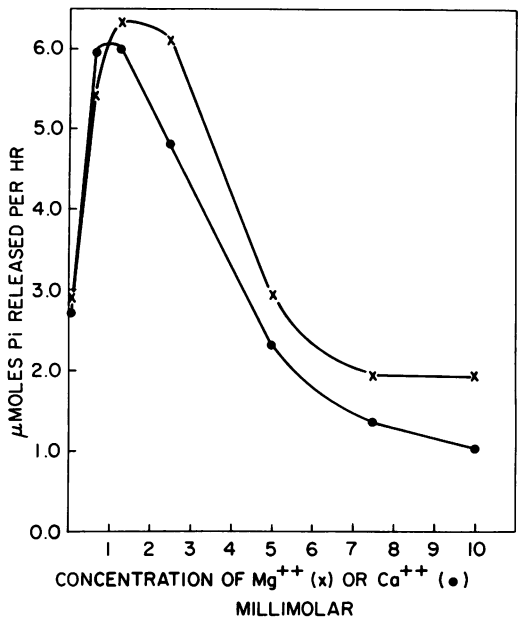

FIG. 1. *Release of orthophosphate from ATP by an E. coli membrane ghost preparation as a function of the concentration of activating ion. Reaction mixtures contained 97 μg of protein and varying amounts of $CaCl_2$ or $MgCl_2$ in a final volume of 1.0 ml. Other reaction conditions are described in Materials and Methods. All reaction mixtures contained a background level of $MgCl_2$ (0.05 mM) added with the enzyme preparation.*

and appeared to be near maximum at *p*H 9.90. It is noteworthy that the Ca^{++} ATPase activity is insignificant below a *p*H value of 8.0 but is greater than Mg^{++} ATPase activity at *p*H values above 9.3.

Assay for E. coli membrane adenosine diphosphatase and monophosphatase activities. Table 1 shows the extent of hydrolysis of ATP, ADP, and AMP by a membrane ghost preparation of *E. coli* under standard assay conditions. ATPase activity in the absence of added Mg^{++} or Ca^{++} was probably due to the concentration of Mg^{++} (0.05 mM) added along with the membrane preparation, although the effect of "endogenous," or bound, ions was not estimated in this instance. Only Mg^{++} elicited adenosine diphosphatase activity, which was only approximately 20% in comparison with ATPase activity. Adenylate kinase activity was not investigated. *E. coli* membrane ghost preparations exhibited no detectable adenosine monophosphatase activity in the presence of either cation.

Influence of various compounds on the membrane ATPase activities of E. coli and of S. faecalis. Numerous compounds are known to inhibit or

stimulate mitochondrial and transport ATPases. In the present investigation, a survey of the effects of several such compounds was made in an attempt to elucidate the relationship between the Mg^{++} ATPase and Ca^{++} ATPase activities of *E. coli* and, on the other hand, to compare the ATPase activities of *E. coli* with the Mg^{++} ATPase of *S. faecalis*. Nine potential inhibitors were tested at four different concentrations from 0.05 to 1.0 mM in otherwise standard reaction mixtures. The *E. coli* and *S. faecalis* ATPases were neither inhibited nor stimulated by ouabain, fluoride, *N*-ethylmaleimide, 2,4-dinitrophenol, cyanide, or dithionite at the concentrations tested. Other results are summarized in Table 2. Only one significant qualitative difference between the responses of the Mg^{++} ATPase of *E. coli* and that of *S. faecalis* was found. *E. coli* Mg^{++} ATPase was stimulated by the addition of 0.5 mM pentachlorophenol to reaction mixtures, whereas that of *S. faecalis* was inhibited. Also, the same concentration of this compound did not influence *E. coli* Ca^{++} ATPase activity, although a concentration of 1.0 mM did inhibit this activity. In general, the responses of the three ATPase activities to the compounds employed were remarkably similar.

Influence of monovalent cations on the enzymatic hydrolysis of ATP by E. coli membrane preparations. It was of interest to investigate the effects of monovalent cations on the membrane ATPase activities of *E. coli*, especially since previous reports concerning this aspect of *E. coli* Mg^{++} ATPase (5, 12, 13) have been inconsistent. The effects of Na^+ and K^+ on the Mg^{++} and Ca^{++} ATPase activities of *E. coli* were investigated as follows. In a series of experiments (Fig. 3), K^+ concentration was varied between 5.0 and 100 mM in the presence of a constant amount (5.0 mM) of Na^+; Na^+ concentration was varied between 5.0 and 100 mM in the presence of 5.0 mM K^+. Mg^{++} and Ca^{++} were employed singly at standard concentrations. Ca^{++} ATPase activity exhibited a striking sensitivity to Na^+ and K^+. The inhibitory response was essentially linear up to a value of approximately 83% at 75 mM Na^+ or K^+. In contrast, the Mg^{++} ATPase was relatively insensitive to inhibition by Na^+ or K^+. Experiments employing various concentrations and ratios of Na^+ and K^+ indicated no cooperative or specific character of the inhibitory effects, although K^+ appeared to be slightly more inhibitory than Na^+ in some instances.

The following experiment demonstrated that a number of monovalent cations duplicate the effects of Na^+ and K^+ on the Mg^{++} and Ca^{++} ATPase of *E. coli* (Table 3). In the standard assay, 50 mM K^+, Li^+, Rb^+, and Cs^+ were tested singly in the presence of 10 mM Na^+. It is noteworthy that Li^+ produced the greatest effect on both ATPase activities and that the observed differences were, for the most part, more quantita-

TABLE 1. *Hydrolytic activity of E. coli membrane preparations on ATP, ADP, and AMP*

Substrate[a]	Addition[b]	Orthophosphate released per hr[c] (μmoles)
ATP	None	2.00
	2.0 mM Mg^{++}	5.14
	1.0 mM Ca^{++}	4.62
ADP	None	0.08
	2.0 mM Mg^{++}	0.94
	1.0 mM Ca^{++}	0.00
AMP	None	0.00
	2.0 mM Mg^{++}	0.00
	1.0 mM Ca^{++}	0.00

[a] Reaction mixtures contained 5.0 mM substrate, 78 μg of protein, $MgCl_2$, or $CaCl_2$ as indicated above, and 100 mM Tris-hydrochloride, *p*H 9.0, in a final volume of 1.0 ml.

[b] All reaction mixtures contained a background level of $MgCl_2$ (0.05 mM) added with the enzyme preparation.

[c] Standard reaction conditions were employed.

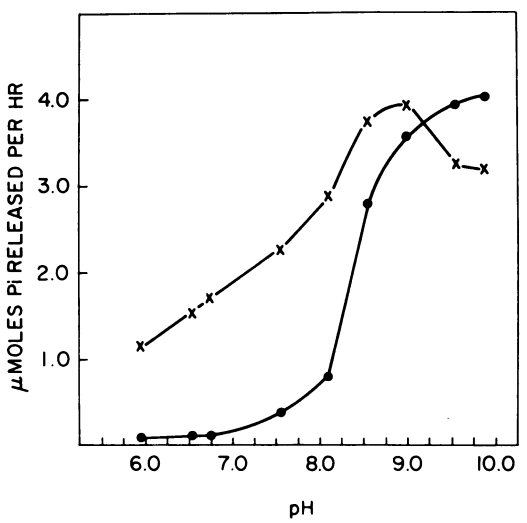

FIG. 2. *Release of orthophosphate from ATP by an E. coli membrane ghost preparation as a function of the pH of the reaction mixture. Reaction mixtures contained 5.0 mM Na_2ATP, 2.0 mM $MgCl_2$ (×) or 1.0 mM $CaCl_2$ (●), 58 μg of protein, and 100 mM Tris-hydrochloride buffer in a final volume of 1.0 ml. The pH values designated by the position of the data points were obtained by direct measurement. Incubation conditions were standard.*

TABLE 2. *Influence of various compounds on the release of orthosphosphate from ATP by E. coli and S. faecalis membrane preparations*

Compound[a] tested	Enzyme source and activating cation[b]	Per cent inhibition[c] at			
		1.0 mM	0.5 mM	0.1 mM	0.05 mM
*p*CMB	*S. faecalis* (Mg^{++})	33.5	33.5	29.8	28.6
	E. coli (Mg^{++})	41.7	41.7	37.8	37.5
	E. coli (Ca^{++})	68.1	68.1	64.7	62.9
Azide	*S. faecalis* (Mg^{++})	30.8	22.1	12.3	10.7
	E. coli (Mg^{++})	90.0	86.5	64.0	49.8
	E. coli (Ca^{++})	71.1	57.1	20.8	12.2
PCP	*S. faecalis* (Mg^{++})	44.1	34.4	11.7	6.2
	E. coli (Mg^{++})	15.9	−29.0[d]	−9.5	−5.5
	E. coli (Ca^{++})	37.5	0.0	−6.8	0.0

[a] Standard assay conditions were employed. *p*CMB = *p*-chloromercuribenzoate; PCP = pentachlorophenol.

[b] *E. coli* reaction mixtures contained 97 μg of protein; *S. faecalis* reaction mixtures contained 140 μg of protein in a final volume of 1.0 ml.

[c] Per cent inhibition was calculated on the basis of the activities of reaction mixtures (controls) with no test compound added.

[d] Negative values denote per cent stimulation.

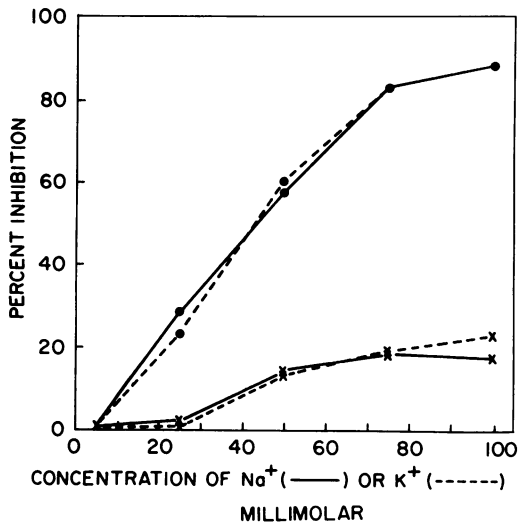

FIG. 3. *Influence of Na^+ and K^+ on the release of orthophosphate from ATP by an E. coli membrane preparation. Reaction mixtures contained 2.5 mM Na_2ATP, 2.5 mM K_2ATP, 78 μg of protein, 2.0 mM $MgCl_2$ (×) or 1.0 mM $CaCl_2$ (●), varying amounts of NaCl or KCl, and 100 mM Tris-hydrochloride buffer, pH 9.0 in a final volume of 1.0 ml. Per cent inhibition was calculated on the basis of the activity of reaction mixtures (controls) without added NaCl or KCl. Other details are presented in Materials and Methods.*

tive than qualitative. This is in contrast to the response of the Mg^{++} ATPase of *S. faecalis*, which was almost completely insensitive to comparable concentrations of Na^+, K^+, and the other monovalent cations tested.

TABLE 3. *Effect of monovalent cations on the release of orthophosphate from ATP by an E. coli membrane preparation*

Cation added[a]	Per cent inhibition[b] of	
	Mg^{++} ATPase	Ca^{++} ATPase
K^+	14.1	62.3
Li^+	29.1	89.1
Rb^+	16.2	64.4
Cs^+	24.4	62.1

[a] KCl, LiCl, RbCl, or CsCl, at a concentration of 50 mM, was employed. All reaction mixtures contained 5.0 mM Na_2ATP, 78 μg of protein, 2.0 mM $MgCl_2$ or 1.0 mM $CaCl_2$, and 100 mM Tris-hydrochloride buffer, pH 9.0, in a final volume of 1.0 ml.

[b] Per cent inhibition was calculated on the basis of the activity of reaction mixtures containing no added monovalent cations.

Close identity of the E. coli Mg^{++}- and Ca^{++}-activated ATPase activities. It was of interest to determine whether the Mg^{++} ATPase and Ca^{++} ATPase activities of *E. coli* were additive, as might be expected if two separate and independent enzymes were present. Therefore, otherwise standard assays were carried out in which Mg^{++} and Ca^{++} were used singly and in combination between 0.4 and 1.6 mM final concentration. The Mg^{++} and Ca^{++} ATPase activities were not additive. As expected, combinations of these ions were inhibitory whenever the final total concentration exceeded 2.0 mM.

Other experiments were performed to determine the extent of Na^+ and K^+ inhibition of *E. coli* ATPase measured in the presence of both activators. In these experiments, inhibition by monovalent cations proved to be directly proportional to the Ca^{++} to Mg^{++} ratio in the reaction mixtures. For example, ATPase activity measured in the presence of 0.8 mM Mg^{++} plus 0.8 mM Ca^{++} was inhibited approximately 45% by 20 mM Na^+ plus 60 mM K^+ (or 20 mM K^+ plus 60 mM Na^+). By reference to Fig. 3, it can be seen that this value is intermediate between those values (20% and 85%, respectively) obtained for the inhibition of ATPase activated by Mg^{++} or Ca^{++} alone by these concentrations of Na^+ plus K^+.

It may be recalled that (i) the Ca^{++} ATPase activity was shown to be approximately four times as sensitive to inhibition by K^+ as the Mg^{++} ATPase activity (Fig. 3), and (ii) the Mg^{++} ATPase was more susceptible to inhibition by low concentrations of azide than was the Ca^{++} ATPase (Table 2). This made it possible to estimate the relative contributions of the Mg^{++} ATPase and of the Ca^{++} ATPase to the total hydrolytic activity in the presence of both activating cations. Table 4 shows the results of an experiment in which both "inhibitors" were tested, alone and in combination, on *E. coli* ATPase activity in the presence of Mg^{++} or Ca^{++}, or both. It is apparent that both cations contribute to the activation of *E. coli* membrane ATPase when these are added, as described, to reaction mixtures. It can be calculated from these data that the total ATPase activity is comprised of 56% Mg^{++} ATPase and 44% Ca^{++} ATPase activity, or a Mg^{++} ATPase to Ca^{++} ATPase activity ratio of 1.3:1.0 when equal concentrations (0.8 mM each) of Mg^{++} and Ca^{++} are added to reaction mixtures at *p*H 9.0. Therefore, the net result is that Mg^{++} and Ca^{++} appear to compete for the same ATPase enzyme(s) in such a manner that the Mg^{++} ATPase and Ca^{++} ATPase activities appear to be mutually exclusive.

Effect of heating on membrane ATPase activities. Two methods were employed to determine the temperatures which would inactivate the *E. coli* and *S. faecalis* membrane ATPase activities by 50%. These temperature values will be referred to as the critical temperatures. The first method was employed to determine the working range of time and temperatures rather than begin with arbitrary values. Samples of enzyme preparation (undiluted) were heat-treated according to the procedures described in Materials and Methods (first method, involving constantly changing temperature). Subsequently, enzyme activities were determined by use of standard ATPase assay conditions. The data (Fig. 4) show that

TABLE 4. *Influence of K^+ and of azide on the Mg^{++}-, Ca^{++}-, and ("Mg^{++} plus Ca^{++}")-activated release of orthophosphate from ATP by an E. coli membrane preparation*

Group[a]	Subgroup[b]	Mg^{++}	Ca^{++}	K^+	Azide	Orthophosphate released per hr (μmoles)	Per cent inhibition[c]
I	A	+	−	−	−	4.52	—
	B	−	+	−	−	3.89	—
	C	+	+	−	−	4.84	—
II	A	+	−	+	−	3.58	20.8
	B	−	+	+	−	1.58	59.4
	C	+	+	+	−	3.16	34.7
III	A	+	−	−	+	1.78	60.6
	B	−	+	−	+	3.47	15.2
	C	+	+	−	+	2.68	44.6
IV	A	+	−	+	+	2.20	51.3
	B	−	+	+	+	1.42	63.5
	C	+	+	+	+	2.10	56.6

[a] Group I reaction mixtures are the three subgroup controls (see below). Group II reaction mixtures contained an additional 50 mM KCl. Group III reaction mixtures contained 0.04 mM sodium azide. To group IV reaction mixtures were added both 50 mM KCl and 0.04 mM sodium azide.

[b] All reaction mixtures contained 2.5 mM Na_2ATP, 2.5 mM K_2ATP, 78 μg of protein, 100 mM Tris-hydrochloride buffer, *p*H 9.0, and 0.8 mM $MgCl_2$ and/or 0.8 mM $CaCl_2$, as indicated in the table, in a final volume of 1.0 ml. Incubation conditions were standard.

[c] Activities of the three controls (group I, subgroups A, B, and C, respectively) were taken as 100%.

the *E. coli* Mg^{++} and Ca^{++} ATPase activities are not only considerably heat-stable but are activated by pretreatment with heat. Both activities were substantially enhanced to about the same extent in samples removed after 30 min (i.e., at 60 C). Both activities exhibited parallel sharp decreases upon further incubation at increasing temperatures. The critical temperatures indicated (68 C) are identical within experimental error. This is in contrast to the behavior of the *S. faecalis* Mg^{++} ATPase activity, which exhibited only a slow rate of inactivation with a critical temperature of approximately 62 C.

Based upon the above information, a second series of experiments was performed by incubating samples of the *E. coli* enzyme preparation for 10 min at the temperatures designated in Fig. 5 and employing standard ATPase assay conditions.

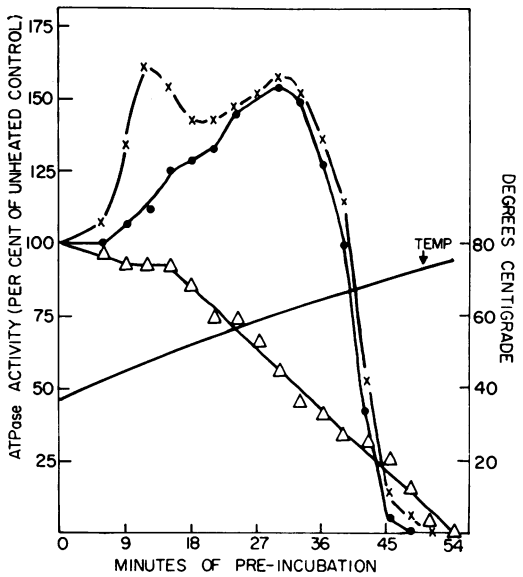

FIG. 4. *Effect of heating, under conditions of increasing temperature, on the ATPase activities of E. coli and S. faecalis membrane preparations. Samples of membrane preparations were heat-treated according to the procedures described in Materials and Methods, and E. coli Mg^{++} ATPase (×), E. coli Ca^{++} ATPase (●), and S. faecalis Mg^{++} ATPase (△) activities were assayed by use of standard assay conditions. Protein concentrations during heat treatment were 1.72 mg/ml (E. coli) and 2.20 mg/ml (S. faecalis). E. coli ATPase reaction mixtures contained 86 μg of protein; those of S. faecalis contained 110 μg of protein.*

Both of the *E. coli* ATPase activities exhibited a slight enhancement after preincubation at 60 C. This was followed by rapid inactivation with increasing temperature, the critical temperature being approximately 66 C. These results are in agreement with those shown in Fig. 4, indicating that the cumulative effects of time and temperature encountered in the first method (Fig. 4) enhance the activation effect of high temperature on the ATPase activities of *E. coli* but do not significantly influence the critical temperature values.

It should be noted that in *E. coli* enzyme preparations exposed to 47 C for 10 min the Mg^{++} and Ca^{++} ATPase activities responded with similar, but slight, enhancement of activity. This indicates that the deviation observed in the Mg^{++} ATPase to Ca^{++} ATPase activity ratios of samples pretreated by slowly increasing the temperature from 37 to 47 C (*see* Fig. 4) is not a serious discrepancy.

DISCUSSION

In general, the results presented in this report concerning the *E. coli* Mg^{++} ATPase are consistent with observations made by previous investigators on the properties of bacterial Mg^{++}-activated ATPases. On the other hand, the *E. coli* Ca^{++} ATPase activity described here presents a unique combination of properties heretofore unreported. Briefly, these are a very high optimal *p*H with little or no activity below neutrality, maximal activation in the presence of a molar Ca^{++} to ATP ratio of 0.2:1.0, heat stability, and sensitivity to inhibition by Na^+, K^+, and other monovalent cations.

It should be noted that very little, if any, of this information would have come to light if a standard reaction *p*H of 9.0 had not been employed in this investigation. It is clear that, in the description of bacterial ATPase activities, major discrepancies in results can, and have, resulted from minor differences in technique and assay methods. Furthermore, the work presented here illustrates the fact that bacterial ATPase activities possessing rather uncommon *p*H optima or ionic requirements and effects, or both, may easily be neglected. Some of these may play important functional roles in the living cell. The possible functional significance of the *E. coli* Ca^{++} ATPase activity will be discussed below. Nonetheless,

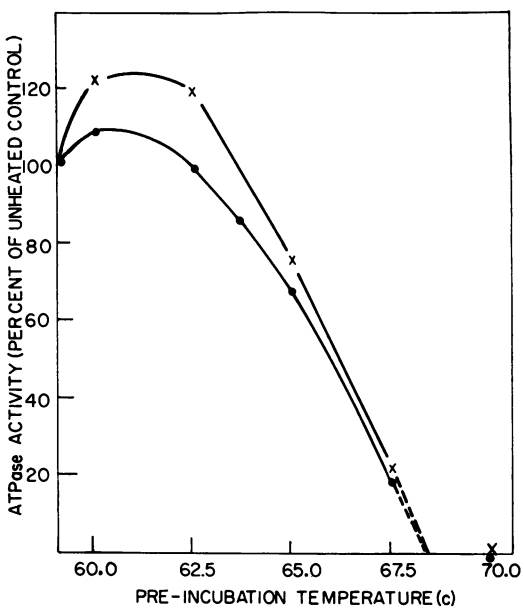

FIG. 5. *Effect of preincubation at various temperatures on the Mg^{++} and Ca^{++} ATPase activities of an E. coli membrane preparation. Samples of an E. coli membrane ghost preparation containing 1.72 mg of protein were preincubated in an equilibrated water bath for 10 min at the designated temperatures (±0.2 C). Mg^{++} ATPase (×) and Ca^{++} ATPase (●) reaction mixtures contained 86 μg of protein/ml. Standard assay conditions were employed.*

further investigation into the kinetics of activation and inhibition of *E. coli* membrane ATPase activities by cations should be rewarding.

With regard to other bacterial ATPase systems in which the effects of Ca^{++} have been investigated, those of *S. faecalis* (1), *Vibrio parahaemolyticus* (14), *Bacillus stearotheromophilus* (18), *Vitreoscilla* (6), and a *Pseudomonas* species (8), all of which possess *p*H optima in the alkaline range, are not activated by Ca^{++}. Slight activation of *S. aureus* ATPase (with a *p*H optima of 6.0) by Ca^{++} was reported by Gross and Coles (11). Ca^{++}-activated ATPases have been found in membrane "ghost" preparations of *Micrococcus lysodeikticus* (15, 19, 20) and in various cell fractions of *B. megaterium* (10, 28). In the case of *M. lysodeikticus*, the Ca^{++} ATPase and Mg^{++} ATPase activities appear to be due to the same enzyme protein, and Mg^{++} acts as an inhibitor of Ca^{++}-activated ATPase (20). Greenawalt et al. (10) concluded that the Mg^{++} ATPase and the Ca^{++} ATPase activities of *B. megaterium* represent two different, but not independent, ATP-hydrolyzing enzyme systems. It is of interest that the Ca^{++} ATPases of *M. lysodeikticus* (20) and of *B. megaterium* (10) are not markedly influenced by Na^+ or K^+ at the concentrations employed in the work reported here.

The effects obtained by adding both Mg^{++} and Ca^{++} to reaction mixtures at different concentrations and the competition observed when both ions are added at equal, but suboptimal, concentrations indicate that under these conditions both Ca^{++} ATPase and Mg^{++} ATPase are almost equally influenced by both activating ions and may, therefore, be the function of the same enzyme. Both activities are increased to an equivalent extent by slowly heating the enzyme preparation from 37 to 60 C, and both exhibit nearly identical heat-inactivation profiles. Both activities are inactivated to the extent of 50% by heating at 66 C for 10 min. These results are also consistent with the idea that the two membrane ATPase activities may be a function of a single species of enzyme protein, although it is possible that both inactivations may actually reflect the denaturation of a structural component common to and prerequisite to the activity of both ATPases. In addition, however, it is noteworthy that the differences in the responses of the Mg^{++} ATPase and Ca^{++} ATPase of *E. coli* to monovalent cations, azide, *p*-chloromercuribenzoate, and, possibly, to *p*H are apparently of a quantitative rather than of a qualitative nature. It is possible that the mechanisms of binding, or activation, are different for Mg^{++} and Ca^{++}, although the same binding sites may be involved. Attempts are being made to procure and purify membrane-free (soluble) enzyme so that the relationship between the Mg^{++} and Ca^{++} ATPase activities of *E. coli* may be further elucidated.

The extreme sensitivity of *E. coli* Ca^{++} ATPase to inhibition by a broad spectrum of monovalent cations is without precedent. It can only be speculated that the basis of this inhibition lies either in the mechanism of activation of this ATPase by Ca^{++} or in its requirement for a high reaction *p*H, or in both. It is interesting that respiration-independent binding of Ca^{++} to phospholipid anionic binding sites on submitochondrial particles is competitively inhibited (22, 23) by both divalent and monovalent cations and by protons (lowered *p*H).

Whatever the mechanism of inhibition of *E. coli* Ca^{++} ATPase by monovalent cations, it should be considered with respect to the possible function(s) of the membrane ATPase in the intact cell. For example, whether or not the *E. coli* Mg^{++} and Ca^{++} ATPase activities are the function of a single enzyme species or of two different but non-independent enzyme systems, there is the possibility that Ca^{++}, in conjunction with monovalent cations such as Na^+ or K^+, may represent a mechanism of regulation of the activity of membrane ATPase in the intact cell. Experiments (for example, *see* Table 4) involving the addition of Mg^{++} plus Ca^{++} and Na^+ or K^+ to ATPase reaction mixtures have demonstrated an analogous situation in vitro. These data suggest that investigations of the influence of Ca^{++} and monovalent cations on the permeability properties of intact spheroplasts would be appropriate.

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