STUDIES ON THE ATP PHOSPHOHYDROLASE ACTIVITIES OF ESCHERICHIA COLI

by

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ABSTRACT

The adenosine triphosphate-hydrolysing activities of Escherichia coli NRC 482 were investigated. These activities could be accounted for by two enzymes, one of which was 5'-nucleotidase and the other was a Ca^{2+}-or Mg^{2+}-activated ATP phosphohydrolase (ATPase). The 5'-nucleotidase and the Ca^{2+}-or Mg^{2+}-activated ATPase could be readily distinguished on the basis of their properties and could be separated from each other by ion exchange chromatography. Both soluble and membrane-bound Ca^{2+}-or Mg^{2+}-activated ATPase activities were present in cell extracts. The membrane-bound ATPase, though firmly attached to the membrane, could be solubilized under conditions in which the membranes were depleted of divalent metal ions. This solubilized ATPase was identical to the ATPase found in the soluble fraction.

The Ca^{2+}-or Mg^{2+}-activated ATPase was partially purified after solubilization from E. coli membranes by gel filtration and ion exchange chromatography. The ATPase activity was shown to correspond to a single protein band after polyacrylamide gel electrophoresis. The enzyme had a molecular weight of 375,000. It was activated by divalent but not by monovalent cations. Optimal activity occured at pH 9.5 and at ion to substrate ratios of 2:5 and 2:3 with Mg^{2+} and Ca^{2+}, respectively. ADP inhibited the ATPase reaction. The ATPase was stable at 22°- 24° but was rapidly

inactivated at 4°. Glycerol stabilized the enzyme at both temperatures. In these, and in other respects, the Ca^{2+}- or Mg^{2+}-activated ATPase of E. coli resembled the ATPase activities of other bacterial strains, which in turn have a resemblance to the mitochondrial and chloroplast ATPases.

To help establish the function of the Ca^{2+}-or Mg^{2+}-activated ATPase the levels of the enzyme were measured in E. coli grown under a variety of conditions. The level of the enzyme in the cell was not affected by the carbon source, phase of growth, aeration of the culture or the extent of catabolite repression, although these are conditions under which the efficiency of oxidative phosphorylation is known to vary in vivo. These findings can be reconciled with the current hypothesis regarding the function of the ATPase in E. coli.

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ABBREVIATIONS USED

ADP	Adenosine-5'-diphosphate
ADPase	ADP phosphohydrolase
AMP	Adenosine-5'-monophosphate
AMPase	AMP phosphohydrolase
AMP-PCP	β,γ Methylene adenosine-5'-triphosphate
ATP	Adenosine-5'-triphosphate
ATPase	ATP phosphohydrolase
Bis-pNPP	Bis-para-nitrophenylphosphate
CCCP	Carbonyl cyanide m-chlorophenylhydrazine
CTP	Cytosine-5'-triphosphate
Cyt	Cytochrome
dATP	Deoxyadenosine-5'-triphosphate
DCCD	N,N'-Dicyclohexylcarbodiimide
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNP	Dinitrophenol
EDTA	Ethylenediamine tetraacetate
FCCP	para-Trifluoromethoxyphenylhydrazone
GTP	Guanosine-5'-triphosphate
ITP	Inosine-5'-triphosphate
IU	International unit
K_3	2-Methyl-1,4-naphthoquinone
K_{av}	$(V_t - V_e)/(V_t - V_o)$
Menadione	2-Methyl-1,4-naphthoquinone
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
Pi	Phosphate
p-NPP	para-Nitrophenylphosphate
psi	Pounds per square inch
RNase	Ribonuclease
TCA	Trichloroacetic acid
TMED	N,N,N',N' tetramethylethylenediamine
TMG	Thiomethyl-ß-D-galactoside
Tris	Tris-(hydroxymethyl)-aminomethane
UTP	Uridine-5'-triphosphate
UV	Ultraviolet
V_e	Elution volume
V_o	Void volume
V_t	Total volumd

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"It is interesting, though, that as time has gone by and the technical developments have become more and more interlocked with one another, the pure exploratory urge has also invaded the scientific sphere. Scientific research - the very name 're-search' gives the game away (and I mean game) - operates very much on the play-principles mentioned earlier. In 'pure' research, the scientist uses his imagination in virtually the same way as the artist. He talks of a beautiful experiment rather than of an expedient one. Like the artist, he is concerned with exploration for exploration's sake. If the results of the studies prove to be useful in the context of some specific survival goal, all to the good, but this is secondary."

Desmond Morris

"The Naked Ape"

INTRODUCTION

Among the many bacterial enzymes that hydrolyze phosphate (Pi) from adenosine-5'-triphosphate (ATP) is ATP phosphohydrolase (EC 3.6.1.4). This enzyme can be distinguished from phosphatases and 5'-nucleotidase in that it degrades ATP only as far as adenosine-5'-diphosphate (ADP). The ATP phosphohydrolases (ATPases) from a number of bacterial strains have been well characterized in terms of their properties (1-4). The similarity of this enzyme from unrelated strains is striking, as is its ubiquity. Though the function of this ATPase is not yet established, the enzyme has been implicated in oxidative phosphorylation (5), active transport (6, 7) and energy-dependent transhydrogenation (8). These roles can be reconciled with the current theories on energy conservation by analogy with the mitochondrial ATP phosphohydrolase (9).

Lack of specificity in the ATPase assay

ATPase activity is normally assayed by measuring the release of inorganic phosphate from ATP. Occasionally the increase in acidity that accompanies this hydrolysis is measured or the loss of label from γ^{32}P-ATP. Thus the distinction between ATPase and other enzymes or groups of enzymes that hydrolyze phosphate from ATP is not immediately apparent from these assay

procedures. Many biosynthetic processes in the cell undoubtedly result in a net hydrolysis of ATP to ADP with the release of inorganic phosphate. However during the fractionation of cell extracts the contribution that they make to the total ATPase activity is minimal due to the loss of cellular organisation. This is not true of the degradative enzymes such as alkaline phosphatase and 5'-nucleotidase. However these enzymes lack the specificity of an ATP phosphohydrolase and can be distinguished by this and other properties.

The early part of this thesis is concerned with evaluating contributions to the total ATPase activity of Escherichia coli.

Enzymes of the periplasmic space

Formation of ATP from ADP in the cell represents an investment of energy. From a teleological outlook this energy store must be protected from dissipation by purely degradative enzymes. One of the ways in which such futile cycles are avoided in the cell is by compartmentation. The possibilities for compartmentation in prokaryotic organisms are somewhat less than for eukaryotes. However it does seem that bacteria are organized such that many of the degradative enzymes are compartmented in the periplasmic space (10). In this way they are separated from the cytoplasm by the inner cell membrane. When E. coli is converted to a spheroplast form by use of ethylenediamine tetraacetate (EDTA) and lysozyme, enzymes of the periplasmic space

are quantitatively released from the cells (11, 12). The same result occurs when the bacteria are osmotically shocked (11). Heppel (13) has listed some enzymes which are released by this method and others that are not. Those that are released include alkaline phosphatase, 5'-nucleotidase, nonspecific acid phosphatase, ribonuclease I and 2',3'-cyclic phosphodiesterase (14, 15). On the other hand β-galactosidase, glucose-6-phosphate dehydrogenase and polynucleotide phosphorylase are retained inside the cell.

Another difference between these two groups of enzymes becomes apparent when whole cells are assayed for the enzyme activities. Enzymes of the periplasmic space are able to show their activity (11, 16) whereas those inside the cell membrane are not. Of the ATPase activity observed during the assay of whole *E. coli*, up to 100% can be displaced into the osmotic shock fluid, which demonstrates its periplasmic nature. Among the periplasmic enzymes that show ATPase activity are acid phosphatase (17), alkaline phosphatase (18) and 5'-nucleotidase (19).

Phosphatases

The acid phosphatase is relatively nonspecific with respect to its substrates which include para-nitrophenyl phosphate (p-NPP) and ATP. However the latter is hydrolysed at only 3% of the rate at which the former is hydrolysed. The

pH optimum for p-NPP hydrolysis lies between pH (4.5 - 5.0) and there is no activity at alkaline pH values (17). Since ATP phosphohydrolase is routinely assayed under alkaline pH conditions (Tables I -IV) the nonspecific acid phosphatase is unlikely to interfere significantly with its assay.

This cannot be said for alkaline phosphatase which has optimal activity between pH (8.0 - 9.0) (16, 20). This enzyme is also nonspecific with respect to its substrates, of which p-NPP is the most often used. ATP is hydrolysed at 75% of the rate at which p-NPP is hydrolysed. Alkaline phosphatase is not a constitutive enzyme. It is synthesized in E. coli only when phosphate is limiting in the growth medium (16, 21).

5'-Nucleotidase

5'-Nucleotidase (19) on the other hand is a constitutive enzyme. It is more specific than the phosphatases being active against 5'-nucleotides but not against 2'- or 3'-nucleotides. The enzyme hydrolyses phosphate from both ribonucleotides and deoxyribonucleotides in the mono, di or triphosphate form. Activity does not vary greatly with the nature of the base. The pH optimum of the enzyme was reported by Neu (19) to be 5.7 - 6.1 for the hydrolysis of adenosine-5'-monophosphate (AMP) and 6.7 - 7.1 for the hydrolysis of ATP. Both activities were assayed in the presence of 5mM $CoCl_2$ together with 10 or 20mM $CaCl_2$.

Glaser et al. (22, 23) have investigated a uridine

diphosphate sugar hydrolase from the periplasmic space of E. coli. This enzyme appears to be identical to 5'-nucleotidase (22, 24). The pH optima of the enzyme varied somewhat according to the nature of the divalent metal ion (22). When Mg^{2+} was used over the range pH 5.1 to 8.0, most activity occurred at pH 8.0. However when Co^{2+} or Mn^{2+} were used there was most activity at pH 7.0.

Of further help in identifying the 5'-nucleotidase is its tendency to complex with a protein inhibitor (23, 24). The activity of the complex is less than 1% of the uninhibited value. This inhibitor is located in the cytoplasm such that the enzyme is not inhibited in the intact cell. However 5'-nucleotidase activity usually decreases after sonication of the whole cell since enzyme and inhibitor can then come into contact . This inhibition can be reversed by heat treatment between 50° and 60° in the presence of divalent metal ions like Co^{2+}, Mg^{2+} or Ca^{2+} (19, 22). The protein inhibitor is very heat-labile while the 5'-nucleotidase is more stable and is protected by the divalent metal ions. Inhibition can also be reversed by the addition of urea to a final concentration of 4M - 6M (24, 25).

The ubiquity of ATP phosphohydrolase in bacteria

ATPase activities with greater specificity than the degradative enzymes listed above have been observed in many

bacteria. These sources include both Gram-positive (26-29) and Gram-negative (30-32) organisms. ATPase activity has been found in photosynthetic bacteria (33, 34), halophilic bacteria (35), chemoautotrophic bacteria (32), and thermophilic bacteria (36, 37) among others.

Cellular location of the ATPases

Much of the earlier work on the bacterial ATPases originated from investigations done on the cell membranes. Weibull *et al.* (38) prepared protoplasts of *Bacillus megaterium* with the aid of lysozyme. After osmotic lysis of the protoplasts they measured the distribution of various enzymes between the protoplasm and the membranes (28). They found that 73% of the ATPase activity present in the lysate remained in the soluble fraction after removing the membranes by centrifugation at 78,000 x g for 25 min (28). After the protoplast membranes had been washed once they retained 29% of the ATPase activity of the lysate. Total recovery of activity was 109%.

Abrams *et al.* (26) prepared protoplasts of *Streptococcus faecalis* in a similar manner. These were then disrupted either by osmotic lysis or by metabolic lysis which resulted from the uptake and metabolism of glucose. In a series of eight preparations the cell membranes retained an average of 82% of the ATPase actiivity while the remaining 18% occurred in the soluble "cytoplasmic" fraction. The specific activity of the

ATPase associated with the membranes remained the same through two washing steps which indicated that it was tightly bound.

During the formation of spheroplast membranes from Lactobacillus fermentii (39, 40) 60% - 80% of the ATPase activity remained in the soluble fraction. In these experiments various proteases were used to make the cells lysozyme-sensitive. These may have had an effect on the overall distribution of ATPase, either releasing the enzyme from the membrane or destroying some of its activity. Comparative experiments with S. faecalis showed that the membrane-bound ATPase was susceptible to the proteases under these conditions. Despite the large proportion of ATPases in the soluble fraction the specific activity of the membrane-bound ATPase in L. fermentii was higher than that of the soluble ATPase. The same result was obtained with E. coli as will be described later.

Most of the ATPase activity was still membrane-bound after sonic disruption of protoplast membranes of Micrococcus lysodeikticus (27). However high speed centrifugation was required to sediment the enzyme because the membranes had been fragmented by the sonication step. In Bacillus sterothermophilus (41) the bulk of the ATPase activity remained bound to membrane particles after extraction of the protoplast membranes with organic solvents. Whereas inorganic pyrophosphatase stayed almost entirely in the soluble fraction.

In general, the evidence obtained from cell fractionation indicates that the ATPase can be considered as a membrane-bound

enzyme with the provision that there may be more than one type of ATPase present.

Localization of ATPase by electron microscopy

Evidence to confirm that the ATPase is located in the cytoplasmic membrane has come from electron microscopy. Voelz (42) fixed E. coli cells in glutaraldehyde, sectioned them, and stained for ATPase activity by following the deposition of lead phosphate in areas where Pi was released from ATP. The stain appeared in the cell wall area and around the cytoplasmic membrane. Kushnarev et al. (43) have also attempted to locate ATPase activity in E. coli using similar techniques. When sections of E. coli B were incubated with ATP most of the phosphate inclusions were observed in, and under, the cytoplasmic membrane, and in the cell wall. However if the substrate was ADP instead of ATP the phosphate granules were located in the cell wall only. They felt that the activity seen in the cell wall was due to 5'-nucleotidase.

These cytochemical experiments confirm that the cell membrane is a major site of ATPase activity. Attempts have been made to further pin down the location of the enzyme within the membrane. In mitochondria, knob-like projections have been observed on the inner surface of the cristae (44-47). These have been shown to contain ATPase activity (48). Similar knob-like projections have been seen in electron micrographs of

bacterial membranes, noteably those of B. sterothermophilus (49), Rhodospirillum rubrum (50), and those of M. lysodeikticus (51). Muñoz et al. (52) have solubilized ATPase activity from membranes of M. lysodeikticus. In these solubilized preparations of the enzyme they have observed spherical particles of approximately 100 Å diameter which show a regular subunit structure. Similar particles were seen in association with the cell membranes. Simakova et al. (53), also working with M. lysodeikticus, have tried to determine if the membrane projections contain the ATPase activity. They found that treatment of spheroplast membranes with subtilopeptidase removed 60% of the projections but did not change the ATPase activity. (Under these conditions of proteolysis no latent ATPase was detected.) Consequently the idea that the membrane projections in bacteria contain ATPase activity as they do in the mitochondria has yet to be proved conclusively.

Properties of the membrane-bound enzyme

In many bacterial systems the properties of the membrane-bound enzyme have been studied as a preliminary to solubilization and purification. These properties may give the best idea of the capabilities of the enzyme in vivo since the enzyme may be closer to its native state when membrane-bound.

Details of some of the better characterized membrane-bound ATPases are presented in Tables I and II. Particular attention

Table I. Properties of membrane-bound ATPases in bacteria. (Activities measured in the presence of Mg^{2+}.)

Organism	Streptococcus faecalis	Bacillus megaterium	Escherichia coli		Bacillus sterothermophilus
Reference	(26)	(28, 55, 56)	(57)	(58)	(36, 37)
Mg^{2+} activation	present	present	present	present	present
Activation by other divalent cations	____	Ca^{2+} equivalent to Mg^{2+}	$Mg^{2+} \equiv Ca^{2+} > Mn^{2+}$	Ca^{2+}	____
Optimum Mg^{2+} : ATP ratio	1:1	1:2	1:2.5	1:2	1:2
pH optimum	____	more active at alkaline pH	pH 9.0	pH 7.5	pH 8.5
Substrate specificity ATP:ADP:AMP	100:3:<1	100:0:0	100:18:0	100:0:0	____
Stimulation by Na^{+}	____	none	slight inhibition	slight inhibition	____
Stimulation by K^{+}	____	none	slight inhibition	none	none

Table II. Properties of membrane-bound ATPases in bacteria. (Activities measured in the presence of Mg^{2+}.)

Organism	Staphylococcus aureus	Vibrio parahaemolyticus	Pseudomonas aeruginosa	Lactobacillus arabinosus
Reference	(54)	(35)	(59)	(29)
Mg^{2+} activation	present	present	present	present
Activation by other divalent cations	$Mg^{2+} > Ca^{2+}$	slight activation by Mn^{2+} and Co^{2+}; Ca^{2+} ineffective	$Mg^{2+} > Mn^{2+} > Ca^{2+}$	——
Optimum Mg^{2+} : ATP ratio	1:1	2:1 and 100:1	1:10	——
pH optimum	pH 5.9 to 6.1	pH 7.2 to 8.5 (dependent on Mg^{2+} concentration)	pH 9.0	pH 6.0
Reaction products of ATPase	ADP + Pi	Adenosine + 3 Pi	ADP + Pi	ADP hydrolysed-rate 20%-30% with respect to ATP
Hydrolysis of other substrates relative to ATP	GTP > UTP > CTP 70% 7% 4%	——	ITP > GTP > UTP > CTP 30% 29% 5% 0%	——
Stimulation by Na^{+}	65% at 0.1M	750% at 1.4M	none; slight inhibition at higher concentration	none
Stimulation by K^{+}	10% at 0.1M	1200% at 1.4M	none; slight inhibition at higher concentration	none

has been paid to these ATPases which have subsequently been solubilized and further characterized. These are listed together in Table I. The format of the tables is based on that of Gross and Coles (54).

Some generalizations can be made on the basis of the data in Tables I and II. All of the ATPases are activated by Mg^{2+}. Some of them are also active with other divalent metal ions, notably Ca^{2+}, Mn^{2+}, and Co^{2+}, though these ions are not as effective as Mg^{2+}. The optimum ion (Mg^{2+}) to substrate (ATP) ratios vary widely from 100:1 to 1:10 but most lie in the range 2:1 to 1:2. With the exceptions of Staphylococcus aureus and Lactobacillus arabinosus the ATPases all bave a pH optimum in the alkaline region. The products of the ATPase reaction (ATP $\longrightarrow$ ADP + Pi) are produced in a 1:1:1 shoichiometry. A small amount of hydrolysis of ADP is observed in S. faecalis, E. coli, and L. arabinosus. However the ATPase of Vibrio parahaemolyticus appears to degrade ATP to adenosine and Pi according to the equation ATP $\longrightarrow$ Adenosine + 3 Pi. Where tested, the ATPases attack other nuclsoside triphosphates in the order guanosine-5'-triphosphate > uridine-5'-triphosphate > cytosine-5'-triphosphate (GTP > UTP > CTP). The effect of Na^{+} and K^{+} on the ATPases is of interest for the bearing it might have on the functions of these enzymes. In most of these organisms there is either a slight inhibition due to these cations or no effect at all, with the exceptions of S. aureus and V. parahaemolyticus. Of these two bacteria the latter is most strongly activated.

However it should be noted that this organism is halophilic.

Other membrane-bound ATPases, about which information is more fragmentary (32, 60-62), seem to fit the general pattern described above. However the ATPase of R. rubrum (60) which is localized in the cytoplasmic membrane and its intracytoplasmic extendions (37) was reported to be more active with Mn^{2+} than Mg^{2+}. The ATPase of Streptococcus pyogenes (61) was more active with GTP (158%) as substrate than ATP (100%). Also CTP (41%) was hydrolyzed faster than UTP (34%).

Bacterial membrane preparations that contain more than one type of ATPase

Marunouchi and Mori have investigated the ATPase activity of Thiobacillus thiooxidans (32), a Gram-negative, chemautotrophic bacterium which is capable of oxidising elementary sulphur to sulphate. This activity required tha presence of Mg^{2+} or Ca^{2+}. It could be stimulated by SO_3^- (3 - 4 fold) but not by Na^+ or K^+. The pH optimum was in the alkaline pH region. There was also a small amount of ADP hydrolysis detectable. However this activity was not affected by SO_3^-. Further investigation showed that the enzyme activity was made up of two different components. One component was activated by Mg^{2+} alone and had a pH optimum of 9.0. The other required SO_3^- in addition to Mg^{2+} and had a pH optimum at pH 7.5 - 8.0. In a subsequent paper Marunouchi (62) reported the separation of the sulphite-dependent

ATPase from the other ATPase activity by ammonium sulphate precipitation. The fraction that they used for this precipitation was solubilized from the membrane by trypsin treatment and contained 50% of the total sulphite-dependent ATPase.

Hafkensheid and Bonting (63) have investigated the ATPase activity of E. coli K12. They found that it required Mg^{2+} and was optimally active at a Mg^{2+}: ATP ratio of 1:1. They resolved the ATPase activity into two components, one which was activated by Na^+ plus K^+ (Na^+-K^+-activated ATPase) and one which was not, (Mg^{2+}-activated ATPase). The former had optimal activity at pH 8.9 and the latter at pH 7.7. The Na^+-K^+-activated ATPase was ouabain-sensitive and was active with ADP as substrate. In order to measure the Na^+-K^+-activated ATPase activity, Hafkensheid and Bonting routinely measured the total ATPase activity in the presence of Na^+ plus K^+ and subtracted from it the value obtained for the Mg^{2+}-activated ATPase assayed in the presence of Na^+ and 10^{-4}M ouabain. The activity of the Na^+-K^+-ATPase was very small in comparison to the Mg^{2+}-ATPase. In order to increase the sensitivity of the subtractive assay the freeze-dried bacteria were routinely pretreated with 1.5M urea which lowered the Mg^{2+}-ATPase activity but did not affect the Na^+-K^+-ATPase.

Gunter and Dorn (64), and Bragg and Hou (65) also reported on the properties of ATPases from E. coli. They were unable to find a Na^+-K^+-activated ATPase. In the light of this work Hafkensheid and Bonting (66) re-evaluated their results and confirmed the existence of the two ATPases. They pointed out that the assays

were performed on freeze-dried bacteria since the Na^+-K^+-activated ATPase was not detectable in sonic extracts or in spheroplasts prepared by either the penicillin method or the lysozyme-EDTA method. Under these conditions only the Mg^{2+}-ATPase was observed.

Substrate specificity

The substrate specificity of the membrane-bound ATPase varies a lot between organisms. This may reflect a difference in the bacterial species or the way in which the fractions were prepared. Protoplast membranes of S. faecalis hydrolysed ADP at 4%, and AMP at less than 1%, of the rate at which ATP was hydrolysed (26). Protoplast membranes of B. megaterium were reported to have no activity with ADP or AMP and only slight activity towards pyrophosphate. Sokawa (61) reported on the substrate specificity of a protoplast membrane fraction from S. pyogenes. AMP was not hydrolysed but ADP was attacked at 31% of the rate at which ATP was hydrolysed. Nucleoside triphosphates were attacked at rates of 158% (GTP), 41% (CTP) and 34% (UTP). These figures are representative of many of the membrane fractions which have been characterized. The limitations of studying the membrane-bound enzyme become apparent in these cases since it is difficult to know if these activities reside in the one enzyme or are the sum of several enzymes. In E. coli, for example, the membrane ghost fraction isolated by Evans (57) is specific for ATP among the adenine ribonucleotides when Ca^{2+} is the activating

ion. However in the presence of Mg^{2+}, ADP is hydrolysed at 18% of the rate at which ATP is attacked. Are there two ATPases, one activated by Ca^{2+} and one, less specific, activated by Mg^{2+}, or is the ADPase activity that of a contaminating enzyme? These, and other problems, have provided the stimulus to solubilize the enzymes from the membranes and to purify them.

Solubilization of membrane-bound ATPase

Various treatments of spheroplast membranes such as extraction with organic solvents (41), addition of urea to a final concentration of 2M (53) or sonication (67) result in the fragmentation of the membranes. Under these conditions much of the ATPase activity can still be sedimented albeit at higher gravitational forces (100,000 x g) than used to sediment the membranes before treatment. This indicates that the enzyme has not been solubilized. Less drastic conditions have, surprisingly, been more successful in solubilizing the ATPase. Ishikawa and Lehninger (67) subjected sonic fragments of M. lysodeikticus protoplast membranes to an "osmotic shock" by resuspending them in cold, distilled water. This was sufficient to release the ATPase from the membrane.

Abrams (1) has investigated the conditions necessary for the release of active ATPase from membrane ghosts of S. faecalis. When the membrane ghosts were prepared in the presence of 1mM Mg^{2+} and were washed many times, also in the presence of

1mM Mg^{2+}, the ATPase remained membrane-bound (26). If these washed membranes were then washed further in Tris-(hydroxymethyl)-aminomethane-HCl (Tris-HCl) (pH 7.5) which contained no multivalent cations, then the bulk of the ATPase was eventually released several washing steps later. In the step where 55% of the enzyme was released the specific activity of the ATPase increased 16-fold which illustrates the specificity of the release process. The ionic strength of the Tris-HCl buffer used in this step was 33mM. Abrams ruled out a simple electrostatic interaction as an explanation for the binding of the enzyme to the membrane since the inclusion of 2M LiCl in the washing steps with Tris-HCl buffer failed to dislodge the ATPase. Instead he suggested that multivalent cations were involved in the binding, and that these were slowly leached from the membranes by repeated washing until a threshold level was reached at which the ATPase was released. Thus merely leaving the Mg^{2+}-washed membranes in Tris-HCl buffer for several days did not fulfil these requirements and consequently no ATPase was released. Similarly the use of smaller volumes in the washing steps was less effective in releasing the enzyme.

The substantial release of ATPase in the later steps of the washing procedure could be blocked by the inclusion of Mg^{2+} in the Tris-HCl buffer. The extent to which the release of the enzyme was blocked was dependent on the ion concentration. At 2mM Mg^{2+} it was nearly complete and at 0.1mM it was still detectable. This effect was not specific for Mg^{2+} since Ca^{2+},

Mn^{2+} and spermidine also blocked the dissociation of ATPase from the membranes.

These studies with S. faecalis have provided a model for the release of ATPases in other bacteria (4, 68, 69). Muñoz et al. (69) subjected protoplast membranes of M. lysodeikticus to various washing procedures and compared the release of ATPase with that of polynucleotide phosphorylase, an enzyme which is also associated with membranes in S. faecalis (70). Washing membranes, that had been prepared in 5mM $MgCl_2$, six times with 0.1M Tris-HCl buffer (pH 7.4) did not release much ATPase. If the first four washing steps were done with 30mM Tris-HCl (pH 7.5) and the last two with 3mM Tris-HCl (pH 7.5), then a substantial release of ATPase occurred on the fifth step. A partial release of ATPase occurred prematurely if the chelating agent EDTA (1mM) was included in the first four washing steps. Ca^{2+} and Mg^{2+} each suppressed the release of the ATPase at a concentration of 50mM. The patterns of release of polynucleotide phosphorylase and of protein do not correspond closely to those of the ATPase. Consequently the release of ATPase is selective and is not the result of a sudden disaggregation of the membrane.

The results obtained with M. lysodeikticus bear out the conclusions arrived at by Abrams. It is of interest that EDTA, which has a strong chelating effect on divalent metal ions, hastens the release of the ATPase. In addition it seems that lowering the ionic strength of the medium achieves the same goal.

Despite the high specific activities obtainable with these

washing techniques other workers have preferred simpler procedures that will release the bulk of the ATPase in one or two steps. Ishida and Mizushima (56) were able to solubilize much of the ATPase from B. megaterium protoplast membranes. They did this by adjusting the pH of the membrane suspension to pH 8.5 after it had been dialyzed overnight against 10mM Tris-HCl (pH 7.5) and for a further 2 h against distilled water.

Half of the membrane-bound ATPase in L. fermentii was released from protoplast membranes by extensive washing with water or 50mM Tris-HCl (pH 7.5) (40). Total release was obtained if the washing was done in the presence of 1mM EDTA.

These gentle washing procedures have been successful in releasing the ATPase in nearly all bacteria; (one exception being S. aureus (54) where the enzyme was not released from the membranes by repeated washing with dilute buffer). The ease of release of the enzyme suggests that it has a peripheral location on the membrane rather than being a part of the stroma as indicated by Simakova et al. (53). Indeed Muñoz et al. (52) reported that M. lysodeikticus membranes that have been depleted of ATPase by EDTA treatment also lose the structured particles and appear as smooth sheets.

Other techniques have been used to solubilize ATPase from bacterial membranes. Bragg and Hou (65) used a variety of methods to obtain ATPase fractions from E. coli. These included extraction with EDTA or the chelating resin Chelex 100, treatment with the detergent sodium dodecyl sulphate (SDS) or a combination

of both approaches.

Evans (71) also used SDS to release the ATPase from E. coli spheroplast membranes. He reported complete solubilization of the ATPase after treatment at 37° for 10 min with 0.04% SDS.

Purification of the ATPase

Purification of an enzyme is the usual preliminary to a thorough determination of its properties. Since the ATPases described previously have been membrane-bound the isolation of a membrane fraction by differential centrifugation gives a starting material with a higher specific activity than if the unfractionated cell extract is used. A second purification step occurs when the ATPase is selectively solubilized from the membrane. Some workers heve studied the properties of the soluble enzyme at this stage of partial purification (56) while others have gone on to further purify the ATPase (2), in some cases, to homogeneity (4, 72-74).

The criteria for homogeneity were the formation of a single peak in the analytical ultracentrifuge and the appearance of a single protein band after polyacrylamide gel electrophoresis. This protein band was shown to be coincident with ATPase activity (72).

The ATPase from B. megaterium (72) was purified by ammonium sulphate precipitation and glycerol gradient centrifugation. The enzymes from S. faecalis (74) and B. sterothermophilus (4)

were put through more rigorous procedures which included gel filtration, repeated chromatography on DEAE-cellulose and either a heat treatment (74) or chromatography on hydroxyapatite (4).

Properties of the solubilized ATPases

It is of interest to compare the properties of the ATPases before and after solubilization to see if release from the membrane has changed the enzymes in any way (Tables I and III).

The comparison reveals essentially no change in the fundamental properties of the enzymes on solubilization. Optimum ion to substrate ratios, pH optima and cation effects remained the same during the transition from the membrane-bound state to the soluble state. This may be due to the fact that gentle conditions were sufficient to solubilize the enzymes. Furthermore it suggests that study of the purified ATPase is relevant to the ATPase *in vivo* and that reconstitution could be successful.

Allotopy

A more detailed study of the soluble ATPases indicates that some of their properties do change on solubilization. "Allotopy" was the term used by Racker (75) to describe the change in the properties of the mitochondrial ATPase on going from the membrane-bound state to the soluble state. Subsequently it has been applied to the bacterial ATPases (71). One of the allotopic properties shown by the mitochondrial ATPase was cold lability. While the

Table III. Properties of the soluble ATPase from bacteria. (Activities measured in the presence of Mg^{2+}.)

Organism	Streptococcus faecalis	Bacillus megaterium	Micrococcus lysodeikticus	Bacillus sterothermophilus
References	(1, 73, 74)	(3, 56, 72)	(2)	(4)
Mg^{2+} activation	present	present	present	present
Activation by other divalent cations	Mn^{2+}	$Ca^{2+} > Mg^{2+}$	$Ca^{2+} > Mg^{2+}$	Mn^{2+}, Cd^{2+} and other divalent metal ions
Optimum Mg^{2+}:ATP ratio	____	1:2.5 at pH 7.2 (56)	1:2	1:2
pH optimum	pH 8.0	pH (7.2-7.8)	____	pH (8.0-8.5)
Substrate specificity ATP:ADP:AMP	100:0:0	100:0:0	100:<1:0	100:0:0
Stimulation by Na^{+}	slight stimulation	none	slight inhibition	none
Stimulation by K^{+}	slight stimulation	none	slight inhibition	none
				no stimulation when added together
Hydrolysis of other substrates relative to ATP	GTP > UTP > CTP 60% 6% 0%	GTP > CTP > UTP 72% 9% 3.5%	ITP > CTP 41% 15%	GTP > UTP > CTP 90% 30% 16%

enzyme was membrane-bound it was stable over a wide temperature range. However once it was released from the membrane it became labile in the cold (0°-4°), though it remained stable at room temperature (76 - 78).

Cold lability of bacterial ATPases

Among the bacterial ATPases cold lability is the commonest allotopic property described (1, 56, 71, 79). Ishida and Mizushima (56) reported that the solubilized ATPase of B. megaterium lost 70% of its activity after 21 h at 4°, whereas the membrane-bound enzyme was stable for at least 2 days at this temperature. ADP, ATP, and ATP in the presence of Ca^{2+} or Mg^{2+} increased the lability of the enzyme. Ammonium sulphate at 70% saturation and methanol (20%) were able to prevent loss of activity. The solubilized ATPase of S. faecalis was labile in the cold only if incubated with ADP. ATP in the presence of Mg^{2+} also inactivated the enzyme in the cold. This was thought to be due to ADP formation, since neither Mg^{2+} nor ATP alone induced the cold lability. Evans (71) has reported on the cold lability of the solubilized ATPase from E. coli. He showed that the ATPase extracted from the membranes with SDS was far more stable at 37° than at 4°. Contrary to these findings Muñoz et al. (2) were unable to find evidence of cold lability in the soluble enzyme of M. lysodeikticus, although they noted that further purification of the ATPase was accompanied by increasing instability of the enzyme during storage in the cold.

Other allotopic properties found in the bacterial ATPases

Many differences have been observed in the effects of inhibitors and uncouplers on the soluble ATPase as compared to the membrane-bound enzyme. However the action of inhibitors will be discussed in more detail later.

Differing requirements for Mg^{2+} and Ca^{2+} in the two states of the ATPase have been reported (2, 56) though these do not appear to be major differences.

A more interesting allotopic property is latency. Ishikawa (79) observed that the membrane-bound ATPase of M. lysodeikticus was not very active unless the membranes were exposed to trypsin. This finding was confirmed by Muñoz et al. (2). These workers also investigated the soluble ATPase to see if it too could be activated by trypsin. They found the converse, that is the soluble enzyme was rapidly inactivated by trypsin unless the constituents of the ATPase assay were also present. ATP (8mM) together with $CaCl_2$ (8mM) or $MgCl_2$ (4mM) gave complete protection to the ATPase.

Molecular weights of the bacterial ATPases

Molecular weight determinations on the soluble ATPases indicate that they are nearly all large enzymes. That of S. faecalis has a molecular weight of 385,000 as determined in the analytical ultracentrifuge by the sedimentation equilibrium method (80). The sedimentation coefficient $S^0_{20,w}$ was 13.4 S.

The molecular weight of the ATPase from M. lysodeikticus has not been reported but the sedimentation coefficient $S^0_{20,w}$ was quoted by Muñoz et al. (2) to lie between 14 - 15 s. Hachimori et al. reported that the molecular weight of the ATPase of B. sterothermophilus was 280,000 and its sedimentation coefficient was 11.9 s (4). The sedimentation coefficient of the ATPase from B. megaterium (72) was very close at 13.6 s to the value obtained for the S. faecalis ATPase.

There is some uniformity in these molecular weights and sedimentation coefficients. This is an addition to the list of similarities which have been observed in the properties of the bacterial ATPases from unrelated organisms. However an exception to the large-size ATPases is the enzyme solubilized by Evans (71) from E. coli membranes with the aid of the detergent SDS. The molecular weight of this enzyme was estimated to be 100,000 by gel filtration on Bio-Gel A 0.5m agarose.

Amino acid composition of the purified ATPases

Amino acid compositions have been reported on only two of the ATPases. These are the enzymes from S. faecalis (80) and B. sterothermophilus (4). In both cases there was no evidence of other components like carbohydrate, lipid or prosthetic groups being present in addition to the amino acids. Nor was there anything unusual about the amino acid compositions, such as a high proportion of hydrophobic amino acids which might be expected

of a membrane-associated protein.

Subunit compositions of the ATPases

Several of the bacterial ATPases have been broken down into subunits by denaturing conditions. The enzyme best characterized in this respect is that from S. faecalis (80). The molecular weight of the subunits was found to be 33,000 by the sedimentation equilibrium method done in the presence of 6M guanidine hydrochloride and 0.1M mercaptoethanol. This value for the molecular weight agreed very well with the minimal molecular weight value of 32,800 calculated on the basis of the amino acid composition. Although the subunits all seemed to have the same or very similar molecular weights, gel electrophoresis in the presence of dithiothreitol and 8M urea gave rise to two protein bands which Shnebli et al. designated α and β . They proposed a model of the enzyme in which the ATPase consists of a hexagonal arrangement of 6 subunits each made up of one α and one β chain. This model was supported by electron micrographs of the purified ATPase which showed uniform, 120 Å diameter particles in the form of hexagonal clusters of globular subunits (diameter 40 Å).

The subunit structure of the ATPase from M. lysodeikticus (52) as seen by electron microscopy also has a hexagonal arrangement, but in this case there is a central subunit surrounded by six others. SDS caused dissociation of this ATPase. Subsequent

polyacrylamide gel electrophoresis of the detergent-treated enzyme in the presence of 0.1% SDS gave rise to two major protein bands. A sedimentation coefficient of 3.5 s was obtained for the subunits by analytical ultracentrifugation. Gel electrophoresis of the ATPase in the presence of urea gave a more complex pattern which included at least 5 protein bands. A more recent report by Salton and Schor (81) confirmed the presence of two major subunits in the ATPase and assigned to them molecular weights of 62,000 and 60,000. In addition to these large subunits there were 1 - 3 smaller proteins associated with the enzyme. The ATPase from B. megaterium has also been subjected to electrophoresis in the presence of 0.1% SDS (3). Under these conditions only one major protein band was seen which corresponded to a molecular weight of 69,000. However gels run in 8M urea gave rise to two bands which suggests that although the subunits may be of the same size there may be at least two kinds which differ in their net charge. This is similar to the situation in S. faecalis. When the ATPase of B. megaterium was inactivated by incubation at 4° the slow-moving enzyme was converted to a faster moving component. This conversion was partial after 8 h at 4° but was complete after 48 h at this temperature.

Rebinding of solubilized ATPase to membranes

The breakdown and reconstitution of a biological system is often an aid to elucidating the function of the components

of the system. The ease with which the bacterial ATPases have been released from the membrane has prompted several workers to attempt the rebinding of the enzyme to the membrane. A simple reversal of the conditions that caused release has had a variable amount of success.

Mirsky and Barlow (3) obtained a soluble ATPase that had been released from protoplast membranes of B. megaterium by washing the membranes with dilute buffer in the absence of divalent metal ions. They then added the enzyme back to the depleted membranes in the presence of 10mM $CaCl_2$. Under these conditions most of the soluble ATPase sedimented with the membrane fraction. They demonstrated the specificity of this rebinding process by repeating the experiment with undepleted membranes in place of the depleted membranes, whereupon increased binding of ATPase was scarcely detectable.

Similar results with B. megaterium were reported in an earlier paper by Ishida and Mizushima (67). Recombination of soluble enzyme to depleted membranes was achieved in the presence of 5mM Mg^{2+} or 5mM Ca^{2+}. However if the soluble ATPase was first purified the extent of rebinding was lower especially in the presence of Mg^{2+}.

Abrams achieved limited success in rebinding solubilized ATPase to depleted membranes in S. faecalis (1). A variety of cations at 1mM concentration were each able to rebind about 30% of the soluble enzyme. A more detailed study (82) showed that the membrane-bound ATPase could be fully reconstituted,

at which point the ATPase accounted for about 2% of the total membrane protein. The binding of ATPase to the membrane was shown by differential centrifugation, sucrose density gradient centrifugation and electrophoresis. At the same time it was shown that the soluble ATPase, an acidic protein, did not bind to ribosomes even in the presence of 20mM Mg^{2+}. The binding of the soluble ATPase to the depleted membrane apparently did not require Mg^{2+} provided that there was an excess of the soluble enzyme. Under these conditions the recombination was reversible. The addition of Mg^{2+} enhanced the binding of ATPase to the membrane either by, or in addition to, preventing the dissociation of the complex. Thus in the presence of Mg^{2+} a lower level of soluble ATPase was sufficient to achieve the same amount of reconstitution. At a non-saturating level of soluble ATPase the optimum Mg^{2+} concentration for reconstitution was reached at 10mM. The specificity of the rebinding process was again demonstrated by the failure of undepleted membranes to bind soluble ATPase. Furthermore the depleted membranes did not bind more ATPase than in the original undepleted state of the membrane. This indicates that there are specific binding sites on the membrane for the ATPase. Also the fact that the original specific activities can be equalled after reconstitution argues that the ATPase has been restored to its native state.

A closer scrutiny of the rebinding process in S. faecalis (202) indicated that an additional protein factor was required for the reconstitution of membrane-bound ATPase. Baron and Abrams

named this factor nectin and demonstrated that it was a protein with a molecular weight of 37,000. They reported that nectin was released from the membranes at the same time and under the same conditions used to solubilize the ATPase. Consequently when the soluble ATPase fraction was added back to the depleted membranes it also contained nectin. However it was possible to separate the ATPase from nectin by gel filtration on Bio-Gel A 1.5 m because of their widely different molecular weights. This separation could only be achieved in the absence of Mg^{2+}. If 10mM Mg^{2+} were present in the eluting solution nectin remained with the ATPase. The nectin-free ATPase did not reassociate with the depleted membranes even in the presence of Mg^{2+} unless nectin was also added. Under these conditions the extent of the binding of the enzyme was directly proportional to the amount of nectin present until all the binding sites for the ATPase had been occupied. Also the rebinding was temperature-dependent occurring at 38° but not at 0°. In contrast to this the unresolved soluble ATPase bound equally well at 0° and 38°. From this finding Baron and Abrams concluded that the association of nectin to the soluble ATPase was the temperature-dependent step.

The existence of a nectin protein in B. megaterium might explain why the partially purified ATPase bound less effectively to depleted membranes than did the unfractionated soluble ATPase (67).

Clearly the physical reassociation of ATPase to the membrane has been well substantiated in these examples of recons-

titution, but how close is the rebound enzyme to its original functional state? Studies with B. megaterium (67) and S. faecalis (82) suggest that the reassociation returns the ATPase to its original state. Not only can full ATPase activity be restored (82) but the allotopic properties that changed on going from the membrane-bound state to the soluble state were reversed on reconstitution. Thus, in B. megaterium (67) 10% - 40% of the rebound ATPase activity could be protected from cold inactivation compared to 0% protection of the unbound enzyme. Similarly the recombined ATPase in S. faecalis (82) became sensitive again to inhibition by dicyclohexylcarbodiimide (DCCD), whereas the soluble enzyme was unaffected (83).

Minor ATPase activities in bacteria

The discussion up to now has dwelt on the membrane-bound ATPases of bacteria since they are quantitatively the most important, they are ubiquitous and they show remarkable similarities between species. Other, minor ATPases have been detected usually in the cytoplasmic fraction. In mentioning them attention will be focussed on reports from E. coli.

Deoxyribonucleic acid (DNA)-dependent ATPases

A DNA-dependent ATPase in M. lysodeikticus has been well characterized by Anai et al. (84, 85). It appears to be an endonuclease which hydrolyses three molecules of ATP to ADP and Pi

for every phosphodiester bond broken. In assays of the soluble form of the membrane-bound ATPase of M. lysodeikticus, Ishikawa (86) found it necessary to add DNase to crude supernatant fractions in order to inhibit DNA-dependent ATPases (87).

In E. coli K12, Oishi et al. (88) observed an ATPase which was completely dependent on DNA for its activity. During the characterization of this enzyme Nobrega et al. found that it was closely associated with an ATP-dependent DNAse (89). Since the two activities co-electrophoresed on polyacrylamide gels and had very similar heat-inactivation profiles, these workers concluded that they were physically and functionally associated. The ATPase activity required Mg^{2+} or Mn^{2+} and converted ATP to ADP and Pi. For each phosphodiester bond cleaved 8 - 9 ATP molecules were hydrolysed. Of the other nucleoside triphosphates tested dATP was also hydrolysed. The pH optimum extended from pH 7.5 - 9.5 and the preference of the enzyme was for double-stranded DNA. Its molecular weight was estimated to be 300,000 - 350,000. Since the ATP-dependent DNAse could not be detected in the recombination-deficient mutant strains recB and recC (90-92) it is thought that both the ATPase and the DNAse activities are involved in genetic recombination.

A different DNA-dependent ATPase has been isolated from E. coli which were infected with the bacteriophage T_4 (93). It also cleaved ATP to ADP and Pi in the presence of DNA but the ATPase activity was not dependent on the hydrolysis of phosphodiester bonds; in fact no exo- or endonucleolytic activity

was detectable with the enzyme. CTP and deoxyadenosine-5'-triphosphate (dATP) were also cleaved, but at a slower rate than ATP. The ATPase required Mg^{2+} for activity as well as DNA. Of the types of DNA tested, native DNA from calf thymus or E. coli, and heat-denatured DNA from the bacteriophages T_4 and T_7 were successful in stimulating the enzyme: the native DNA of the bacteriophages was ineffective. The molecular weight of the enzyme was found by gel filtration to be 15,000. Its function is unknown, but since the enzyme was not detectable in uninfected E. coli it is probably not of general importance.

Paetkau and Coy (94) reported the removal of an ATPase activity from DNA-dependent RNA polymerase during the purification of the latter enzyme from the cytoplasmic fraction of E. coli. Under the denaturing conditions of SDS gel electrophoresis the molecular weight of the ATPase protein was 68,000. The ATPase had a tendency to aggregate during centrifugation in a glycerol gradient and during dialysis against 50mM Tris-HCl buffer (pH 8.0). Analytical ultracentrifugation of the aggregated ATPase protein gave a sedimentation coefficient of 22.2 s for the major protein peak. Its molecular weight was estimated by gel filtration to lie between 400,000 and 490,000. The enzyme hydrolysed CTP, UTP, and GTP at 2.6%, 0.57% and 0.16% respectively of the rate at which ATP was hydrolysed. From the results that Paetkau and Coy obtained they were unable to say whether the ATPase was normally in association with itself or with the RNA polymerase.

Functions of the bacterial ATPases

The study of the properties of bacterial ATPases has progressed faster than the determination of the function(s) of the enzyme(s). For many enzymes their role in catalysing a specific chemical reaction is their function in the cell. However the likelihood of the bacterial ATPase existing solely to break down ATP to ADP and Pi is remote. It is more likely that the ATPase reaction either normally acts in a reverse direction to bring about the synthesis of ATP or it is used to drive an endergonic process. In line with this reasoning the ATPase has most frequently been linked to oxidative phosphorylation or ion transport.

In assigning a role to the ATPase activity, investigators have tried to draw analogies with the better characterized ATPases from eukaryotes. Thus the existence in eukaryotes of a $(Na^+ + K^+)$-activated ATPase which is responsible for active transport of Na^+ and K^+ (95-97) has prompted many workers to look at the effects of Na^+ and K^+ on the bacterial ATPases. Similarly, many of the properties of the mitochondrial and chloroplast ATPases, such as cold-lability (76-78) have been looked for in bacterial ATPases.

From these studies it has become obvious that the membrane-bound bacterial ATPases show a marked resemblance to the mitochondrial ATPase. In order to compare these enzymes it is necessary to briefly outline some of the properties of the mito-

chondrial ATPase. Several more detailed reviews are available (9, 75, 98, 99).

The properties of the mitochondrial ATPase

The best characterized mitochondrial ATPases are from beef heart and rat liver. The properties listed in Table IV are those of the beef heart enzyme, though often no distinction is made between the two enzymes since they are so similar to each other. The ATPase of the mitochondrion is bound to the inner membrane. The particles seen on the inner surface of this membrane after negative staining (44-47) have been shown to be the ATPase itself (100-102). These 85 Å diameter particles are usually released from the membrane by sonication. Catteral and Pedersen (103) found that sonication of rat liver mitochondrial membranes alone gave a low yield of solubilized ATPase (less than 0.5%). However if the membranes were washed thoroughly in a buffer of low ionic strength, followed by incubation with ATP and ethylene glycol for 16 h and then sonicated, 30% - 55% of the ATPase was solubilized.

The purified, solubilized enzyme from beef heart mitochondria was activated by Mg^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}, or Ca^{2+} (104). The greatest activation was seen with Mg^{2+}. The products of the reaction were ADP and Pi only. Other nucleoside triphosphates were attacked. Their rates of hydrolysis compared to a 100% value for ATP were: Inosine-5'-triphosphate (ITP) (125%),

Table IV. Comparison of the properties of ATPases from a variety of sources. (Activities measured in the presence of Mg^{2+}.)

Source	*S. faecalis*	*B. megaterium*	*M. lysodeikticus*	*B. stearothermophilus*	Mitochondria (beef heart)
References	(1, 73, 74)	(3, 56, 72)	(2)	(4)	(104, 105)
Mg^{2+} activation	present	present	present	present	present
Activation by other divalent cations	Mn^{2+}	$Ca^{2+} > Mg^{2+}$	$Ca^{2+} > Mg^{2+}$	Mn^{2+}, Cd^{2+}, etc.	Co^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}
Optimum Mg^{2+}:ATP ratio	___	1 : 2.5	1 : 2	1 : 2	1 : 1.5
pH optimum	pH 8.0	pH (7.2 - 7.8)	___	pH (8 - 8.5)	pH (8.5 - 9.3)
Substrate specificity ATP:ADP:AMP	100:0:0	100: 0:0	100:<1:0	100:0:0	100:0:0
Effect of Na^+ and K^+	slight stimulation	none	slight inhibition	none	none
Hydrolysis of other substrates relative to ATP (%)	GTP>UTP>CTP 60%: 6%: 0%	GTP>CTP>UTP 72%: 9%:3.5%	ITP>CTP 41%:15%	GTP>UTP>CTP 90%:30%:16%	ITP>GTP>UTP>CTP 125%:75%:63%: 0%
Molecular weight or sedimentation coefficient	385,000 $S^0_{20,w}$=13.4 S	$S^0_{20,w}$=13.6 S	$S^0_{20,w}$=14 - 15 S	280,000 $S^0_{20,w}$=11.9 S	360,000 (105)

GTP (75%), UTP (63%), and CTP (0%). Nucleoside diphosphates and monophosphates were not hydrolyzed. ADP alone among the nucleoside diphosphates was inhibitory to the ATPase reaction. There was no inhibition by nucleoside monophosphates. The optimum ion to substrate ratio (Mg^{2+}:ATP) was 1:1.5, and the optimum pH lay between pH 8.5 and 9.3. These properties of the solubilized enzyme are very similar to those of the membrane-bound enzyme (106-108). In this respect and in regard to the other properties listed the mitochondrial ATPase bears a close resemblance to the bacterial ATPases (Table IV).

Further similarities include molecular weight values and amino acid compositions of the enzymes. Initially the molecular weight of the ATPase from beef heart mitochondria was put at 284,000 by Penefsky and Warner (109). A similar value was obtained by Forrest and Edelstein (110). This figure has been re-evaluated by Lambeth et al. (105) who gave 360,000 as the molecular weight. The molecular weight of the ATPase from rat liver mitochondria was found to be 384,000 by Catterall and Pedersen (103) and 360,000 by Lambeth and Lardy (111), while the yeast mitochondrial ATPase had a molecular weight of 340,000 (112). These molecular weight values for the various mitochondrial ATPases are all very similar to the values obtained for the bacterial ATPases as listed in Table IV.

The amino acid composition of the ATPase from S. faecalis is quite similar to those of rat liver and bovine heart mitochondrial ATPases (103). It is even more like that of spinach

chloroplast ATPase (113, 114), an enzyme which bears a very close physical and functional resemblance to the mitochondrial ATPases (99).

Function of the mitochondrial ATPase

The function of the mitochondrial ATPase has been well established. Besides having a structural role to play in the inner mitochondrial membrane (75, 98), the enzyme catalyzes the terminal reaction of oxidative phosphorylation in which ATP is synthesized from ADP and Pi (115). The energy to drive this reaction comes from the oxidation of substrates in the electron transport chain. At the present time the mechanism by which the energy is conserved is not fully understood. There are two major hypotheses which attempt to explain the process. One is the chemical hypothesis of Slater (116), the other is the chemiosmotic hypothesis of Mitchell (117, 118). In the former hypothesis, "high energy intermediates" are generated at specific coupling sites during the transfer of electrons between certain carriers of the electron transport chain. These high energy intermediates can then be used in turn to generate ATP by way of the ATPase. In the chemiosmotic hypothesis electron transfer down the respiratory chain is used to extrude protons on the outer or C-side (98) of the membrane. The resulting proton gradient exerts a force known as the "proton motive force" which can bring about the synthesis of ATP again by way of the

ATPase. Fortunately in discussing the function of the mitochondrial ATPase it is not important to know thich mechanism is the correct one since both mechanisms require an ATPase and since the net results are the same.

The energy-dependent synthesis of ATP can be reversed. In this way ATP hydrolysis can generate "high-energy intermediates" or a "proton motive force" which can be used to drive various endergonic processes associated with the inner mitochondrial membrane. These processes include active transport, energy-dependent transhydrogenation and the reversal of the direction of oxidation in the respiratory chain.

When the ATPase is detached from the membrane it is not possible to drive the above processes with ATP, nor is it possible to generate ATP by oxidative phosphorylation. However, under these circumstances, respiration can continue and can still generate a "proton motive force" or "high-energy intermediates". These can then be used directly to drive active transport, energy-dependent transhydrogenation or the reversal of the direction of oxidation in the respiratory chain. Under the right conditions the ATPase can be rebound to the membrane to restore the ATP-driven functions. Thus the ATPase is one of several "coupling factors" from the soluble fraction which are needed for the reconstitution of oxidative phosphorylation.

Inhibitor studies

Inhibitors have played a major part in working out the

details of energy conservation in the mitochondrion. One type of inhibitor is the "uncoupler", a compound which abolishes phosphorylation without inhibiting respiration. This loss of phosphorylation is accompanied by an increase in ATPase activity. In the chemical hypothesis the uncoupler might dissipate the "high-energy intermediates" by promoting their hydrolysis (9). In the chemiosmotic hypothesis the uncouplers are postulated to dissipate the "proton motive force" by conducting protons across the membrane (119). Frequently used uncouplers include dinitrophenol (DNP), carbonylcyanide m-chlorophenylhydrazone (CCCP) and gramicidin.

Other inhibitors have a more specific effect on the ATPase. Oligomycin and rutamycin both inhibit the ATPase (120, 121) either when the enzyme is membrane-bound, or after solubilization when the enzyme is associated with other membrane proteins, one of which is the "oligomycin-sensitivity-conferring-protein" (OSCP) (122, 123). This inhibition of the ATPase also blocks respiration. DCCD has a similar effect to oligomycin (124). It binds irreversibly to the oligomycin-sensitive ATPase complex. This fact has been made use of by Strekhoven et al. (125) who isolated the DCCD-binding protein from bovine heart mitochondrial ATPase. Dio 9 is an effective inhibitor of the mitochondrial ATPase though its mode of action is not well characterized (126-128).

The effects of inhibitors on bacterial ATPases

Many workers have tested bacterial ATPases with the same compounds that give activation or inhibition of the mitochondrial ATPase. Amongst the uncoupling reagents, DNP, which stimulates the mitochondrial ATPase, also stimulated the ATPase of Desulphovibrio gigas (129) though in most organisms it either had no effect or was mildly inhibitory (4, 55-59). CCCP stimulated the ATPase of R. rubrum (60) but not that of E. coli (58). Gramicidin S showed allotopic properties with respect to its effect on the ATPase of B. megaterium (56). It stimulated the soluble enzyme but inhibited the membrane-bound form. Oligomycin had little, if any, effect when tested (55, 56, 59) though it did inhibit the membrane-bound ATPase of R. rubrum (60).

The most potent and most universal inhibitor of bacterial ATPases is azide (4, 52, 56, 57, 59). In B. megaterium (56) 5mM Na azide gave 93% inhibition of the membrane-bound ATPase and 89% inhibition of the soluble ATPase. In B. sterothermophilus 82% inhibition of the soluble ATPase was obtained with 0.5mM sodium azide. Iodine at the same concentration inhibited the enzyme activity by 87%. Azide and iodine are both strong inhibitors of the mitochondrial ATPase (104, 130).

Several of the ATPases were inhibited by sulphydryl reagents (59, 60, 131) but others (4, 52, 54) like the mitochondrial ATPase (104) were not affected. Dithionite stimulated the ATPases of B. megaterium (55, 56), and R. rubrum (60) but not the ATPase of E. coli (57). Dio 9, an inhibitor of the mito-

chondrial ATPase, also inhibited the ATPases of E. coli (58) and S. faecalis (132). DCCD inhibits the membrane-bound, mitochondrial ATPase by reacting with a component in the membrane. The soluble enzyme is not inhibited. The same effect was observed with the ATPase of S. faecalis (6, 132, 133) and the coupling factor ATPase of M. phlei (134). DCCD inhibited both enzymes when they were bound to the membrane but not after they had been solubilized. However in E. coli, inhibition with DCCD has been reported for both the membrane-bound (58, 71) and the soluble ATPases (71).

Clearly then there are many similarities between mitochondrial and bacterial ATPases with regards to the way in which they respond to inhibitors. This might be because they fulfil the same functions in their respective systems.

ATPase as a coupling factor for bacterial oxidative phosphorylation

The extensive similarities in respiration and energy conservation between bacteria and the mitochondria have been reviewed (9, 51, 135). Early attempts to demonstrate oxidative phosphorylation in cell-free extracts of micro-organisms produced very low P/O ratios, perhaps as a result of the harsher conditions needed to produce a cell-free extract in bacteria. These studies were confused by contributions from substrate-level phosphorylation via glycolysis. Pinchot (136) avoided the latter problem by looking at oxidative phosphorylation in Alcaligenes faecalis,

an organism which is deficient in the glycolytic pathway. He obtained P/O ratios of 0.1 to 0.3 which were sensitive to DNP only at concentrations somewhat higher than those used to inhibit mitochondrial oxidative phosphorylation. (Generally, bacterial phosphorylation is less sensitive to the inhibitors of mitochondrial phosphorylation.)

Subsequently oxidative phosphorylation has been looked at in a variety of bacteria (27, 137-139) including E. coli (140). The P/O ratios found in E. coli tend to be low (140) whereas values approaching 2 have been obtained in cell extracts of Mycobacterium phlei (137, 141).

When bacterial cell extracts which are capable of oxidative phosphorylation have been separated by centrifugation into soluble and particulate fractions, the phosphorylation efficiency of the particles tends to be lower than that of the cell extract. However, as in the mitochondria this efficiency can often be increased by the addition to the particles of factors from the soluble fraction (27, 136, 141). These factors are frequently classified into heat-labile and heat-stable factors. Some of these soluble, heat-labile factors appear to have ATPase activity associated with them (27, 142, 143) though often it is latent and requires activation by trypsin, heat treatment or sulphydryl reagents (79, 86, 142, 143). On the other hand, Ota (144), working with E. coli and Asano and Brodie (145) using M. phlei found no parallel between the ATPase of the soluble fraction and coupling factor activity. However Bogin et al.

(146) observed in M. phlei a particulate-bound coupling factor with ATPase activity which after solubilization could act at all coupling sites to stimulate oxidative phosphorylation. This ATPase activity was also latent.

The latency of the bacterial coupling factors has its parallels with the ATPase of M. lysodeikticus (2, 79) and with the chloroplast (114) and mitochondrial ATPases (75). Submitochondrial particles from beef heart have variable ATPase activity. However if they are subjected to proteolysis by trypsin (147) or passed through Sephadex, the activity increases 4 - 10 times (75). This activation is thought to be due to the destruction or removal of a small molecular weight protein inhibitor (148), which normally masks the ATPase activity and also protects it from cold inactivation.

In addition to latency the bacterial coupling factors have many other properties in common with the bacterial and mitochondrial ATPases. In A. faecalis (142) the coupling factor ATPase activity was Mg^{2+}- or Ca^{2+}-dependent, with Mg^{2+} being the better activator. It had a sedimentation coefficient of 13 s and after purification appeared as a 90 Å diameter particle that was indistinguishable by electron microscopy from the mitochondrial ATPase.

In the heat-labile coupling factor fraction of M. lysodeikticus, Ishikawa et al. (87) observed a DNA-stimulated ATPase activity. Total ATPase activity was reduced by 90% in the absence of DNA. Sucrose density gradient centrifugation

of the coupling factor fraction gave 3 peaks of ATPase activity with sedimentation coefficients of 13 s, 9.8 s, and 4.5 s, respectively. The latter two activities required DNA while the 13 s component was DNA-independent. Of the three ATPases only the 13 s enzyme also had coupling factor activity. Since its ATPase activity could be markedly increased by trypsin, latency probably accounts for its activity being overshadowed by the DNA-dependent ATPases. The coupling factor ATPase appears to be very similar to the ATPase of M. lysodeikticus as described by Salton's group (2, 52, 69). It was activated by Ca^{2+} and Mg^{2+} and inhibited by ADP. The coupling factor ATPase showed allotopic behaviour (86). For example, Gramicidin A stimulated the soluble ATPase but inhibited the membrane-bound enzyme. The effect of arsenate was just the opposite. The reconstitution of oxidative phosphorylation in M. lysodeikticus (86) involved the rebinding of the coupling factor ATPase to the depleted membranes. Once the ATPase was rebound it became sensitive again to inhibition by Gramicidin A.

Like the soluble ATPase of S. faecalis (1) the coupling factor ATPase of M. lysodeikticus was cold-labile in the presence of ADP.

In Azotobacter vinelandii (143) the coupling factor ATPase activity was Mg^{2+}- or Ca^{2+}-dependent, with Mg^{2+} being the better activator. The optimum Mg^{2+} to ATP ratio was 1. The reaction was inhibited by ADP but not by AMP. The ATPase was affected by inhibitors in the same manner as the mitochondrial

ATPase, being stimulated by uncouplers like CCCP and inhibited by Dio 9.

Similarities in properties between the bacterial coupling factors and the bacterial and mitochondrial ATPases extend to the way in which these proteins are bound to the membrane. Catteral and Pedersen (103) were able to increase the solubilization of the mitochondrial ATPase by a pretreatment which included washing the membranes exhaustively with low ionic strength buffer. In A. faecalis (142) the coupling factor was bound less strongly in a medium of low ionic strength. The coupling factor of M. lysodeikticus was solubilized by washing the membrane with distilled water (27). Divalent metal ions were required for its reattachment (86). In membrane particles of the photosynthetic bacterium Rhodopseudomonas capsulata (149) the loss of photophosphorylation after various treatments parallels the loss of ATPase. This loss was greatest when EDTA was present during sonication of the membranes but could be largely prevented or reversed by the addition of Mg^{2+}. Similar results were obtained with R. rubrum (150).

The coupling factor which was responsible for the restoration of photophosphorylation in R. capsulata has been partially characterized (151). Like the other coupling factors described previously it had ATPase activity which could be activated by Mg^{2+}, Mn^{2+} or Ca^{2+}. This enzyme activity was stimulated by DNP and by p-trifluoromethoxyphenylhydrazone (FCCP). FCCP, which is structurally similar to CCCP, is an

even stronger uncoupler of oxidative phosphorylation in the mitochondria than is CCCP (152). The molecular weight of the ATPase coupling factor was estimated to be 280,000 by gel filtration. However the most interesting point about this coupling factor is the observation that it will restore oxidative phosphorylation as well as photophosphorylation (153, 154). In view of this dual role of the coupling factor ATPase in a photosynthetic bacterium it is not so surprising that the coupling factor ATPases of the chloroplast and the mitochondria are so alike (99, 103, 104, 113-115). Indeed it is possible that all the enzymes that conserve energy from a respiratory chain in the form of ATP have a common origin and perhaps a common mechanism. For this reason the bacterial coupling factor ATPases would be similar to each other and to those of the mitochondria and chloroplast.

Evidence that the mitochondrial and bacterial coupling factor ATPases fulfil the same role in their respective systems is supported by the work of Bogin *et al.* (155). These workers were able to interchange coupling factors from the bacterium *M. phlei* and beef heart mitochondria and still reconstitute oxidative phosphorylation in either system. Although the P/O ratios obtained with the foreign coupling factors were lower than with native coupling factors the phosphorylation reconstituted was sensitive to the uncoupling agents Gramicidin A and DNP.

Use of mutants deficient in ATPase

Clearly there is a wealth of evidence that points to the common identity of the bacterial ATPases and the bacterial coupling factor ATPase, and indicates a common function for the bacterial and mitochondrial ATPases. However much of this evidence was circumstantial and it is only very recently that these postulates have been proven.

In much biochemical research bacterial systems have been investigated first and the results obtained have aided the study of eukaryotic systems. One major reason why this is so, is the ease with which mutant strains of bacteria can be isolated. However in energy conservation the study of the eukaryotic system has led the way. Perhaps this is because there has been little work done with mutants in this area of biochemistry. Recently, however, mutants of E. coli were isolated which were deficient in electron transport or oxidative phosphorylation. These mutants were able to grow on glucose, though with a lower growth yield, but were not able to grow on lactate or intermediates of the tricarboxylic acid cycle. The latter property provided a basis for the initial selection of colonies of the mutants. Amongst the strains isolated were two that lacked the Ca^{2+}, Mg^{2+}-activated ATPase (5) (designated unc A^-). The uncA^- allele was transduced into another strain which was subsequently compared to the parent strain. The comparison showed that the lesion in the ATPase gene abolished oxidative phosphorylation. A subsequent study (156)

showed that this mutation had also abolished the ATP-driven, energy-dependent transhydrogenase without diminishing the non-energy-linked transhydrogenase activity.

A similar mutant was isolated by Kanner and Gutnick (157). They substantiated the findings of Cox et al. with respect to the transhydrogenase activities (8). In addition, they showed that although the energy-dependent transhydrogenase could not be driven by ATP, it was present, and could derive the necessary energy from respiration. DCCD, which could inhibit the ATP-driven, energy-dependent transhydrogenase in the parent strain, had no effect on the respiration-driven, energy-dependent transhydrogenase.

The role of ATPase in active transport

From these results it can be seen that the ATPase of E. coli is the terminal coupling factor in oxidative phosphorylation and fulfils the same role as the mitochondrial ATPase. There is good reason to believe that the same is true in other bacteria. However S. faecalis is essentially a fermentative organism despite the demonstration that it can to a limited extent couple NADH oxidation to ATP synthesis (158). Consequently it relies on substrate-level phosphorylation for ATP generation. In view of this the role of the ATPase in active transport has been examined more closely in this organism.

Harold et al. (6) found that the addition of DCCD to intact cells inhibited active transport processes such as

the uptake of alanine and phosphate and the exchange of H^+ and Na^+ for K^+. Since DCCD also inhibited the degradation of ATP *in vivo* it was inferred that the ATPase mediated the active transport using ATP generated by substrate-level phosphorylation. Similar findings were obtained with the ATPase inhibitors Dio 9 and chlorhexidine (132). Abrams *et al.* (159) have isolated a mutant of *S. faecalis* in which the ATPase is 100 times more resistant to DCCD inhibition than the wild type. They found that the active transport processes were similarly resistant to DCCD. At the same time they identified the membrane as the site of action of the inhibitor, since reconstituted membrane-bound ATPase made up of ATPase and nectin from the mutant, and ATPase-depleted membranes from the wild type, was sensitive to DCCD. Whereas if the ATPase and nectin came from the wild type and the membranes from the mutant then the reconstituted membrane-bound ATPase was no longer sensitive to DCCD.

Further evidence to link the ATPase to active transport was obtained by Abrams and Smith (160). They grew *S. faecalis* on limiting amounts of K^+ and found that the bacteria produced 1.5 - 2.1 times more ATPase per cell. Redwood *et al.* noticed an increased conductance across a lipid bilayer after the addition of ATPase from *S. faecalis* (161). This increased conductance was dependent on Mg^{2+}, ATP and the concentrations of Na^+ and K^+. It was abolished after treatment of the ATPase with pronase.

The bacterial ATPases are very different from the Na^++K^+-

activated ATPases of mammalian plasma membranes which are involved in Na^+ and K^+ transport (9). The mammalian enzyme is difficult to solubilize from the membrane, it has a requirement for phospholipid and is stimulated by $Na^+ + K^+$. The enzyme is strongly inhibited by the steroid glycoside ouabain, but not by DCCD. Apart from a very minor amount of ATPase activity in *E. coli* which was sensitive to the inhibitor ouabain (63), this compound has not been found to inhibit bacterial ATPases (4, 35, 52, 54, 57, 129). The bacterial ATPases are not markedly stimulated by monovalent cations apart from the ATPases of *V. parahaemolyticus* which may be a special example since the organism is halophilic (35).

The demonstration that the ATPase of *S. faecalis* mediates active transport is timely since there does not appear to be an alternative enzyme for these processes. This is probably true for all bacteria. In *E. coli* K12 another unc A^- mutant has been isolated and tested for its ability to carry out active transport of thiomethyl-β-D galactoside (TMG) (7). The rate of accumulation of this compound was the same in the wild type and the unc A^- mutant. However if respiration was inhibited by cyanide the transport was abolished in the mutant but only decreased by 35% in the wild type. Schairer and Haddock conclude that the uptake of TMG is driven either by respiration or by hydrolysis of ATP by the ATPase. So that if respiration is inhibited the ATPase is the only remaining route by which energy can be generated for the active transport of TMG.

Summary

In summary, these findings indicate that the bacterial ATPase, like the mitochondrial ATPase, catalyzes the terminal reaction of energy conservation in which ATP is synthesized from ADP and Pi. During the complete combustion of carbon sources such as glucose, lactate and succinate, oxidative phosphorylation supplies a large portion of the cell's requirement for ATP. However ATP synthesis is just one possible use for the "high-energy intermediates" or "proton motive force" that are generated during substrate oxidation via the electron transport chain. Other uses are active transport and energy-dependent transhydrogenation. These processes can be driven directly by the energy of respiration without having to first generate ATP. Since the oxidative phosphorylation process is reversible, ATP hydrolysis via the ATPase will generate the "high-energy intermediate" or "proton motive force" that is necessary to drive active transport or transhydrogenation of nicotinamide adenine dinucleotide phosphate from the oxidized (NADP) to the reduced (NADPH) form. This reverse reaction would be important during glycolysis for example, and particularly in an organism like S. faecalis which does not appear to conserve energy by way of respiration.

METHODS AND MATERIALS

Reagents

All chemicals used were of reagent-grade purity. Crystalline bovine serum albumin, bovine catalase, yeast hexokinase, yeast alcohol dehydrogenase, yeast glucose-6-phosphate dehydrogenase, equine hemoglobin and soybean trypsin inhibitor were obtained from Calbiochem. Porcine thyroglobulin and equine apoferritin were products of Mann Research Laboratories. Deoxyribonuclease was obtained from Nutritional Biochemicals Corporation, trypsin from Boehringer (Mannheim, Germany), and Glucostat reagents from Worthington Biochemical Corporation. Egg-white lysozyme was obtained from both Worthington Biochemical Corporation and Calbiochem.

Nucleotides were purchased from P.L. Biochemicals Ltd., or from Calbiochem with the exceptions of the Tris salt of ATP, which came from Sigma Chemical Company and tritiated ADP (20 μCi/ml) which came from Schwarz. NAD(H), NADP(H), p-NPP, bis-p-NPP and penicillin G (potassium salt) were from Calbiochem. Glucose-6-phosphate came from Boehringer (Mannheim, Germany), 2-methyl-1,4-napthoquinone from Nutritional Biochemicals Corporation, N,N,N',N',tetramethylethylenediamine (TMED) from Eastman Kodak Company and adenine trinucleotide from Miles Laboratories.

Treatment of diethylaminoethyl-cellulose (DEAE-cellulose) prior to use

DEAE-cellulose (40 g of Cellex D) was mixed with 0.5N hydrochloric acid (3.5 l.) and allowed to settle for 30 min. The supernatant was removed by suction, and the sediment was resuspended in distilled water to a volume of 4 l. After 30 - 60 min the supernatant was again removed by suction. This washing step was repeated several times until the pH of the supernatant was 4. At this stage 0.5N NaOH (3.5 l.) was added with stirring to the DEAE-cellulose and a similar cycle of sedimentation and washing with distilled water was continued until the pH of the supernatant reached 8. The exchanger used for nucleotide chromatography was freshly prepared by this method.

Paper chromatography of nucleotides

Preparations of ATP and ADP were examined after paper chromatography on Whatman #40 paper for ultra violet (UV) light-absorbing impurities. The solvent system used was a mixture of M ammonium hydroxide (30 ml), isobutyric acid (50 ml) and 0.2M disodium EDTA (0.4 ml). Chromatograms were run in the descending manner for 22 h. The nucleotide samples and standards were converted to the ammonium ion form prior to chromatography. This was done by mixing a 0.1M solution of the nucleotide (1 ml) with wet DOWEX 50WX2 (0.5 ml) that had previously been converted to the ammonium ion form. Spots were seen when the

dried paper was examined under the short wavelength UV light. To quantitate the results strips of paper, each containing one of the spots, were cut out and eluted as described by Heppel (162).

Chromatography of commercial ADP

ADP (sodium salt) supplied by P.L. Biochemicals Inc. was freed from traces of ATP and glucose-6-phosphate by ion exchange chromatography on freshly precycled DEAE-cellulose (40 g). The exchanger was converted to the carbonate form by the addition of 2M ammonium carbonate (700 ml), and by packing and washing the column in more of the same solution. The column of bed volume 2.5 cm x 38 cm was then washed with water until the effluent was neutral to pH indicator paper. ADP from 3 different batches was combined (Lots #s 632, 634 and 635) to give a total weight of 856 mg. Lot # 632 (307 mg) was reported by P.L. Biochemicals Inc. to contain 1% - 2% by weight of glucose-6-phosphate as an impurity. The sample was dissolved in water (210 ml) and applied to the column at a flow rate of 30 ml per h. This was followed by water until 490 ml of effluent had been collected.

The sample was eluted by application of a linear gradient of ammonium bicarbonate. This was set up by having 0.25M ammonium bicarbonate (2 l.) in the salt chamber and water (2 l.) in the mixing chamber. Fractions (10 ml) were

collected at the rate of 5 - 6 per h and their absorbance at 260 nm determined. ADP in the ammonium ion form was recovered from pooled fractions by evaporation in the presence of 20% ethanol under reduced pressure at 37° in a rotatory evaporator. The residue in the flask was taken up in fresh 20% ethanol and again evaporated to dryness. This procedure was repeated several times until the ammonium bicarbonate (white residue) had volatilized. Finally the nucleotide was dissolved in distilled water and lyophilized.

Determination of protein

Protein concentrations were determined by the method of Lowry *et al.* (163), over the range 0 - 500 µg/ml. Crystalline bovine serum albumin was used as a standard.

Determination of glucose

Glucose was determined by the "Glucostat" method. Immediately prior to use, one vial of lyophilized enzyme preparation (Glucostat) and one vial of Glucostat chromagen were dissolved together in less than 50 ml of water. The pH was measured and, if necessary, was adjusted to 7.0 with 0.1M potassium phosphate buffer (pH 7.0). The volume was then made up to 50 ml with water. Two and a half ml of 0.1M potassium phosphate buffer (pH 7.0) were added to a 0.5 ml sample followed by 2.0 ml of Glucostat reagent. The samples were incubated for

30 min in the dark at 37°. At the end of this period 2 drops of 6N HCl were added to each tube, followed by 5.0 ml of water. The samples were mixed rapidly on a Vortex mixer and were kept at 22° for 5 min before reading the absorbance of the solutions at 420 nm. The glucose concentrations in the samples were determined from a standard curve ranging from 0 - 250 µg glucose per ml.

Determination of inorganic phosphate

In the earlier work described here phosphate was measured by a modification of Sumner's method (164). The assay mixture contained 6.6% ammonium molybdate:4 H_2O (0.3 ml), 7.5N H_2SO_4 (0.3 ml), water (2.4 ml) and $FeSO_4$:7 H_2O (25 mg) to which was added 0.15 ml of the phosphate sample (0-0.75 umoles). After 5 min at 22° the absorbance at 660 nm was recorded and the phosphate content of the sample was read off from a calibration curve. Most of the phosphate determinations were done by the method of Chen et al. (165) as modified by Ames (166), in which the phospho-molybdate complex is reduced by ascorbic acid rather than $FeSO_4$. Stock solutions of ascorbic acid (10%) and ammonium molybdate tetrahydrate (4.2 g plus 28.6 ml of 36N sulphuric acid/l.) were mixed in the ratio of 1 part of ascorbic acid reagent to 6 parts of ammonium molybdate reagent. This assay solution was kept in ice and discarded after 24 h, while the ascorbic acid stock solution could be kept at 4° for

1 month. To a 0.9 ml sample (0-0.3 μmoles) was added 2.1 ml of the assay solution. The resulting mixture was heated at 45° for 20 min then cooled to 22°, before the absorbance was read at 660 nm. The phosphate content of the sample was determined from a calibration curve.

Determination of glucose-6-phosphate

The assay for glucose-6-phosphate was done in a volume of 3.0 ml which included 600 μmoles of glycylglycine-NaOH buffer (pH 7.6), 1.25 μmoles of NADP, 2.5 μl of a crystalline suspension of glucose-6-phosphate debydrogenase in 3M ammonium sulphate (0.5 IU at 30°) and the glucose-6-phosphate sample. The reaction was followed to completion at which point there was no further increase in the absorbance at 340 nm. The overall increase in absorbance at 340 nm during the assay was used as a measure of glucose-6-phosphate content.

Determination of cytochrome b_1

The height of the Soret band at 427 nm relative to 410 nm in the reduced minus oxidized difference spectrum was used to determine the cytochrome b_1 content in various cell extracts. An extinction coefficient of 1.53×10^5 $l.moles^{-1}.cm^{-1}$ was used for the Soret band (167). The determinations were done in a Cary 15 spectrophotometer at 22° with 1 ml of cell extract in both the reference and sample cuvettes. After scanning between

600 nm and 400 nm to obtain a baseline one drop of 0.3% hydrogen peroxide was added to the reference cuvette while excess sodium dithionite was added to the sample cuvette. The spectrum was rescanned over the baseline between 600 nm and 400 nm.

Enzyme assay procedures

One unit of enzyme activity is defined as the reaction of 1 μmole of substrate per min at 37°. Specific activity is defined as the reaction of 1 μmole of substrate per minute per mg of protein at 37°. When enzyme activity was used to determine the elution position of the enzyme from a column then the activity was not quantitated. The assays for ATPase, ADPase, AMPase, 5'-nucleotidase, alkaline phosphatase, polynucleotide phosphorylase and (Ca^{2+}-Mg^{2+}) ATPase were done at 37° unless otherwise stated. The other assays were routinely done at 22°. In every assay the initial reaction rate was used to determine enzyme activity.

Enzyme assays on whole cells

When intact cells were assayed for an enzyme activity precautions were taken to prevent cold shock (168-170) by doing all manipulations, subsequent to harvesting the cells and prior to the assay incubation, at 22°. The cells were routinely suspended in 10 mM glycylglycine-NaOH buffer (pH 8.0)

containing 10 mM $MgCl_2$ and 0.2M sucrose. The latter was added to prevent osmotic shock of the cells.

Catalase

This assay procedure is based on that described by Beers and Sizer (171). The assay mixture contained 1.2 ml of 3% hydrogen peroxide and 5 ml of 0.2M potassium phosphate buffer (pH 7.1) to which was added 15 ml of water. Of this mixture 1.2 ml were pipetted into a cuvette followed by the enzyme sample plus water to a final volume of 2 ml. The enzyme sample was added last, and the change in absorbance at 240 nm with respect to time was followed. In each assay the initial reaction rates were measured. Since this activity was used to determine the elution position of catalase during gel filtration or to monitor the lysis of spheroplasts it was not quantitated.

Hexokinase

This assay was done in a final volume of 1 ml which contained 0.18 µmoles of NADP, 3.3 µmoles ATP, 10 µmoles of glucose, 6.6 µmoles of $MgCl_2$, 66 µmoles of Tris-HCl buffer (pH 7.4) and 5 µl of a crystalline suspension of glucose-6-phosphate dehydrogenase in 3M ammonium sulphate (1 IU at 30°). The reaction was started by the addition of the enzyme sample. The change in absorbance at 340 nm was recorded as a function of time and the initial reaction rate was found for each assay.

Alcohol dehydrogenase

Alcohol dehydrogenase was assayed in a final volume of 1 ml that contained 1.4 μmoles of NAD, 1.14% v/v ethanol and 50 μmoles of sodium pyrophosphate-HCl buffer (pH 8.8). After addition of the enzyme the rate of change of absorbance at 340 nm was monitored.

Glucose-6-phosphate dehydrogenase

The assay method of Coukell and Polglase (172) was used with the modification that the pH of the glycylglycine-NaOH buffer was 8.0 and not 7.5. Contained in a final volume of 1 ml were 5 μmoles of glucose-6-phosphate, 10 μmoles of $MgCl_2$, 0.14 μmoles of NADP and 50 μmoles of glycylglycine-NaOH buffer (pH 8.0). The change in absorbance at 340 nm with respect to time was followed after the addition of the enzyme sample.

Adenylate kinase

The basis of the adenylate kinase assay is the determination of the rate of ATP production with ADP as the substrate. ATP was determined in a coupled assay system similar to that used in the hexokinase assay described above, where the net result is an increase in absorbance at 340 nm due to NADPH formation. The assay components were present in excess so that

the reaction was only limited by ATP production and hence adenylate kinase activity. This was verified by experiment. The order of addition of the reagents was critical since the commercial ADP contained small amounts of ATP (about 2%) which gave an endogenous reaction. This endogenous reaction was allowed to go to completion before the adenylate kinase sample was added. The components of the assay and their order of addition to the cuvette were as follows: 115 µmoles of glycyl-glycine-NaOH buffer (pH 7.6), 5.8 µmoles of $MgCl_2$, 58 µmoles of glucose, 5 µl of a hexokinase suspension in 3.3M ammonium sulphate (2.5 IU at 30°), 5 µl of a crystalline suspension of glucose-6-phosphate dehydrogenase in 3M ammonium sulphate (1 IU at 30°) and 1.2 µmoles of NADP. At this point the mixture in the cuvette (volume 2.35 ml) was scanned at 340 nm to obtain a baseline. Then 2.9 µmoles of ADP were added in 0.15 ml of water and the endogenous reaction followed at the same wavelength. When there was no further change with time in the absorbance at 340 nm the adenylate kinase sample plus water was added to give a final volume of 2.6 ml. The reaction rate of the enzyme at 22° was determined and the activity expressed as µmoles of ADP changed per min per mg of protein at this temperature.

Polynucleotide phosphorylase

The method for determining polynucleotide phosphorylase

activity was based on the incorporation of labelled nucleotide into acid insoluble material. A series of conical test tubes (12 ml) were set up each containing 10 µl of adenine trinucleotide solution (5 mg/ml), 10 µmoles of Tris-HCl buffer (pH 8.0), 1 µmole of $MgCl_2$ and 1 µmole of ADP which included 0.4 µCi of ^{3}H-ADP. The reaction was started by the addition of 30 µl of enzyme plus distilled water to give a final volume of 100 µl. The mixtures were incubated for 2.5 h at 37°. At the end of this period 2 ml of 5% trichloroacetic acid (TCA) (0°) were added to each tube followed by 250 µg of bovine serum albumin. The assay mixtures, which were faintly turbid at this point, were filtered on millipore filters (0.45 µ pore size) that had been presoaked in 5% TCA and placed on Whatman paper supports. Each sample was washed three times on the filter with 5 ml of 5% TCA and finally with 5 ml of ethanol. The filters were dried and then placed in scintillation vials together with 5 ml of toluene plus "omnifluor" (New England Nuclear) (4 g per l. of toluene). The radioactivity of the samples was measured in a Nuclear Chicago MK 1 liquid scintillation counter with 25% efficiency for two 10 min periods. The counts per min were averaged after correcting for blank values (assay done without enzyme present) and for background radioactivity.

Determination of NAD(P)H oxidase and NAD(P)H-K_3 reductase

These assays were performed as described by Bragg and

Hou (173) with the exception that the 5 min preincubation of the enzyme with the assay mixture was done at 22°. The standard assay contained 50 μmoles of glycylglycine-NaOH buffer (pH 8.0), 10 μmoles of $MgCl_2$ and the enzyme sample in a volume of 0.95 ml. After preincubating this mixture for 5 min at 22° the reaction was started by the addition of 0.32 μmoles of NADH or NADPH (50 μl). The oxidase activity was given by the rate of change in absorbance at 340 nm. When sufficient reaction had occurred to establish the oxidase activity, 50 nmoles of 2-methyl-1,4, naphthoquinone (menadione) in 5 μl of ethanol were added and the rate of change in absorbance at 340 nm was again measured. The difference between the reaction rates before and after the addition of menadione (K_3) was taken to be a measure of the NAD(P)H-K_3 reductase activity. Both the oxidase and reductase activities were expressed as specific activities at 22°, using a molar extinction coefficient for NAD(P)H of 6.22×10^3 l. $mole^{-1}.cm^{-1}$.

5'-Nucleotidase

The assays for 5'-nucleotidase were derived from those described by Neu (19). The standard assay mixture contained 2.5 μmoles of AMP, 50 μmoles of sodium acetate buffer (pH 6.0), 2.5 μmoles of $CoCl_2$, 50 μg of bovine serum albumin and the enzyme sample in a volume of 0.5 ml. The reaction was started by the addition of the enzyme sample. When necessary

the enzyme was diluted in a solution of bovine serum albumin (100 μg/ml). After 30 - 60 min at 37° the reaction was stopped by the addition of 10% TCA to each tube, The resulting precipitate was pelleted by centrifugation at 14,500 x g for 10 min at 2°- 5°. Samples of the supernatant were assayed for phosphate as described elsewhere in METHODS AND MATERIALS.

Variations in 5'-nucleotidase assay

Variations in the assay were made as follows. Divalent metal ions like Mg^{2+}, Ca^{2+}, Zn^{2+}, and Mn^{2+} sometimes replaced Co^{2+} at the same final concentration of 5mM. All were used in the chloride form. When the assays were done at pH 8.0, glycylglycine-NaOH buffer (pH 8.0) replaced the sodium acetate buffer again at the same final concentration. When the substrate was ATP rather than AMP the assay mixture contained 2.5 μmoles of ATP and 50 μmoles of Tris-maleic acid buffer (pH 7.0) in 0.5 ml.

The fractions obtained from hydroxylapatite by elution with a gradient of potassium phosphate during the purification of 5'-nucleotidase were assayed using bis-p-nitrophenyl phosphate (bis-pNPP) as the substrate. The hydrolysis product p-nitrophenol was measured by its absorbance at 410 nm in alkaline solution. The assay volume of 0.5 ml contained 1.5 μmoles of bis-pNPP, 50 μmoles of Tris-maleic acid buffer (pH 7.0) and the enzyme sample. After a 30 - 60 min incubation at 37° the reaction was

stopped by addition of 2.5 ml of 0.1N NaOH and the absorbance at 410 nm was recorded. When using bis-pNPP as the substrate the enzyme activity was not quantitated; with AMP and ATP, specific activities and units were calculated as defined.

Assay for 'masked' 5'-nucleotidase

Where 5'-nucleotidase inhibitor was suspected of masking the full enzyme activity samples were pretreated at 50° for 10 min, cooled to 22°, and then assayed for 5'-nucleotidase or AMPase activity as described in "Enzyme assay procedures". The enzyme activities before and after heat treatment were compared.

AMPase, ADPase and ATPase

Assays for the hydrolysis of these three nucleotide substrates were done in the presence of 100mM glycylglycine-NaOH buffer (pH 8.0) and 5mM $MgCl_2$. The nucleotides were all used at a final concentration of 5mM. The samples were routinely assayed at 37° for 30 - 60 min in a final volume of 0.5 ml. The reaction was stopped by the addition of 10% TCA (0.25 ml) as described in the 5'-nucleotidase assay. However when there was not enough protein in the assay to give a precipitate, the phosphate assay reagent was added with the 10% TCA. Specificity activity and units were calculated as defined.

Alkaline phosphatase

The substrate used in the alkaline phosphatase assay was p-NPP. The release of phosphate was measured rather than the formation of p-nitrophenol. The assay conditions were the same as those described above for the AMPase, ADPase and ATPase assays with p-NPP also at 5mM concentration.

Ca^{2+}- or Mg^{2+}-activated ATPase

Once the properties of the Ca^{2+}- or Mg^{2+}- activated ATPase had been elucidated the ATPase assay was modified to make the assay conditions more specific for this enzyme. $CaCl_2$ at 5mM final concentration was used in place of the $MgCl_2$, and 100mM Tris-HCl (pH 9.0) replaced the 100mM glycyl-glycine-NaOH buffer (pH 8.0).

Variations in the (Ca^{2+}-Mg^{2+})-ATPase assay

When the effects of monovalent cations on the (Ca^{2+}-Mg^{2+})-ATPase were tested the Tris salt of ATP was used instead of the disodium salt. When the optimum ion to substrate ratio was investigated the concentration of substrate was held at 5mM while the concentrations of Ca^{2+} and Mg^{2+} were varied from 0 to 10 mM. For studies on the pH optimum of the enzyme the Tris-HCl buffer (pH 9.0) was replaced by 100mM Tris-acetate buffers of pH values ranging from 4.5 to 9.0. Solutions of pH 9.5

and 10.0 were obtained by using 100mM glycine adjusted to the required pH with NaOH. For studies on the substrate specificity of the enzyme, ATP was replaced by other substrates at the same concentration of 5mM. Other variations of the assay are mentioned as they occur in the legends to tables and figures.

Column chromatography

Column chromatography was routinely done at 0°- 4°. However once the cold lability of Ca^{2+} or Mg^{2+}-activated ATPase was established the chromatography of this enzyme was done at 22° unless 10% v/v glycerol was included in the eluting solutions to stabilize the ATPase.

Adsorption chromatography on hydroxylapatite

Hydroxylapatite powder (Bio-gel HTP) was a Bio-Rad product supplied by Calbiochem. After presoaking the powder in 0.01M potassium phosphate buffer (pH 7.1), a column of dimensions 1.4 cm x 14.5 cm was packed and washed with 200 ml of the phosphate buffer. The sample (3 mg protein) in 0.01M potassium phosphate buffer (pH 7.1) (38 ml) was applied to the column and washed in with more buffer until 70 ml of eluate had been collected. The sample was eluted by a linear gradient of phosphate. This was set up by having 50 ml of 0.01M

potassium phosphate buffer (pH 7.1) in the mixing chamber and 50 ml of 0.2M potassium phosphate buffer (pH 7.1) in the salt chamber. One ml fractions were collected at a flow rate of 25 ml/h.

Ion-exchange chromatography of protein on DEAE-cellulose

DEAE-cellulose from Calbiochem (Cellex D) was either freshly treated as described in METHODS AND MATERIALS or was reused after storage at 4° in the presence of 0.1% butanol. Butanol was removed from the exchanger by washing with several volumes of the equilibrating buffer. The DEAE-cellulose was used in the chloride ion form and prior to use was equilibrated with 2 - 10mM Tris-HCl buffer (pH 7 - 8) until the eluate had the same pH as the buffer. Samples were applied in Tris-HCl buffer of the same pH and molarity. Elution was by stepwise increases in NaCl concentration or by a linear gradient of increasing NaCl concentration. DEAE-cellulose (DE52) from Whatman was sometimes used to obtain better resolution of protein components. It was not pretreated with 0.5N HCl and 0.5N NaOH but other manipulations were done as described above.

Gel filtration

Gel filtration through Sepharose 4B or 6B (Pharmacia) was done in Pharmacia columns (2.5 cm x 45 cm). Prior to packing the column the pre-swollen gel was brought to the

temperature at which the chromatography was to be carried out and then diluted with an equal volume of the solution that would be used to elute the sample. After packing the column to the desired height the gel was equilibrated by washing with at least two column volumes of the eluting solution before the sample was applied. Elution was by downward flow at a rate of 15 - 30 ml per h. The total volume (V_t) of the column was found by including 0.1 ml of 0.2M phosphate buffer (pH 7.1) in the sample and subsequently testing the fractions collected for inorganic phosphate. When not in use the Sepharose gels were stored in the presence of 0.02% sodium azide at 4°. Packed columns were preserved from bacterial attack by equilibrating them with the 0.02% sodium azide solution at the temperature previously used for the chromatography.

Determination of molecular weight by gel filtration

A column of Sepharose 6B (2.5 cm x 38 cm) was prepared for calibration with molecular weight markers. A buffer containing 0.2mM disodium EDTA in 50mM Tris-HCl (pH 7.4) was used for equilibration and elution of the column. Fractions (1.7 ml) were collected at a flow rate of 30 ml per h. Yeast hexokinase (mol. wt. 102,000 (174)), yeast alcohol dehydrogenase (mol. wt. 150,000 (174)), bovine catalase (true mol. wt. 248,000 (175); apparent mol. wt. 195,000 (175)), equine apoferritin (mol. wt. 480,000 (174)), equine hemoglobin (mol. wt. 64,500 (174)),

and porcine thyroglobulin (mol. wt. 670,000 (174)) were used as molecular weight markers. The elution positions (V_e) of the last three proteins were measured by their absorbance (apoferritin and thyroglobulin at 280 nm or 230 nm and hemoglobin at 415 nm) while the other proteins were estimated by the enzyme activities. The void volume (V_o) of the column was determined with blue dextran (10 mg in 2 ml sample) by reading the absorbance at 256 nm of the fractions collected. The calibration curve was obtained by plotting the $\log_{10}$ (molecular weight) against K_{av}, where

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \quad (176).$$

Polyacrylamide gel electrophoresis

Routine analysis of the purity of fractions containing the Ca^{2+}- or Mg^{2+}-activated ATPase was done by electrophoresis on 5% polyacrylamide gels at pH 8.7.

Preparation of disc gels

The gel mixture was prepared by dissolving cyanogum (0.5 g) (E-C apparatus Corporation) in 10 ml of 50mM Tris-glycine buffer (pH 8.7). This solution was de-aerated by suction and then 10 µl of TMED were added followed by ammonium persulphate (10 mg). After the latter had been dissolved the gel mixture

was quickly dispensed into glass tubes (7.5 cm x 0.5 cm or 8.5 cm x 0.6 cm) which were held vertically in a rack and which had their ends sealed with parafilm. The tubes were filled to a uniform height (80% - 90% full). A flat gel surface was obtained by gently layering de-aerated Tris-glycine buffer on top of the gel mixture as soon as it had been introduced into the tube. At this stage the gels were left to polymerize for 2 h at 37°.

Electrophoresis

Electrophoresis was carried out in a Shandon disc electrophoresis apparatus with the 50mM Tris-glycine buffer (pH 8.7) in the upper and lower reservoirs. The parafilm covering was removed from the bottom of the gel tube and fresh Tris-glycine buffer was placed on top. After putting the requisite number of gels into the apparatus, charged impurities were removed by pre-electrophoresis for 20 min at 2.5 mA per tube with the positive terminal in the bottom reservoir. The sample (10 µl - 50 µl) containing 25 µg - 200 µg of protein was then layered on the gel surface underneath the buffer with the aid of a micropipette. The electrophoresis of the sample was done for 30 - 45 min again at a constant current of 2.5 mA per tube. At the end of this period the current was switched off and the gels were removed from the tubes by inserting a fine needle down the inside of the glass while rotating the

tube and irrigating the surface of the gel with tap water. The gels were then stained for protein or for enzyme activity.

Sample preparation

Preparation of a sample for electrophoresis usually involved concentrating it until the protein content was between 2.5 mg/ml and 20 mg/ml. Volumes greater than 20 ml were concentrated by ultrafiltration using a PM 10 ultrafilter in a Model 402 cell (Amicon Corporation). Volumes less than 20 ml were placed in dialysis tubing which had been pre-soaked in water and were concentrated against carbowax 6000 (Union Carbide). The concentrated sample was made 10% - 20% in sucrose to avoid dispersion when placed on the gel surface.

Detection of protein

Gels were stained for protein with Coomassie brilliant blue (Schwarz Mann). The staining solution consisted of Coomassie brilliant blue (0.125 g), methanol (22.7 ml), glacial acetic acid (4.6 ml) and distilled water (22.7 ml). This mixture was stirred for 2 h and then filtered. The gels were left in this solution for at least 2 h after which they were washed once with 7% acetic acid. Excess stain was removed from the gels electrolytically in destaining tubes. These were plugged at the bottom with a short section of 7.5% polyacrylamide gel which had been polymerized in the presence of 35% acetic acid in a manner

similar to that described above. After the gels had been introduced into the destaining tubes they were surrounded with a polyacrylamide destaining solution. This was made up by combining acrylamide (3 g) (Eastman Kodak Co.), water (50 ml), TMED (50 µl), and finally ammonium persulphate (50 mg). After the addition of the ammonium persulphate the mixture was allowed to polymerize at 22° for 10 min before 50 ml of 14% acetic acid were added. The destaining was done overnight at 7.5 mA - 10 mA per tube with 10% acetic acid in the upper and lower reservoirs.

Detection of the $(Ca^{2+}$-$Mg^{2+})$-ATPase

The $(Ca^{2+}$-$Mg^{2+})$-ATPase activity was located on the gels after electrophoresis by using the routine $(Ca^{2+}$-$Mg^{2+})$-ATPase assay. The gel was placed in a test tube (10 cm x 1.1 cm) and surrounded by $(Ca^{2+}$-$Mg^{2+})$-ATPase assay mixture. After 30 min at 22° the gel was removed and quickly washed with water before placing it in a tube containing the phosphate assay mixture at 22°. After 5 - 10 min in this solution a blue band was observed and in view of its transitory nature it was marked by stabbing the gel with a fine needle dipped in India ink. Having identified the band of $(Ca^{2+}$-$Mg^{2+})$-ATPase activity the gel was washed again with distilled water and then stained for protein with Coomassie brilliant blue as described above.

Alternatively the gel was placed on parafilm immediately after electrophoresis and cut into 32 approximately equal

sections. Each section was placed in a separate tube and assayed in the usual way for (Ca^{2+}-Mg^{2+})-ATPase activity.

Organisms and their maintenance

The bulk of the work described here was done with Escherichia coli - strain 482 of the culture collection of the National Research Council of Canada. Wild type E. coli B (ATCC 11303) was sometimes used for comparative purposes. These strains were maintained on nutrient agar slopes formed by dissolving Fisher bacteriological culture medium (1.6 g) and agar (0.9 g) in 60 ml of boiling water, dispensing 10 ml of the solution per test tube for autoclaving, and finally leaving the nutrient agar to solidify while the tubes were slanted. Every month the two strains were streaked out onto fresh slopes, grown for 24 h in an incubator at 37° and then stored at 4°. The cultures were checked for contamination by using the Gram stain (177).

Growth conditions

Except where indicated cells were grown on a minimal medium of glucose (0.4%) plus salts: K_2HPO_4 (0.7%), KH_2PO_4 (0.3%), $MgSO_4.7H_2O$ (0.02%), $(NH_4)_2SO_4$ (0.1%), and sodium citrate.2 H_2O (0.05%). Prior to growing up a batch of cells a culture tube containing 10 ml of the minimal medium was inoculated from

the agar slope and placed at 37° in an incubator. This initial liquid culture was subcultured several times at 12 - 24 h intervals to obtain an inoculum of rapidly growing cells. The size of the inoculum varied rom 1% to 10% v/v. Flask cultures (300 ml - 4 l.) were grown at 37° with vigorous aeration through a sintered glass sparger. Larger batches of cells (20 l.) were grown up in a "Biogen" (American Sterilizer Company, Brampton, Ontario) at an agitation setting of 142 and aeration at 4 cu. ft/min. Growth was monitored by following the absorbance at 420 nm of the culture, unless a complex medium was used in which case the absorbance at 600 nm was recorded instead. The cells were harvested by centrifugation at 6,000 x g for 10 min at 2°- 5° and were washed by resuspension in 0.9% NaCl (0°) at 50 mg wet wt/ml. The washed cells were recovered by repeating the centrifugation step above. Cultures from the "Biogen" were harvested by passage through a water-cooled Sharples centrifuge prior to the washing step.

Osmotic shock procedure

The osmotic shock procedure used was based on that of Neu and Heppel (11). E. coli B or NRC 482 were harvested in the late log phase of growth. The cells (2 g wet weight) were suspended at 22°in 160 ml of 20% sucrose in 30 mM Tris-HCl (pH 8.0). When suspension was complete 20mM disodium EDTA (8 ml) was added with swirling. After 10 min the cells were recovered by centri-

fugation at 13,200 x g for 10 min at 2°- 5°. The supernatant fluid was decanted and the cell pellet was rapidly resuspended in water (160 ml) at 0°. This was the stage at which the osmotic shock occurred. "Shocked" cells could then be recovered after centrifugation at 13,200 x g for 10 min at 2°- 5° and, if required for an assay, were resuspended in 18 ml of 20% sucrose in 30mM Tris-HCl (pH 8.0). The clear supernatant from the latter centrifugation was termed the osmotic shockate.

Determination of viable organisms

Determination of the number of viable organisms in a liquid sample was done by the "pour plate" method as described in "Laboratory Methods in Microbiology" (178). Sterile conditions were maintained throughout. The diluent used was quarter-strength Ringer's solution which contained 38.5 mmoles of NaCl, 1.4 mmoles of KCl, 0.55 mmoles of $CaCl_2.6H_2O$ and 0.46 mmoles of $NaHCO_3$ per litre. Serial dilutions of the sample were made by pipetting 1 ml of the sample into 9 ml of diluent, mixing this thoroughly and then pipetting 1 ml of it into the next 9 ml of diluent. After 6 - 10 dilutions 1 ml samples were plated out onto Petri-dishes containing 10 - 15 ml of solidified 1.5% Agar (Difco) made up in the glucose plus minimal salts medium. Sterile, liquid agar (10 ml of a 1% solution in glucose plus salts minimal medium) at 50° was added to the 1 ml sample and thoroughly mixed with it. Once the agar had set the plates were inverted and

placed in an incubator at 37° for 1 to 2 days. An initial count of the colonies was made after 12 h and a second count after 36 h. Samples were done in triplicate and the counts averaged. Plates containing less than 50 colonies were discarded.

Preparation and lysis of spheroplasts

E. coli NRC 482 was converted to spheroplast form either by digestion with lysozyme or by the addition of penicillin to an exponentially growing culture. In each case the spheroplasts were lysed osmotically.

The lysozyme method

Cells (late log phase) were grown, harvested and washed as described in METHODS AND MATERIALS. Washed cells were suspended at 22° to a concentration of 25 mg wet wt/ml in 20% sucrose solution made 30mM in Tris-HCl (pH 8.0). Lysozyme (5 mg/g of cells) was then added as a solution in a few drops of 0.05% NaCl and the suspension was stirred gently. After 30 min at 22° the suspension was diluted 10-fold into 0.2mM $MgCl_2$ which contained a small amount of deoxyribonuclease (DNAse).

The resulting lysate was stirred rapidly and the decrease in turbidity was followed at 420 nm. After 20 - 40 min when the absorbance at 420 nm had reached a constant, minimum value the lysate was centrifuged at 15,000 x g for 30 min at 18° to recover

the spheroplast membranes as an opalescent, reddish-brown pellet.

Investigation of the extent of lysis

During osmotic lysis of the spheroplasts the lysate was sampled at timed intervals and immediately filtered at 22° through a Millipore apparatus containing a filter of pore size 0.65 μ and a fibreglass prefilter disc. The filtrates were kept at 0°. Part of the lysate (30 ml) was disrupted for 10 min in an ice-cooled 20-kHz Bronwill sonic oscillator. The filtrates and the sonicate (not filtered) were assayed for two soluble enzymes, catalase and NADH-menadione reductase. The level of each enzyme in the filtrate was compared to the value for the sonicate and was used as an approximate indicator of lysis.

After 40 min a sample of the lysate was removed for determination of the number of viable organisms. The viability counts were done by the "pour plate" method (178).

The penicillin method

Using this technique E. coli NRC 482 was converted to spheroplast form during growth by the addition of penicillin to the culture. The medium was made hypertonic to prevent premature lysis of the spheroplasts. The cells were grown on Difco #3 penicillin assay broth (5.34 g/300 ml) at 37° with gentle aeration (1:2 l./min). The inoculum (7% v/v) had been subcultured in the Difco #3 broth. The turbidity of the culture was read at 600 nm

and when the absorbance at 600 nm reached 0.8 a solution containing sucrose (68.4 g), $MgSO_4.7H_2O$ (1.479 g) and the potassium salt of penicillin G (0.405 g) dissolved in water (100 ml) was added. Growth was allowed to continue for a further 3.5 h at which point the absorbance at 600 nm was 0.6. The spheroplasts were recovered by centrifugation at 2°- 5° for 15 min at 13,000 x g. Osmotic fragility was monitored during the formation of the spheroplasts by diluting a sample of the culture ten-fold in water and comparing its absorbance at 600 nm with that of the undiluted culture.

Cell breakage

Cells were broken by sonication or by passage through a French press. In the former technique 15 - 30 ml of cell suspension (0.067 g wet wt/ml of buffer solution) were disrupted for 10 min in a 20-KHz Bronwill sonic oscillator, the chamber of which had been pre-cooled to 0°. Disruption in a French press was done at 20,000 psi in a pre-cooled (0°) French pressure cell (Aminco). Up to 40 ml of cell suspension were treated at a time. The cell concentration was varied between 0.067 - 0.2 g wet wt/ml of buffer solution. The more concentrated suspensions were passed through the press twice.

Preparation of cell extracts

The lysate resulting from osmotic lysis of lysozyme-induced spheroplasts was fractionated to obtain a "lysate soluble

fraction" and "washed spheroplast membranes". The "lysate soluble fraction" was the supernatant obtained by centrifuging down spheroplast membranes at 15,000 x g for 30 min at 18°. "Washed spheroplast membranes" was the pellet obtained by resuspension of the spheroplast membranes in 10mM Tris-HCl buffer (pH 7.4) containing 0.5mM $MgCl_2$ at 0.7 mg of protein per ml, followed by recentrifugation at 23,700 x g for 30 min at 2°- 5°. Cells that were broken in the French press were fractionated in a variety of ways described as they occur in RESULTS.

Release of $(Ca^{2+}$-$Mg^{2+})$-ATPase from membranes

$(Ca^{2+}$-$Mg^{2+})$-ATPase was released from cell membranes by one of two methods. In the first method, spheroplast membranes (prepared by the lysozyme technique) were put through a washing procedure similar to that used by Abrams (1). The spheroplast membranes (170 mg protein) from 10 g of cells were suspended in 250 ml of 10mM Tris-HCl buffer (pH 7.4) containing 0.5mM $MgCl_2$ using a glass homogenizer with a teflon plunger. They were then sedimented at 23,700 x g for 30 min and the resulting supernatant was set aside. This same procedure was followed through 5 more washings. The resuspension media were 2M LiCl in 50mM Tris-HCl (pH 7.4) for the second and third washings, 50mM Tris-HCl (pH 7.4) for the fourth, and 1mM Tris-HCl (pH 7.4) for the fifth and sixth washings. The membrane pellet which remained after the sixth washing was resuspended in 250 ml of 1mM Tris-HCl (pH 7.4). These

steps were done at 0° - 4°, but subsequent steps and storage of samples were done at 22°. The supernatant from the fifth washing was used as a source of soluble (Ca^{2+}-Mg^{2+})-ATPase.

The second method was a single step procedure in which membrane fractions from E. coli NRC 482 were dialysed against EDTA. The membrane fractions were obtained either by osmotic lysis of lysozyme-induced spheroplasts as described elsewhere in METHODS AND MATERIALS or by differential centrifugation after disruption of cells in a French pressure cell. For French pressing, the cell concentration was 0.1 g wet wt/ml of 100mM Tris-HCl buffer (pH 7.4) containing 10mM $MgCl_2$. Differential centrifugation involved an initial centrifugation at 6,000 x g for 10 min at 2°- 5°. The pellet from this step was resuspended in Tris-HCl/ $MgCl_2$ solution to 40% of the pressate volume and recentrifuged under the same conditions. The first supernatant was retained and combined with that from the washing step, and together they were centrifuged at 23,700 x g for 30 min at 2°- 5°. The pellet from this centrifugation was resuspended in the Tris-HCl/$MgCl_2$ solution to 40% of the pressate volume and recentrifuged at 23,700 x g for 30 min at 2°- 5° to give washed membranes. The washed membranes were resuspended at 22° in 1mM Tris-HCl buffer (pH 9.0) containing 0.5mM di Na EDTA. The volume of Tris-HCl/EDTA solution used for the resuspension was calculated on the basis of 5 ml for every g wet wt of cells originally French pressed. This suspension was dialysed at 22° against the Tris-HCl/EDTA solution (1-4 l.) for 10 - 15 h during which time the solution

was renewed once. The dialysate was then centrifuged at 23,700 x g for 30 min at 18° to give a supernatant fraction enriched in soluble (Ca^{2+}-Mg^{2+})-ATPase and a pellet containing "depleted" membranes. "Washed spheroplast membranes" were put through this same procedure.

Preparation of samples for electron microscopy

Spheroplast membranes from 2.1 g wet wt of E. coli NRC 482 were prepared in the usual manner with the exception that all procedures subsequent to the lysis of the spheroplasts were carried out at 0° - 4°. The membranes were washed by resuspension in 100 ml of 5mM Tris-HCl (pH 7.4) containing 0.2mM $MgCl_2$ followed by centrifugation at 17,600 x g. Four pellets of equal size were recovered. Two of these were combined and resuspended in 5 ml of the Tris-HCl/$MgCl_2$ buffer. Two ml of this sample were retained and the other 3 ml were sonicated for 30 seconds at 110 watts using a model W185C sonic oscillator (Heatsystems-Ultrasonics Inc.). Samples of the membrane suspension and the sonicate were fixed with phosphotungstic acid (1%) and laid on carbon-coated, copper grids. The grids were examined in a Siemens Elmiskop I electron microscope. Photographs were taken at magnifications between 1900 X and 17,000 X.

Treatment of E. coli NRC 482 membranes with trypsin

Washed spheroplast membranes (prepared by the lysozyme

method) were treated with trypsin at a ratio of 12 µg of trypsin per mg of membrane protein in 0.5 ml. The reactions were stopped after time intervals (0 - 60 min) by the addition of trypsin inhibitor (2 µg per µg of trypsin) and the membrane samples were assayed for (Ca^{2+}-Mg^{2+})-ATPase activity.

Membranes from a French pressate of E. coli NRC 482 were depleted of (Ca^{2+}-Mg^{2+})-ATPase activity as described in "Release of (Ca^{2+}-Mg^{2+})-ATPase from membranes" (METHODS AND MATERIALS). These depleted membranes were treated with trypsin in an attempt to expose latent (Ca^{2+}-Mg^{2+})-ATPase activity. To 1 mg of membrane protein in 0.3 ml was added 330 µg of trypsin. Reactions were stopped at zero time or after five and ten min by addition of 330 µg of trypsin inhibitor. Then the membranes were assayed for (Ca^{2+}-Mg^{2+})-ATPase.

Partial purification of Co^{2+}-stimulated 5'-nucleotidase according to the method of Neu (19)

An osmotic shockate was prepared (as described earlier) from 10 g of freshly-grown E. coli NRC 482. The shockate (500 ml) was next heat-treated by warming it to 56°- 57° and keeping it at this temperature for 4 min before cooling it in ice. Prior to the heat-treatment, the pH of the shockate was adjusted to pH 6.0 by the addition of 20 ml of 0.5M Na acetate buffer (pH 6.0). $MgCl_2$ and $CoCl_2$ were also added to give a final concentration of 5mM and 0.25mM respectively. The heat-treated shockate was

then centrifuged at 23,000 x g for 20 min at 4° to sediment denatured protein.

During the next stage in the purification of the Co^{2+}-stimulated 5'-nucleotidase the heat-treated osmotic shockate was adsorbed onto a DEAE-cellulose column (25 cm x 5 sq cm) which had previously been equilibrated with 5mM Tris-HCl buffer (pH 7.4). The column was then washed with a further 50 ml of the same buffer. A linear gradient of increasing NaCl concentration ranging from 0 to 0.25M (total volume 900 ml) was used to elute the sample. Fractions (6 ml) from the column were collected and assayed for Co^{2+}-stimulated 5'-nucleotidase. Fractions 59 to 72 inclusive were combined and dialysed overnight against 1.5 l. of 10mM potassium phosphate buffer (pH 7.1) with three changes of the buffer in preparation for adsorption chromatography on hydroxylapatite.

After retaining approximately half of the dialysed material the remainder (38 ml) was run into a hydroxylapatite column (14.5 cm x 1.4 cm). This column had been equilibrated by washing with 10mM potassium phosphate buffer (pH 7.1). The sample was washed into the column with 30 ml of the same buffer and then eluted by a linear gradient of increasing potassium phosphate concentration at a constant pH of 7.1 (Fig. 10). The gradient ranged from 10mM to 200mM potassium phosphate with a total volume of 100 ml. Following the procedure of Neu (19) the fractions (1 ml) from the hydroxylapatite column were assayed for 5'-nucleotidase activity by using bis-pNPP as the substrate instead of AMP. (The 5'-nucleotidase has a slight phosphodiesterase

activity and will release p-nitrophenol from bis-pNPP.) This hydrolysis product was measured by its characteristic yellow colour in alkaline solution. This assay avoided the need to dialyse each fraction to remove the phosphate of the gradient that would interfere in the regular 5'-nucleotidase assay. However after the identification of the bis-pNPPasee activity the active fractions (68-73 inclusive) were combined and dialysed overnight against 1 l. of 5mM Tris-HCl (pH 7.4) with three changes of the buffer. Other fractions which were in regions of peak absorbance at 280 nm were combined (44-48 inclusive; 74-78 inclusive; and 79-84 inclusive) and similarly dialysed. After dialysis each of these pooled fractions was assayed by the usual 5'-nucleotidase assay method.

Large-scale isolation of ATPase

Freshly grown E. coli NRC 482 (20 g) were suspended in 200 ml of 20mM Tris-HCl buffer (pH 7.4) containing 10mM $MgCl_2$. This cell suspension was passed through the French press twice before being centrifuged at 6,000 x g for 10 min at 18° to sediment unbroken cells. The supernatant from this centrifugation step was in turn centrifuged at 140,000 x g for 90 min at 18° to sediment the membrane fragments. The pellets from this second centrifugation were combined and then washed by resuspension in 70 ml of the above buffer followed by centrifugation at 140,000 x g for 90 min at 18°. The washed membranes were resuspended in 200 ml of 0.5mM di Na EDTA containing 1mM Tris-HCl (pH 9.0) and were

dialysed overnight at 22°- 24° against 4 l. of the same solution.

The dialysed membrane suspension was centrifuged at 140,000 x g for 90 min at 18° to recover the solubilized ATPase in the supernatant fraction (208 ml). This volume was reduced to 44 ml by ultrafiltration at 40 psi using a PM10 ultrafilter. Glycerol (5 ml) and 0.2M Tris-HCl (pH 7.4) (1.25 ml) were added to this solution to make it approximately 10% in glycerol and 5mM in Tris-HCl. This solution was then run into a column of DEAE-cellulose (18 cm x 1.2 cm) which had previously been equilibrated at 22°- 24° with 2mM Tris-HCl (pH 7.4) containing 10% glycerol. Fractions (2.6 ml) were collected. Once the sample had been run into the column it was followed by equilibrating buffer until 80 ml of effluent had been collected. The sample was eluted from the column by a sequence of four solutions of increasing Tris-HCl concentration. These were respectively 0.15M, 0.20M, 0.25M and 0.50M in Tris-HCl (pH 7.4) and each contained 10% glycerol. Approximately 50 ml of each solution was run into the column in turn.

Fractions 84 to 89 inclusive were combined (15 ml) and concentrated to a volume of 3.8 ml by placing the fractions in a dialysis bag and covering the latter with carbowax 6000. This sample was chromatographed on Sepharose 6B at 22°- 24° in the usual manner using 5mM Tris-HCl (pH 7.4) containing 0.2mM di Na EDTA to equilibrate the column (38 cm x 2.5 cm) and to elute the sample into 3 ml fractions. Fractions 27 to 31 inclusive were combined and concentrated to a volume of 0.4 ml using

carbowax 6000.

To this concentrated material was added 0.1 g of sucrose and two drops of 0.05M Tris-glycine buffer (pH 8.7). For preparative purposes 50 µl of this sample was placed on top of each of 8 large-size disc gels (8.5 x 0.6 cm). These samples were resolved by electrophoresis as described earlier with the following modifications. The current was increased to 4 ma per tube and the electrophoresis was carried out for 70 min because the larger sized disc gels were used. At the end of this period one gel was removed and stained for ATPase activity. Another was stained for protein. Once the ATPase had been located on the gel (length 7.4 cm) as a band 4.8 cm from the bottom, sections lying between 4.5 cm to 5.3 cm from the bottoms of the other gels were cut out. These sections were crushed with a glass rod and extracted four times with distilled water over a period of 12 h. The combined extracts were lyophilised. The uncrushed gel segments were stained for protein in the usual manner.

RESULTS

Preparation for the assay of 5'-nucleotidase and ATPase

These enzyme activities were assayed by the release of Pi from adenine nucleotides. The Pi in turn was measured by one of two methods. In earlier work (65) Sumner's assay method (164) was used but this was superceded by the method of Chen *et al.* (165) as modified by Ames (166). The latter procedure was found to be three times more sensitive than the former, and gave a more linear relationship between absorbance and phosphate concentration (Fig. 1).

The commercial preparations of ATP contained substantial amounts of Pi (Table V). A correction was made for this in the enzyme assays. The amount of Pi present was proportional to the ATP concentration (74 mmoles Pi/mole ATP) and was only slightly increased by the addition of TCA to a final concentration of 3.3% v/v (Fig. 2). There was little difference in Pi content between the various commercial preparations tested (Table V). Commercial ADP contained lesser amounts of Pi (30 - 50 mmoles/mole ATP). This again was corrected for in the enzyme assays.

Besides Pi the commercial ATP and ADP also contained UV-absorbing impurities which were observed after paper chromatography of the nucleotides (Table VI). The two minor, UV-absorbing spots seen in the ATP sample had the same mobilities as the ADP

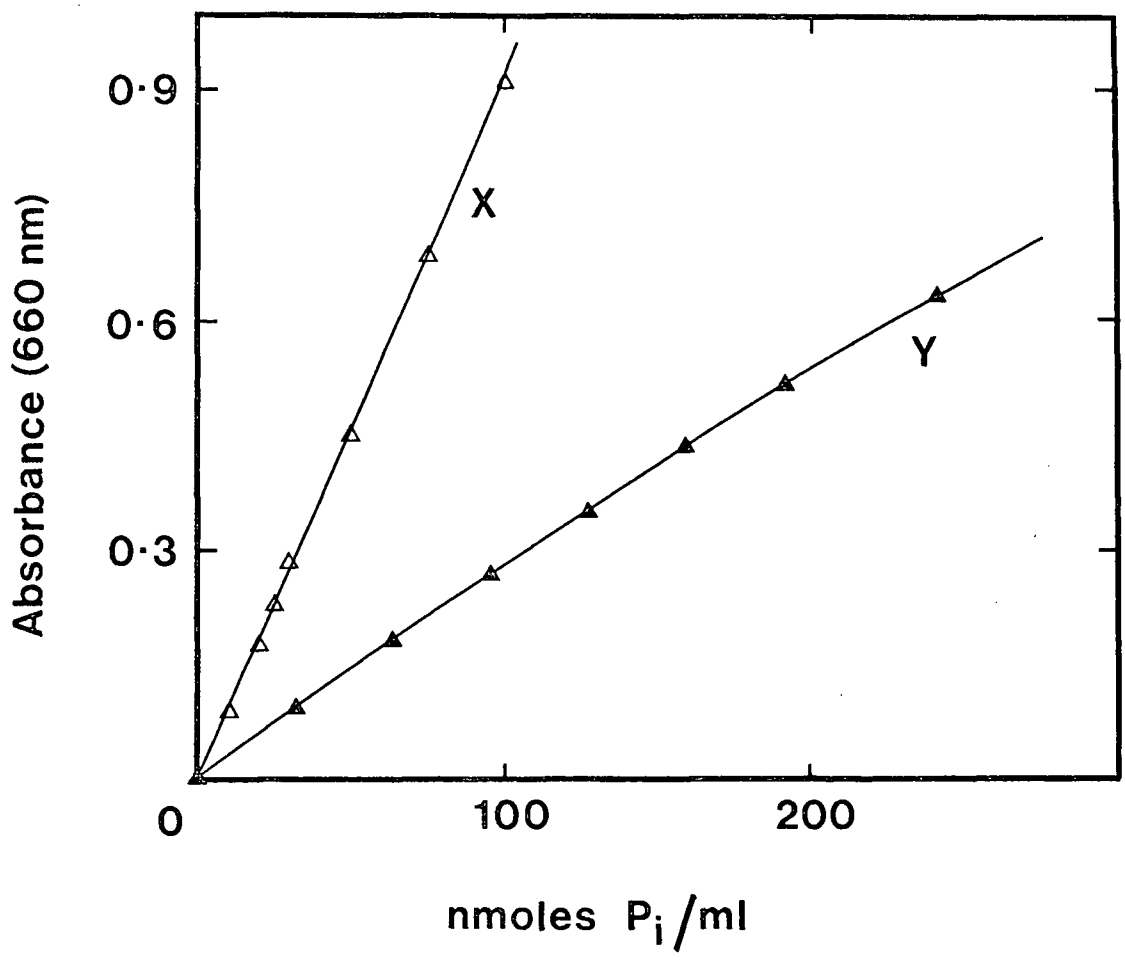


Fig. 1. Standard curves for Pi determination.

The assays were done as described in METHODS AND MATERIALS. Standard curves X and Y were obtained by the methods of Ames (166) and Sumner (164) respectively. $Na_2HPO_4.7\ H_2O$ was used as the phosphate standard.

Table V. Content of Pi in commercial preparations of ATP.

All samples of ATP had previously been stored at -20°. They were dissolved in the standard ATPase assay mixture at a final concentration of 5mM and 0.5 ml samples were assayed for Pi as described in METHODS AND MATERIALS.

Source of ATP	Lot #	Pi content (mmoles of Pi/mole ATP)
P-L. Biochemicals	186-A	74.3
P-L. Biochemicals	185-A	84.4
Calbiochem A grade	71105	69.6
Calbiochem A grade	900635	81.3

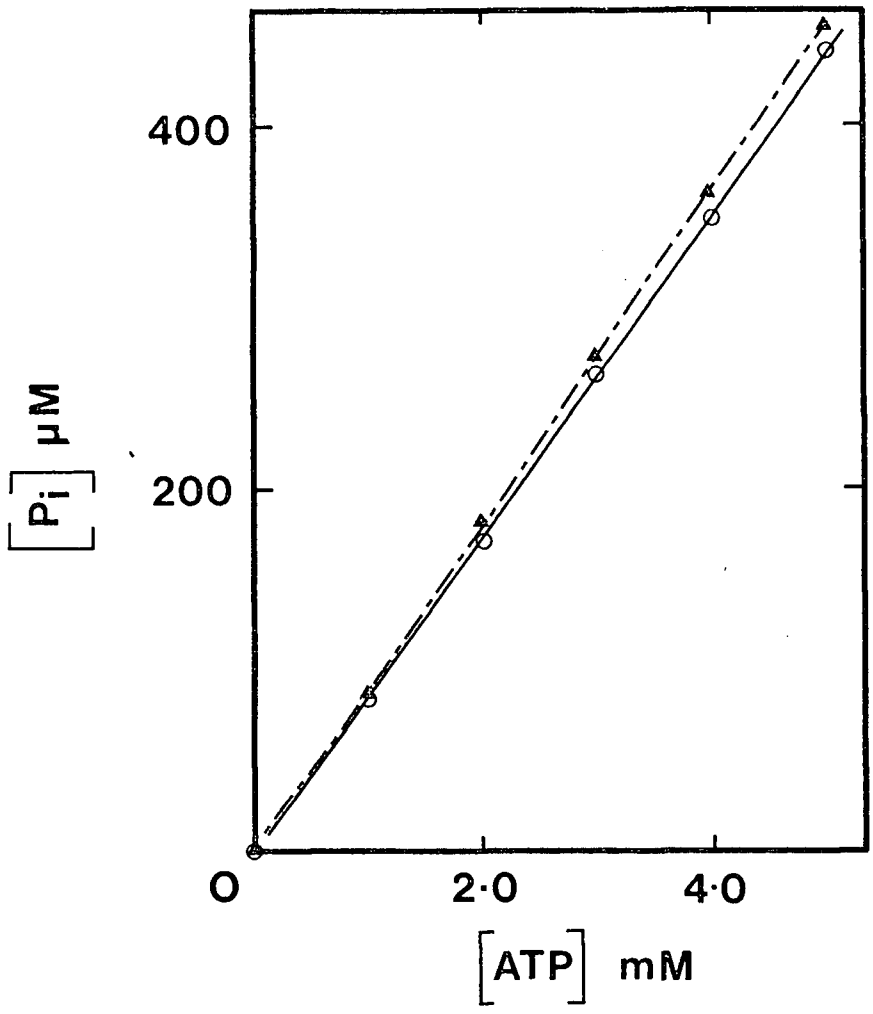


Fig. 2. Free phosphate content of ATP and effect of TCA.

The free inorganic phosphate content of commercial ATP was determined after the ATP had been dissolved in the standard ATPase assay mixture to form a range of concentration from 0 - 5mM. Samples were assayed for phosphate after incubation at 22° for 1 h (—o—) or after an additional 30 min incubation in the presence of TCA (3.3%) (—▲--).

Table VI. Paper chromatography of adenine nucleotides.

Commercial ATP, ADP and purified ADP fractions 1 and 2 were chromatographed on paper as described in METHODS AND MATERIALS. One unit is defined as the amount of nucleotide which gives an absorbance of 1 at 260 nm when dissolved in a volume of 1 ml. (nd: not detected.)

Sample	Units on chromatogram	Units in ATP spot	Units in ADP spot	Units in AMP spot
ATP	28.4	26.2	0.53	0.09
ADP	28.6	0.52	21.0	1.27
Fraction 1	148.0	nd	98.0	10.8
Fraction 2	136.1	nd	106.4	0.73

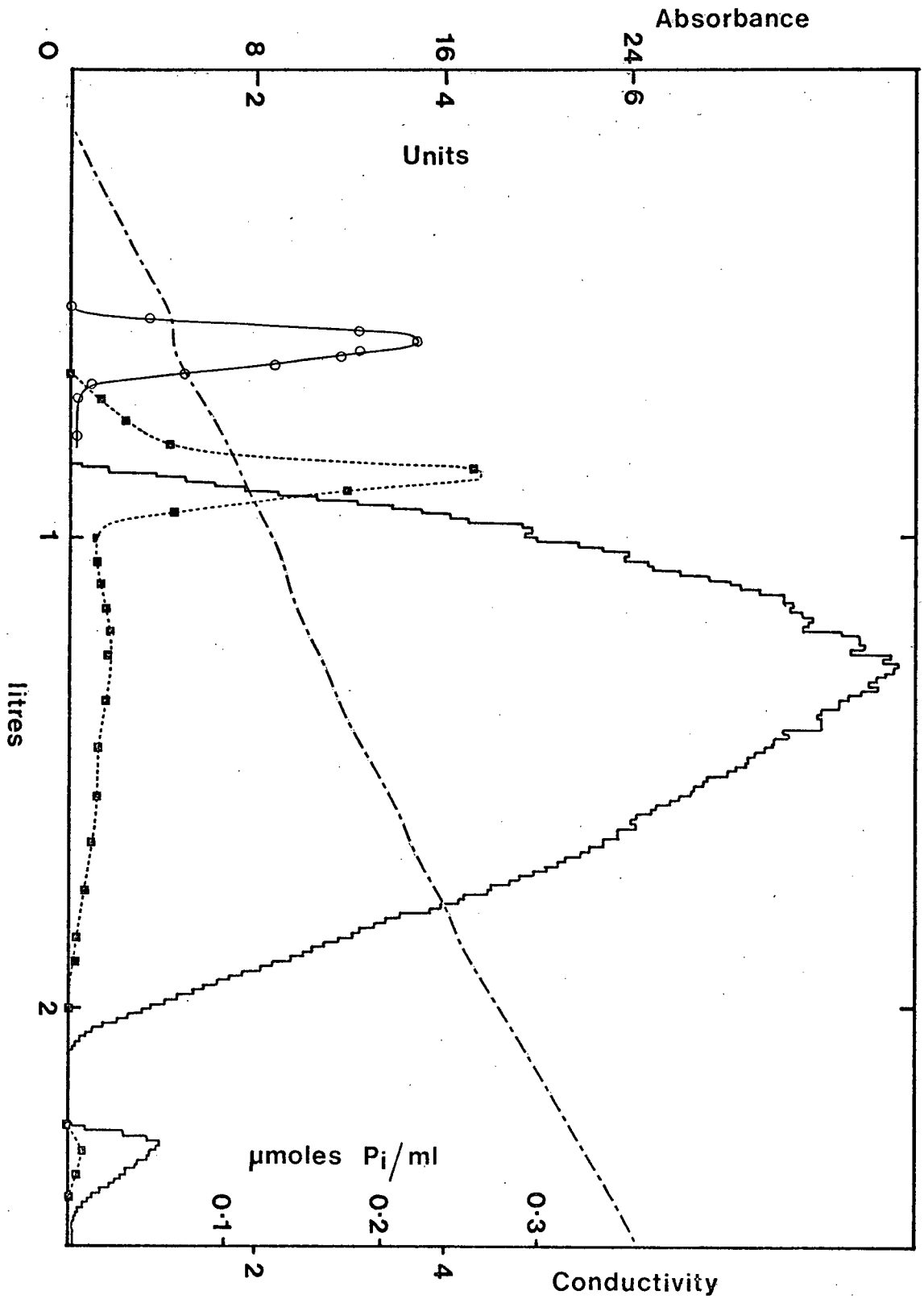
and AMP standards, while the two seen in the ADP sample had the same mobilities as ATP and AMP. The extent of contamination was measured by separately eluting the materials from the paper chromatogram as described in METHODS AND MATERIALS. The number of absorbance units placed on the chromatogram amd the number recovered in each spot were recorded (Table VI). In the ADP sample 2% of the absorbance units recovered were in the ATP spot. This was felt to be the most important contaminant to remove since in subsequent specificity studies the ATPase might appear to hydrolyze ADP when in fact it could be hydrolyzing the ATP contaminant in the ADP.

Purification of commercial ADP by ion exchange chromatography

ADP was purified on freshly prepared DEAE-cellulose as described in METHODS AND MATERIALS (Fig. 3). A total of 2.24×10^4 absorbance units were placed on the column. Elution by the application of a linear gradient of increasing ammonium bicarbonate concentration was unable to resolve AMP from ADP. The major peak of material that absorbed at 260 nm contained both nucleotides, though AMP was concentrated in the earlier fractions. The material eluting between 950 ml and 2,090 ml was divided into two major fractions, #1 and #2. Fraction #1 contained those fractions eluting between 950 ml to 1,300 ml while fraction #2 was made up of the remainder. The material in these fractions was analyzed by paper chromatography (Table VI). Fraction #1

Fig. 3. Chromatography of commercial ADP on DEAE-cellulose.

Chromatography was done as described in METHODS AND MATERIALS. Absorbance was measured at 260 nm (——). The fractions were assayed for Pi (·····■·····) and glucose-6-phosphate (—⊖—). Units refer to the content of glucose-6-phosphate as measured by the change in absorbance at 340 nm/ml of eluate during the standard assay. Conductivity (– – –) was measured in mΩ.


Absorbance
0
8
16
24
2
4
6
Units
litres
1
2
µmoles Pi/ml
0·1
0·2
0·3
2
4
Conductivity

contained 11% AMP and 89% ADP. Fraction #2 contained 0.7% AMP and 99.3% ADP, and was subsequently used in the specificity studies. In the eluate from the DEAE-cellulose column (Fig. 3) the minor peak of material that absorbed at 260 nm contained 0.4×10^4 absorbance units, which is 1.8% of the total units placed on the column. On paper chromatography this material corresponded to ATP. Pi eluted from the column at the front of the major nucleotide peak while glucose-6-phosphate was eluted well ahead of this peak. Thus the ADP in fraction #2 was freed of glucose-6-phosphate, ATP and the bulk of Pi and AMP.

Characterization of the periplasmic ATPase activity

Intact E. coli B and E. coli NRC 482 were observed to have appreciable ATPase, ADPase and AMPase activity (Table VII). Since nucleotides are not able to enter the cell (179), these enzyme activities probably occur outside the plasma membrane in the periplasmic space.

A number of enzymes have been located in this area of the cell. All of them seem to be involved in the breakdown of substrates rather than in biosynthesis. The location of the enzyme activities was confirmed by the fact that, like other periplasmic enzymes, much of the ATPase and AMPase activities could be released from the cells by an osmotic shock (Table VIII) (i.e., the rapid transition from a hypertonic to a hypotonic

Table VII. ATPase, ADPase and AMPase activities in intact E. coli and in cell extracts.

Freshly grown cells (2 g) were suspended at 22°- 24° in 40 ml of 0.2M sucrose containing 10mM $MgCl_2$ and 10mM glycylglycine-NaOH (pH 8.0). The cells were assayed for the three enzyme activities before and after sonication. The enzyme units are expressed as nmoles/min/mg protein. Identical experiments were done with E. coli B and E. coli NRC 482.

E. coli B

Enzyme activity	Whole cell (units)	Sonicate (units)
ATPase	13.2	92
ADPase	9.1	36
AMPase	5.5	4.8

E. coli NRC 482

Enzyme activity	Whole cell (units)	Sonicate (units)
ATPase	58	119
ADPase	57	53
AMPase	45	5.5

Table VIII. Distribution of AMPase and ATPase activities before and after osmotic shock of *E. coli* NRC 482.

"Shocked" cells and osmotic shockate were prepared as described in METHODS AND MATERIALS from whole cells (2 g). AMPase and ATPase assays were done on samples of each preparation. The units of enzyme activity refer to the total activity originating from 2 g of cells.

Enzyme activity	Whole cells	Shocked cells	Osmotic shockate
ATPase (units)	33.0	7.8	10.7
AMPase (units)	23.9	4.8	11.3
ATPase (units per mg protein)	0.123	0.040	0.877
AMPase (units per mg protein)	0.089	0.025	0.919

medium). The release was accompanied by an increase in the specific activity of these enzymes. The pH optima of the ATPase and AMPase activities of the osmotic shockate were measured in the presence of Mg^{2+} and found to be pH 9.0 for both activities (Fig. 8). (The osmotic shockate is defined as the supernatant fraction that remains when whole cells have been sedimented after being subjected to the osmotic shock procedure.)

In order to gauge what proportion of the total ATPase, ADPase and AMPase activities of the cell were periplasmic these activities were measured before and after sonication (Table VII). Significant portions of all three activities were expressed by the intact cells, particularly in the NRC 482 strain. However, while the ATPase activity of the cells increased on sonication, the AMPase activity actually decreased. This decrease was most noticeable in the NRC 482 strain. The effect on ADPase activity was different between the two strains. ADPase activity increased in the B strain but decreased slightly in the NRC 482 strain.

The enzymes of the periplasmic space in E. coli that have ATPase activity at alkaline pH values are alkaline phosphatase and 5'-nucleotidase. It was felt unlikely that the ATPase activity was due to alkaline phosphatase since the latter is not synthesized when cells are grown in the presence of phosphate (16, 21). The medium on which these cells were grown contained K_2HPO_4 (0.7%) and KH_2PO_4 (0.3%). In order to see if the cells had escaped this normal repression and were synthesizing alkaline phosphatase, the experiment shown in Table VII was repeated

using p-NPP in place of ADP (Table IX). There was very little hydrolysis of this substrate either by the intact cells or by the sonic-extract, which indicated that alkaline phosphatase was absent. (The observed stoichiometry of inorganic phosphate release from ATP, p-NPP and AMP by alkaline phosphatase is 1.05:1:1 respectively (180)). Furthermore the AMPase activity of intact E. coli is dependent on the addition of a divalent metal ion as shown in Fig. 4, whereas alkaline phosphatase does not require exogenous cations for activity (180).

5'-Nucleotidase, the other periplasmic enzyme that has ATPase activity, can also hydrolyze AMP and ADP (19), and might account for all three activities shown by intact cells (Table VII). The 5'-nucleotidase is known to have a protein inhibitor (23, 24) which is found in the cytoplasm. This could explain the dramatic decrease in AMPase activity when E. coli NRC 482 was sonicated (Table VII), since sonication would enable the inhibitor to come into contact with the enzyme. The protein inhibitor is easily denatured by heating to 50°- 60° (19, 22), while the 5'-nucleotidase is unaffected. To see if such an inhibitor was masking the AMPase activity of cell extracts prepared as in Table VII, two different experiments were done. In the first of these, aliquots of the cell extract were preincubated at 50° for various time periods from 0 to 60 min. After heating the aliquots, they were chilled in ice and then assayed for AMPase at 30°. The results of this experiment (Fig. 5) showed that 5'-nucleotidase activity was markedly increased by heating. This activation is essentially

Table IX. Hydrolysis of p-NPP relative to that of ATP and AMP by intact E. coli and in cell extracts.

E. coli NRC 482 (2 g) were suspended at 22°- 24° in 30 ml of 0.2M sucrose containing 10mM $MgCl_2$ and 10mM glycylglycine-NaOH buffer (pH 8.0). The other details of the experiment are as described in the legend to Table VII.

Enzyme activity	Whole cell (units)	Sonicate (units)
ATPase	37	124
AMPase	22	4.8
p-NPPase	0.9	0.8

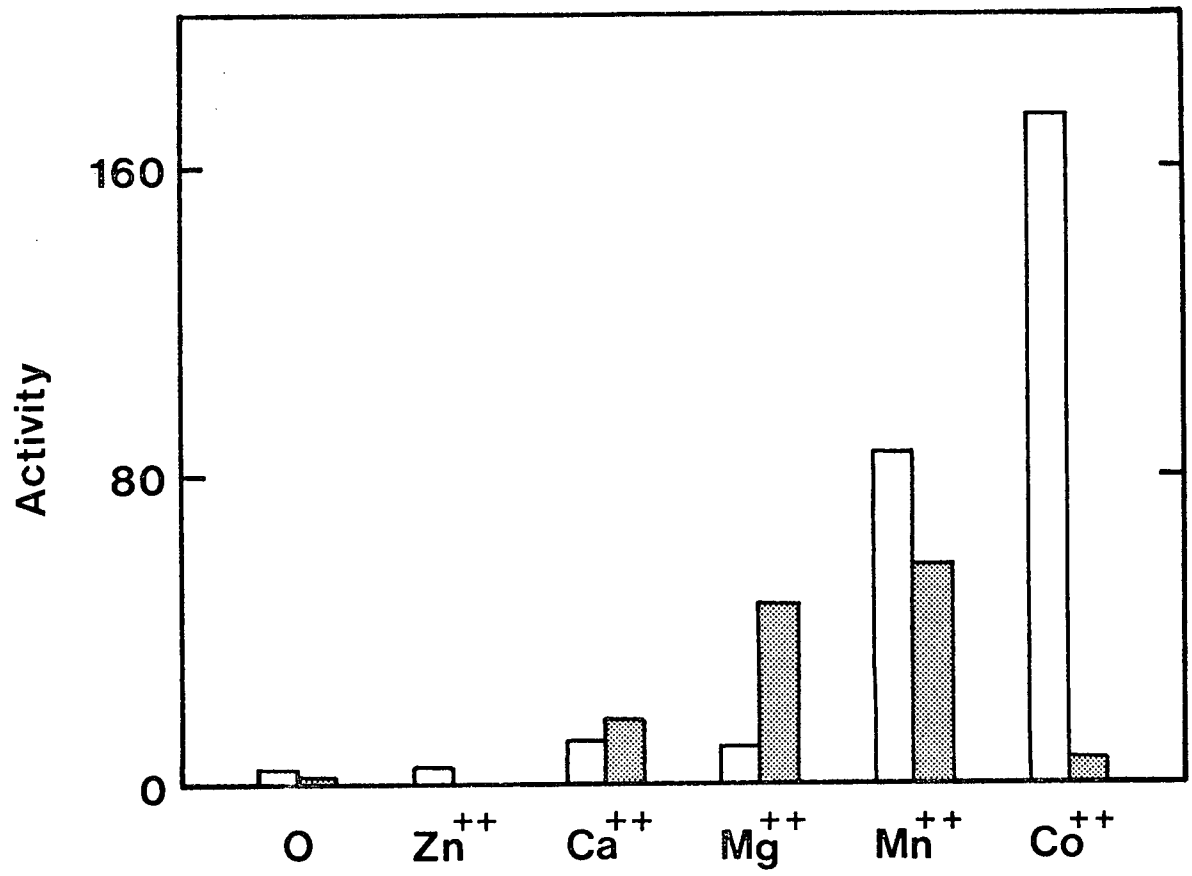


Fig. 4. Effect of divalent metal ions on AMPase activity of intact E. coli.

E. coli NRC 482 (0.5 g) were suspended at 22°- 24° in 50 ml of 0.2M sucrose containing 10mM glycylglycine-NaOH buffer (pH 8.0). Aliquots of the cell suspension were assayed for AMPase activity at pH 8.0 (shaded) as described in METHODS AND MATERIALS. Assays were also done at pH 6.0 (unshaded) by replacing the 0.1M glycylglycine-NaOH buffer (pH 8.0) with 0.1M Na-acetate buffer (pH 6.0). Mg^{2+} was replaced in turn at a concentration of 5mM by each of the divalent metal ions listed. Enzyme activity is given by nmoles/min/mg protein.

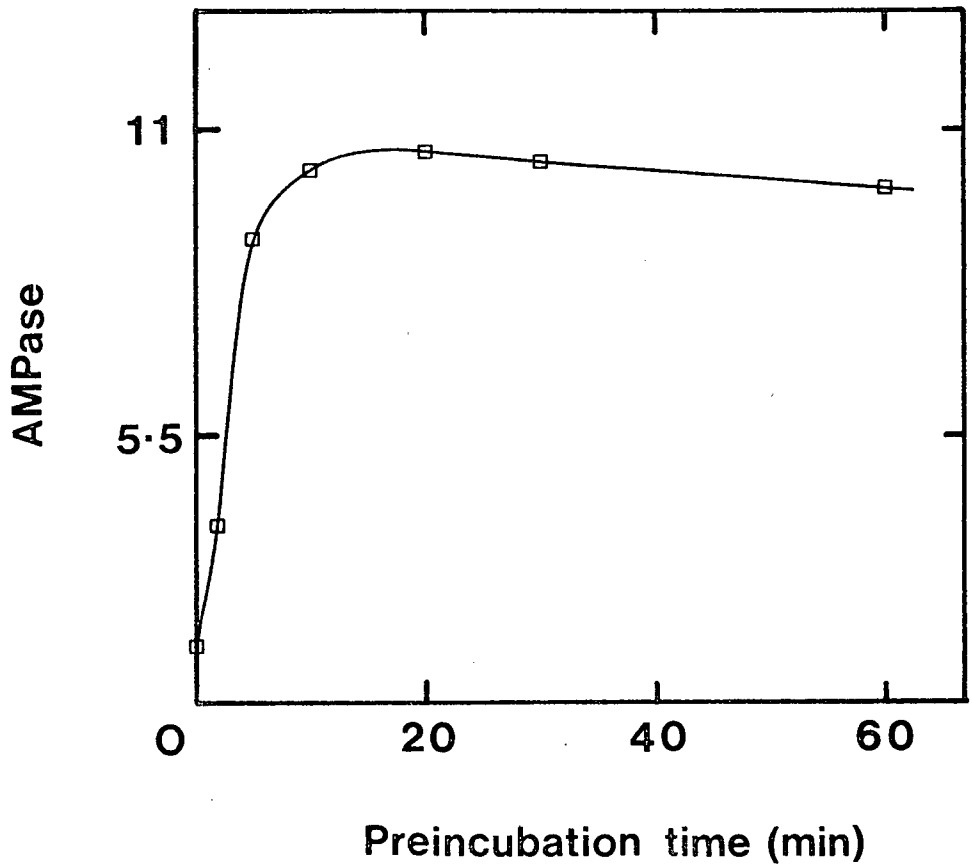


Fig. 5. Heat activation of AMPase activity in cell extracts of E. coli.

E. coli NRC 482 (3.33 g) were sonicated as described in METHODS AND MATERIALS. Aliquots (0.5 ml) of this cell extract (1.73 mg protein/ml) were heated for various times at 50° before cooling in ice. These preincubated samples were then assayed for AMPase activity at 30°. The enzyme activity is expressed as units/ml of cell extract at 30°.

complete after 10 min at 50°. The AMPase assay was done at 30° instead of the usual 37°, in case the latter temperature was sufficient to cause denaturation of the inhibitor during the assay. In another experiment an Arrhenius plot was made of the variation with temperature of the AMPase activity in the E. coli cell extract. Most chemical reactions double their rate for every 10° rise in temperature. This is also true of reactions catalyzed by enzymes. However the AMPase activity of the cell extract increased 3.5 times for every 10° rise in temperature (Fig. 6). This higher rate of increased activity with temperature could be due to the denaturation of the inhibitor. The activation energy of this reaction was calculated to be 21.5 Kcal/mole.

Another property of 5'-nucleotidase reported by Neu (19) is its stimulation by Co^{2+}. Of the divalent metal ions tested, Co^{2+} was the most effective in activating the enzyme. The activation of the AMPase of E. coli osmotic shockate by Co^{2+} was measured at pH 6.0 and pH 8.0, and compared to the activation produced by Mg^{2+} (Fig. 7). Co^{2+} stimulated the AMPase much more than Mg^{2+}. In the presence of Co^{2+} the AMPase was more active at pH 6.0 than at pH 8.0. However, in the presence of Mg^{2+} the opposite was true (Figs. 7 and 8). Similar effects of pH and metal ion activation were observed with the ATPase activity of the shockate (Figs. 7 and 8).

The effects of pH and metal ion activation on purified 5'-nucleotidase have been investigated by Glaser et al. (22) and by Neu (19). Both groups reported the effectiveness of activation

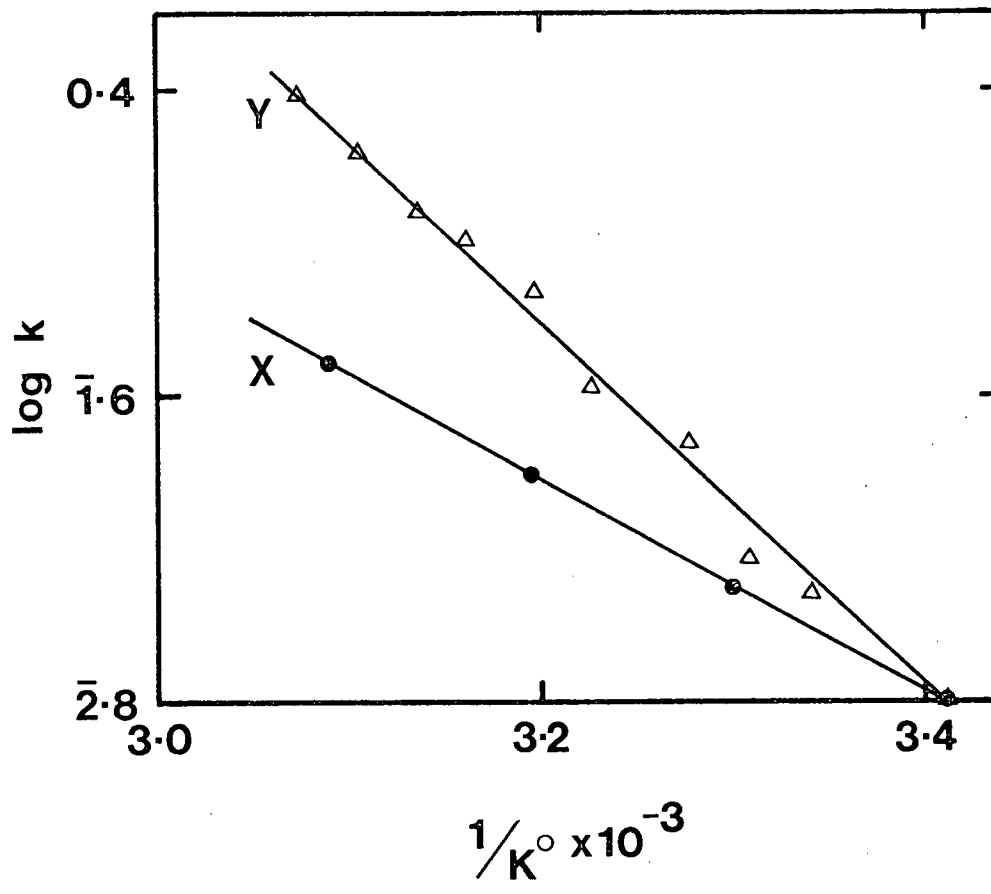


Fig. 6. Arrhenius plot for hydrolysis of AMP by a cell extract of E. coli.

The cell extract (1.73 mg protein/ml) was prepared as described in the legend to Fig. 5. The AMPase assay was done on 0.5 ml of extract as described in METHODS AND MATERIALS. k, rate of hydrolysis of AMP (units/ml extract x 10^2); T, incubation temperature (°K). The observed Arrhenius plot of the AMPase activity (Y) was compared to an idealised Arrhenius plot in which the activity expressed at 20.0° was doubled for every 10° rise in temperature (X).

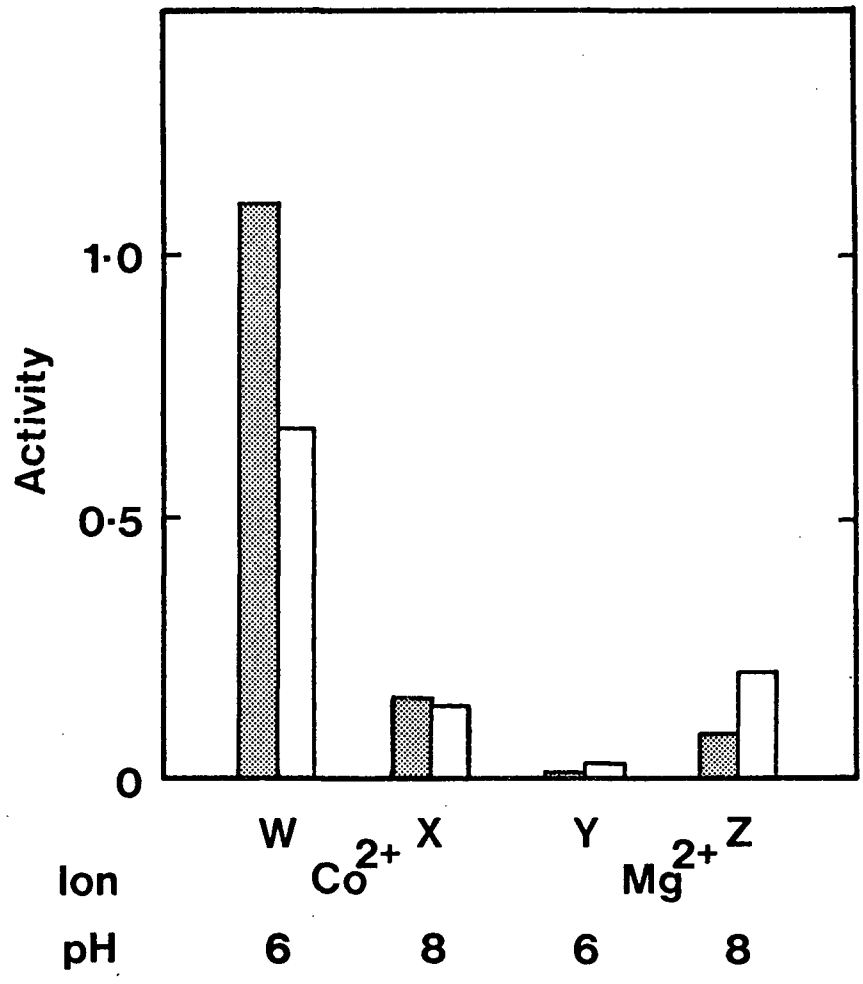


Fig. 7. Ion effects at pH 6.0 and 8.0 on AMPase and ATPase activities of osmotic shockate of E. coli B.

The osmotic shockate from E. coli B (1 g) was prepared as described in METHODS AND MATERIALS. It was stored at 0° for 4 days after the addition of $MgCl_2$ and bovine serum albumin to final concentrations of 1mM and 1 mg/ml respectively. The AMPase or ATPase assays were done at 25° for 60 min in the presence of 2.5mM AMP or ATP. The final assay volume was 1 ml which included 0.5 ml of the enzyme preparation (osmotic shockate). Solutions of pH 6.0 (W,Y) and pH 8.0 (X,Z) were produced by 100mM Na-acetate and 100mM glycylglycine-NaOH buffers respectively. The assays were done in the presence of 2.5mM $MgCl_2$ (Y,Z) or 5mM $CoCl_2$ (W,X). The activities of AMPase (shaded) and ATPase (unshaded) are expressed as nmoles/min/ml of osmotic shockate.

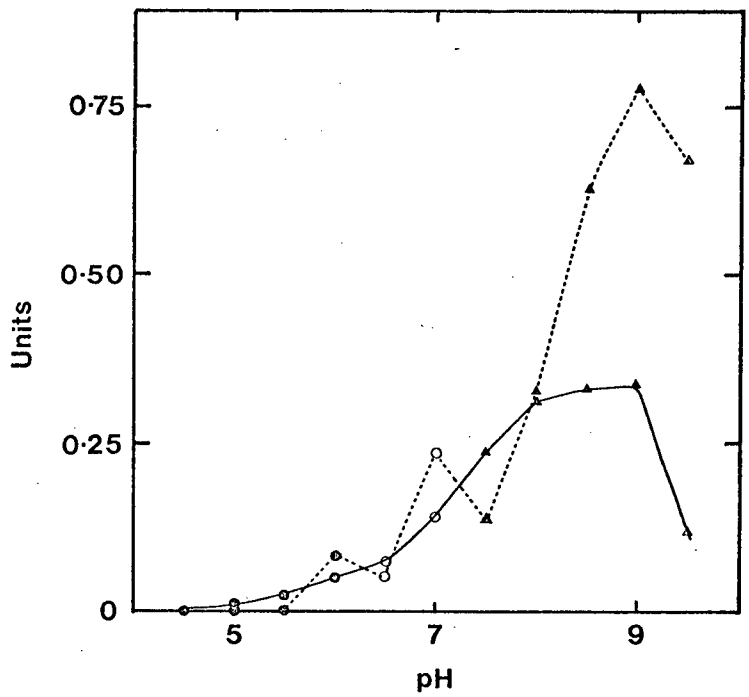


Fig. 8. pH optima of AMPase and ATPase activities of osmotic shockate from E. coli B.

The osmotic shockate was prepared and stored as described in the legend to Fig. 7. The pH optima of both enzyme activities were determined in the presence of 5mM $MgCl_2$. Na-acetate buffers were used to obtain pH 4.5, 5.0, 5.5 and 6.0 (●); Na-cacodylate buffers were used for pH 6.5 and 7.0 (○); Tris-HCl for pH 7.5, 8.0, 8.5 and 9.0 (▲) and glycine-NaOH buffer for pH 9.5 (△). All buffers were used at a final concentration of 0.1M. Units of enzyme activity are expressed as nmoles/min/ml of osmotic shockate at 25°. AMPase (——), ATPase (-------).

of the enzyme by cations was in the order $Co^{2+} > Mn^{2+} > Mg^{2+}$. With Co^{2+}, Neu reported that the enzyme had a pH optimum of pH 5.7 to 6.1, while Glaser *et al.* found pH 7 to be optimal. Neu reported that there was no pH optimum at an alkaline pH whereas Glaser *et al.* found Mg^{2+} to give most activation at pH 8.0.

In view of the discrepancies between these two reports, and because the AMPase and ATPase activities of the osmotic shockate had a pH optimum with Co^{2+} at an acid pH and with Mg^{2+} at an alkaline pH, it was considered possible that there was more than one enzyme responsible for these properties. To investigate this possibility the Co^{2+}-stimulated 5'-nucleotidase was partially purified according to Neu's procedure (19) in order to see if the other activity (alkaline pH optimum with Mg^{2+}) remained with it.

Partial purification of Co^{2+}-stimulated 5'-nucleotidase

The partial purification of Co^{2+}-stimulated 5'-nucleotidase was carried out as described in METHODS AND MATERIALS. The specific activity of the 5'-nucleotidase was increased only two-fold by the osmotic shock procedure (Table X). This was a much smaller increase than that obtained by Neu (19). One explanation for this small increase in specific activity lies in the incomplete (55%) release of enzyme from the "shocked" cells. Another possible explanation is that some of the cells lysed during the

Table X. Partial purification of 5'-nucleotidase from E. coli NRC 482.

The procedure for the partial purification of 5'-nucleotidase from E. coli NRC 482 (10 g) is described in METHODS AND MATERIALS.

Step	Fraction	Units /ml	Protein mg/ml	Units/ mg protein	Units	Yield %
	Whole cells	0.211	1.61	0.131	120	100
1	Osmotic shockate	0.111	0.453	0.245	66.6	55
2	Heat-treated osmotic shockate	0.145	0.244	0.595	73.8	61.5
3	Pooled fractions from DEAE-cellulose	0.793	0.077	10.3	66.6	55
4	Pooled fractions from hydroxyl-apatite	1.51	0.025	60.3	27.2	22.5

osmotic shock procedure and released 5'-nucleotidase inhibitor into the shockate. This latter hypothesis was later confirmed by the results of the second purification step, in which the shockate was heat-treated. After heat-treatment the shockate was found to have an increased number of units of 5'-nucleotidase activity. At the same time the specific activity of the 5'-nucleotidase rose from 0.245 μmoles/min/mg of protein to 0.595 μmoles/min/mg of protein (Table X).

After the next step in the purification procedure, which was a separation on DEAE-cellulose, fractions 59 to 72 inclusive contained most of the enzyme activity (Fig. 9) and were subsequently combined (total volume 84 ml). During this ion exchange chromatography the specific activity of the 5'-nucleotidase was increased 17-fold, while 89% of the total enzyme activity was recovered.

The purification procedure was stopped after the third step, that of adsorption chromatography on hydroxylapatite. Only one peak of bis-pNPPase activity was seen in the elution profile after this separation (Fig. 10). This activity occurred in fractions 68 - 73 inclusive. After dialysis these fractions were shown to have 5'-nucleotidase activity. Fractions 44 - 48 inclusive, 74 - 78 inclusive, and 79 - 84 inclusive had no 5'-nucleotidase activity.

The total recovery of 5'-nucleotidase activity after the four purification steps was 22.5% while the specific activity had been increased 450 times (Table X). The effects of pH and metal ion activation on this partially purified enzyme activity

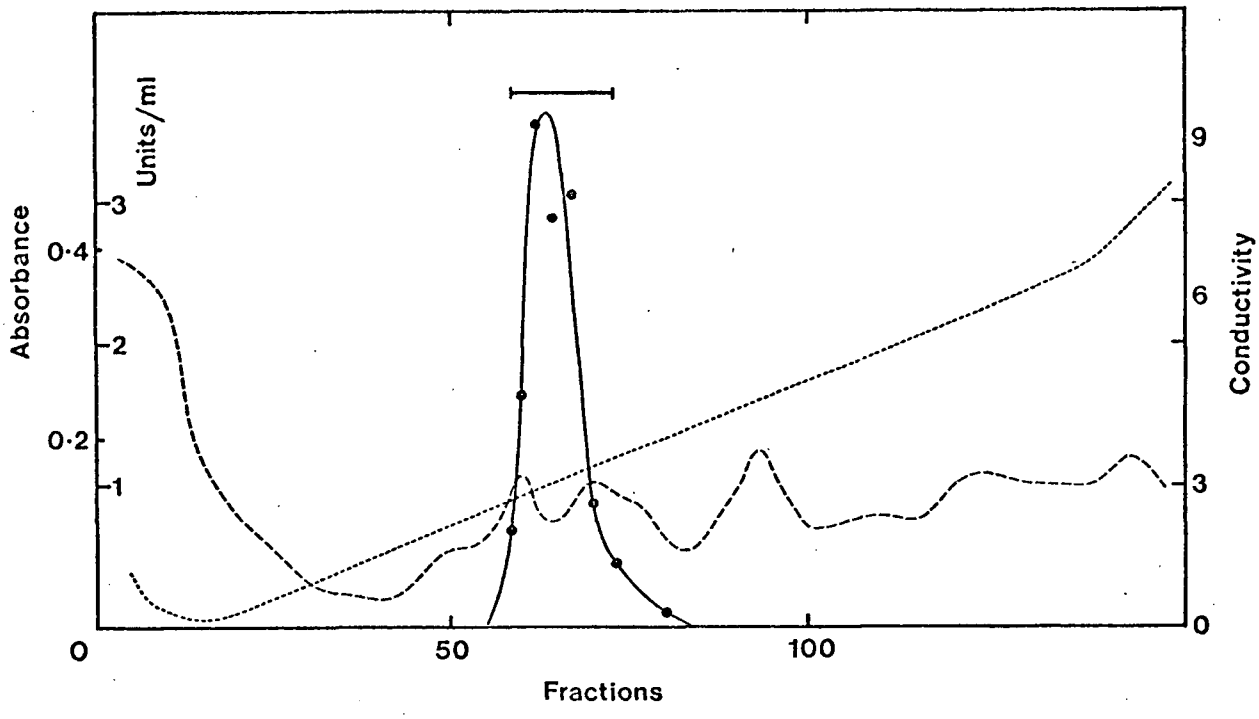


Fig. 9. Chromatography of heat-treated osmotic shockate on DEAE-cellulose.

This chromatographic procedure is described in METHODS AND MATERIALS. The fractions (6 ml) were tested for conductivity (mℜ) (·····), absorbance at 280 nm (– – –), and 5'-nucleotidase activity (—●—). Enzyme activity is expressed as units/ml fraction. Fractions covered by the bar (59 to 72 inclusive) were pooled.

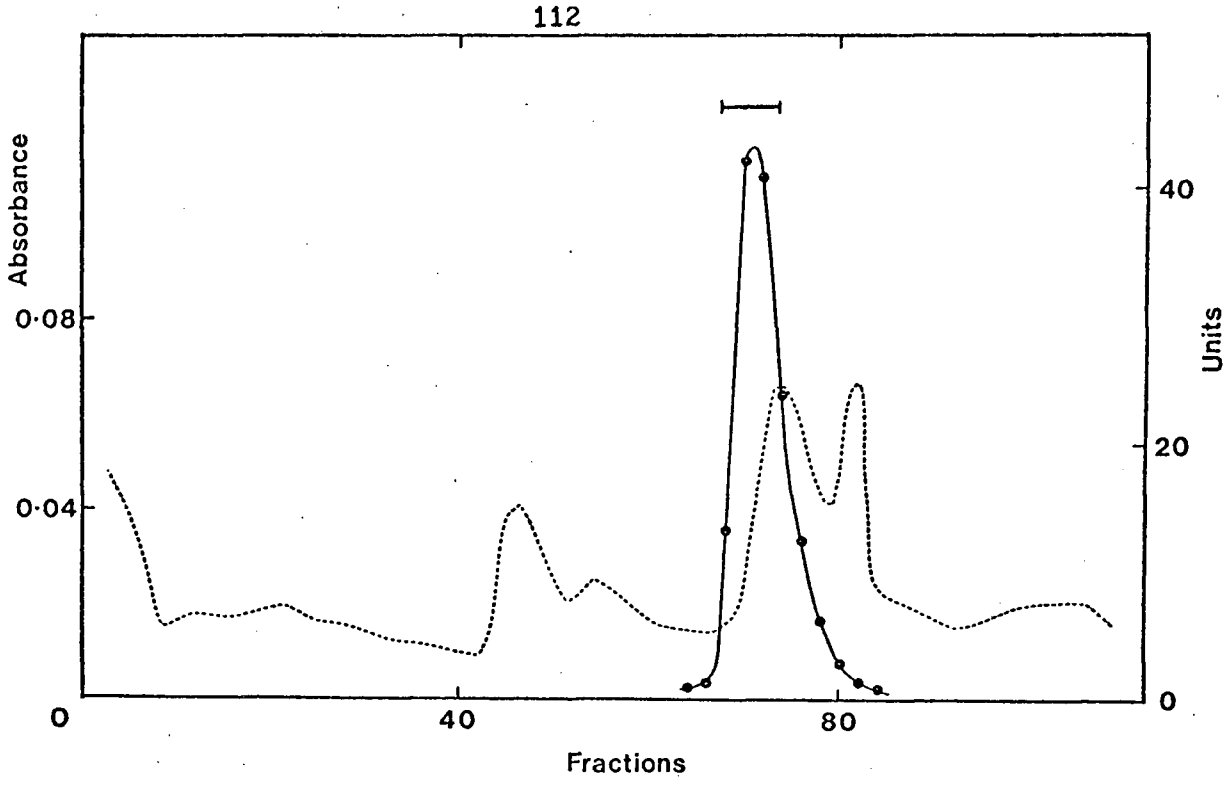


Fig. 10. Chromatography of partially-purified 5'-nucleotidase on hydroxylapatite.

This chromatographic procedure is described in METHODS AND MATERIALS. Fractions (1 ml) were tested for absorbance at 280 nm (·····) and bis-pNPPase activity (—●—). Units of enzyme activity are expressed as nmoles/min/ml fraction based on a molar extinction coefficient for p-nitrophenol at 410 nm of 17 x 10^3 l. $moles^{-1}.cm^{-1}$. Fractions covered by the bar were combined and were shown to have 5'-nucleotidase activity.

were studied. It was of particular interest to see if the enzyme had retained its widely different pH optima with Co^{2+} and Mg^{2+}. The variation in 5'-nucleotidase activity with pH was measured in the presence of 5mM Co^{2+}, Mn^{2+}, and Mg^{2+} (Fig. 11). Optimum activity with Co^{2+} occurred at pH 5.5, while with Mg^{2+} the pH for optimum activity was greater than or equal to 9.5. The pH-activity curve with Mn^{2+} was very broad but had a peak at pH 7.5, between the optima for Co^{2+} and Mg^{2+}. This intermediate effect of Mn^{2+} was also seen when whole cells were assayed for AMPase and ATPase activities (Figs. 4 and 12).

The pH optimum of an enzyme is a composite of several variables which include the effect of pH on the charge of the substrate, the net charge on the protein, and the stability of its conformation with respect to pH. Particularly important in determining the pH optimum of the reaction are the amino acids at the active site. Since these amino acids cannot change, it is surprising that the 5'-nucleotidase can have such variable pH optima.

In the assays with Co^{2+} and Mn^{2+} at an alkaline pH there was often a slight precipitate which formed in the assay mixture before the addition of enzyme to start the reaction. Although the chloride salts of these two divalent metal ions are very soluble, the metal hydroxides are not. The solubility product of $Co(OH)_2$ is 2×10^{-16} (gram ions/l.)^3 and that of $Mn(OH)_2$ is 1.9×10^{-13} (gram ions/l.)^3 (181). It is conceivable that at an alkaline pH the $[OH^-]$ is elevated to such an extent

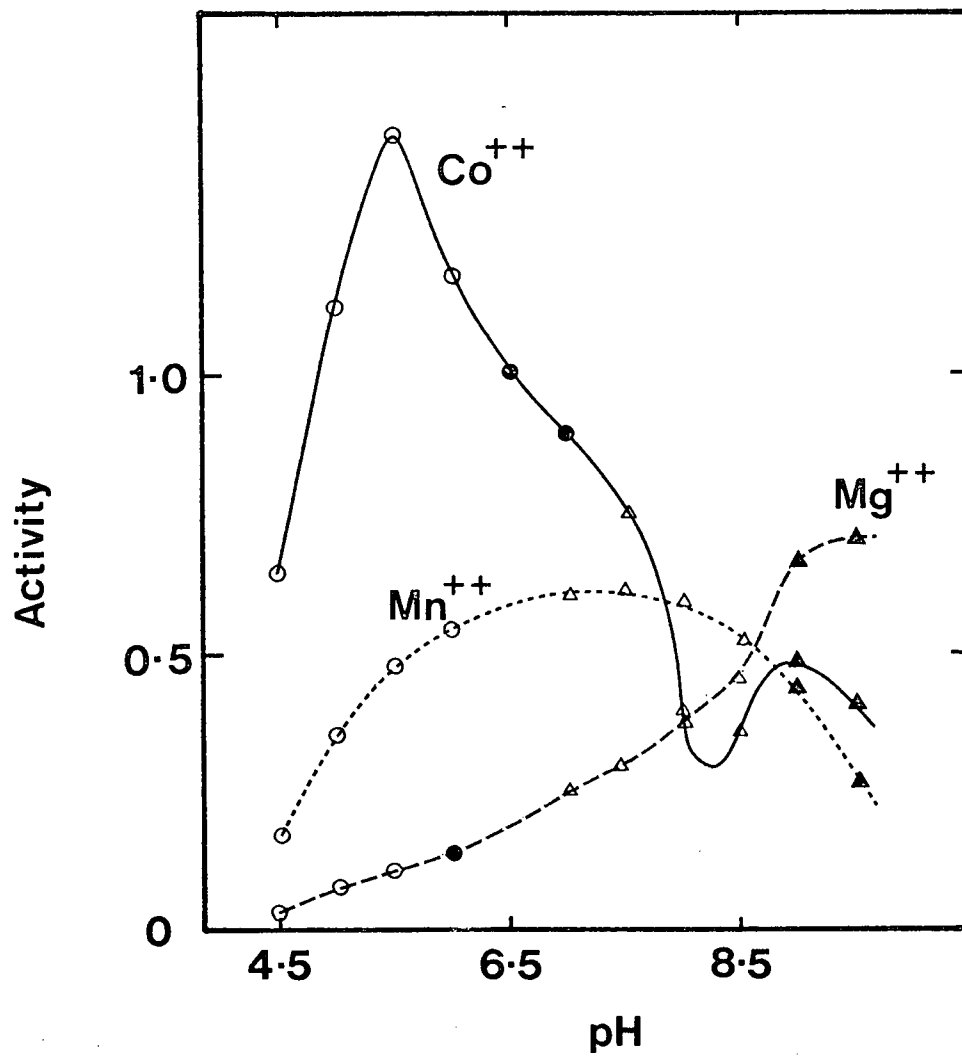


Fig. 11. pH optima of AMP hydrolysis by 5'-nucleotidase.

The pH optima of 5'-nucleotidase were determined in the presence of Co^{2+} (———), Mn^{2+} (·-·-·-·), or Mg^{2+} (– – – –) all at a final concentration of 5mM. The enzyme preparation was the combined fractions from hydroxylapatite (Fig. 10) (25 µg protein/ml). Na-acetate buffers were used to obtain pH 4.5, 5.0, 5.5 and 6.0 (○); Na-cacodylate buffers were used for pH 6.0, 6.5 and 7.0 (●); Glycylglycine-NaOH buffers for pH 7.0, 7.5, 8.0 and 8.5 (△) and alanine-NaOH buffers for pH 9.0 and 9.5 (▲). All buffers were used at a final concentration of 0.1M. The enzyme assay was done as described in METHODS AND MATERIALS but with the variations in metal ions and buffers as described above. Enzyme activity is expressed in units/ml of enzyme preparation.

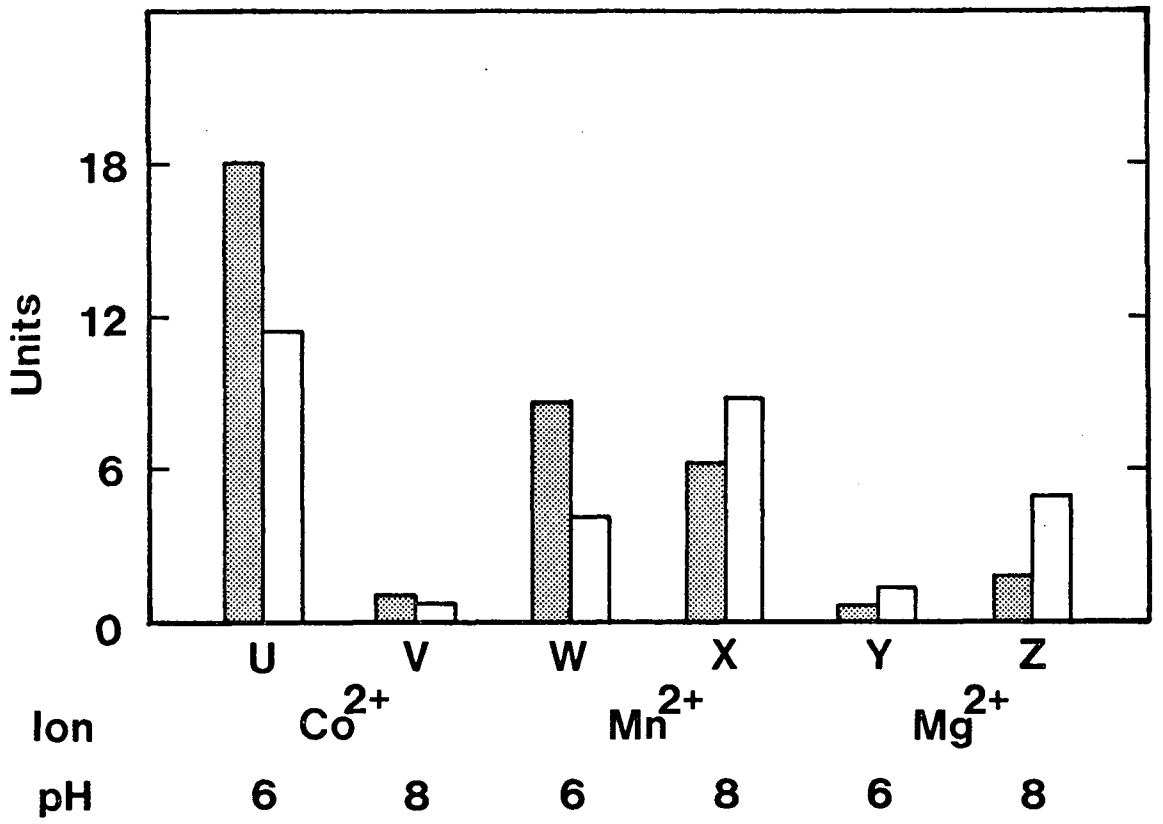


Fig. 12. Ion effects on the AMPase and ATPase activities of intact E. coli NRC 482.

Freshly grown E. coli NRC 482 (0.5 g) were suspended in 10mM glycylglycine-NaOH buffer (pH 8.0) made 0.2M in sucrose (50 ml). The AMPase (shaded) and ATPase (unshaded) activities of the cell suspension were determined at pH 6.0 (U,W,Y) and pH 8.0 (V,X,Z) in the presence of Co^{2+} (U,V), Mn^{2+} (W,X) or Mg^{2+} (Y,Z). Each of the divalent metal ions were used at a concentration of 5mM. The buffer solutions for pH 6.0 and 8.0 were Na-acetate and glycylglycine-NaOH buffers respectively, both at a final concentration of 0.1M. The units of enzyme activity are expressed as (μmoles/min/mg protein) x 10^2 at 25°.

that Co^{2+} and Mn^{2+} are removed from solution as insoluble hydroxides. The removal of the activating ions at alkaline pH might alter the shape of the pH profile and shift the optimum pH towards the acid region. The fact that $Co(OH)_2$ is less soluble than $Mn(OH)_2$ is in line with the observation that the pH optimum of 5'-nucleotidase with Co^{2+} is lower than with Mn^{2+} (Fig. 11 and reference 22).

This hypothesis reconciles the widely different pH optima with one enzyme. Furthermore no evidence appeared during the partial purification of the 5'-nucleotidase to indicate that there might be more than one enzyme responsible for the AMPase activity of the intact E. coli. Since the Co^{2+}-stimulated 5'-nucleotidase of E. coli hydrolyzes ATP and ADP in addition to AMP, and since the periplasmic ATPase has the same pH and metal ion activation properties as the AMPase, it is likely that the periplasmic ATPase activity is due to Co^{2+}-stimulated 5'-nucleotidase. That these two enzymes have a common identity is suggested by the ratios of ATPase : ADPase : AMPase of intact E. coli (Table VII) being very similar to the ratios seen for 5'-nucleotidase activity after DEAE-cellulose chromatography (Figs 18, 25 and 26). Presumably the periplasmic ATPase and ADPase activities are also inhibited after sonication of the whole cells in the same manner as the periplasmic AMPase. However this inhibition is hidden in the sonicate by the considerable ATPase and ADPase activities that originate from inside the cell (Table VII).

Removal of 5'-nucleotidase from E. coli

Even though the 5'-nucleotidase was largely inhibited in cell extracts it still retained the potential to interfere with the assay of other ATPase activities, since the 5'-nucleotidase inhibitor is very labile. Two procedures were considered for reducing the 5'-nucleotidase content of the E. coli prior to the formation of a cell extract. The first of these was spheroplast formation using lysozyme as described in METHODS AND MATERIALS. Under conditions in which there is essentially complete conversion of whole cells to spheroplasts there was no release of 5'-nucleotidase (Table XI). This is in contrast to the results of Malamy and Horecker (182), Neu and Heppel (183), and Melo and Glaser (184) working with alkaline phosphatase, RNase and nucleotide diphosphate hexose pyrophosphatases respectively. (One of the nucleotide diphosphate hexose pyrophosphatases that Melo and Glaser studied (184) is uridine diphosphate sugar hydrolase which has been shown to be identical to 5'-nucleotidase (19, 22).) All these periplasmic enzymes were completely released from the cells during spheroplast formation. The only difference between the procedures is that with E. coli NRC 482, EDTA was omitted during spheroplast formation whereas with the other bacteria (182-184) it was present. It would seem that the presence of the chelating agent is essential for the release of periplasmic enzymes during lysozyme-induced spheroplast formation. That this release is not simply a matter of spheroplast formation is shown by the fact that there is essentially no release of periplasmic

Table XI. Release of 5'-nucleotidase after lysozyme treatment of E. coli NRC 482.

E. coli NRC 482 (2 g) were converted to spheroplasts with the aid of lysozyme as described in METHODS AND MATERIALS. The whole cell suspension was sampled immediately prior to lysozyme addition. After 20 min of treatment with lysozyme the digest was centrifuged at 14,500 x g for 20 min at 2°- 5°. The supernatant fraction was retained and the pellets were resuspended in 30mM Tris-HCl (pH 8.0) made 20% (w/v) in sucrose (80 ml) to give the "lysozyme-treated whole cell" suspension. The three fractions were each assayed for 5'-nucleotidase in the usual way.

Fraction	Units/mg protein	Units recovered (%)
Whole cells	0.121	100
Lysozyme-treated whole cells	0.114	94
Supernatant	0	0

enzymes during penicillin-induced spheroplast formation (13). The use of EDTA during spheroplast formation in E. coli NRC 482 caused some premature lysis. In the light of reports (1, 69) about divalent metal ions being involved in the binding of ATPase to the membrane it was thought that inclusion of EDTA during spheroplast formation might cause premature release of the enzyme.

The second method for removing 5'-nucleotidase prior to the formation of cell extracts was by osmotic shocking. Under ideal conditions it was possible to get 99% release of 5'-nucleotidase into the shockate (Table XII). This procedure was not routinely used since the amount of enzyme released was often well below 99%. In practice the 5'-nucleotidase did not interfere greatly with the study of other ATPase activities. In cell extracts the enzyme occurred in the soluble fraction rather than with the membranes (Table XIV). Furthermore during chromatography of the soluble fraction on DEAE-cellulose it was possible to separate the 5'-nucleotidase and the complex of 5'-nucleotidase with inhibitor from other ATPase activities (Figs. 25 and 26).

Studies with the ATP analogue β,γ methylene adenosine triphosphate

β,γ Methylene adenosine triphosphate (AMP-PCP) is an analogue of ATP in which the β and γ phosphate groups are linked by a methylene bridge instead of by an oxygen atom. The similarity of its structure to that of ATP suggested that it might be useful

Table XII. Release of 5'-nucleotidase from *E. coli* NRC 482 after osmotic shock treatment.

E. coli NRC 482 (2 g) were osmotically "shocked" as described in METHODS AND MATERIALS. The whole cell suspension in the buffered sucrose and the osmotic shockate were assayed for 5'-nucleotidase in the usual way.

Fraction	Units/mg protein	Units recovered (%)
Whole cells	0.108	100
Osmotic shockate	1.28	99

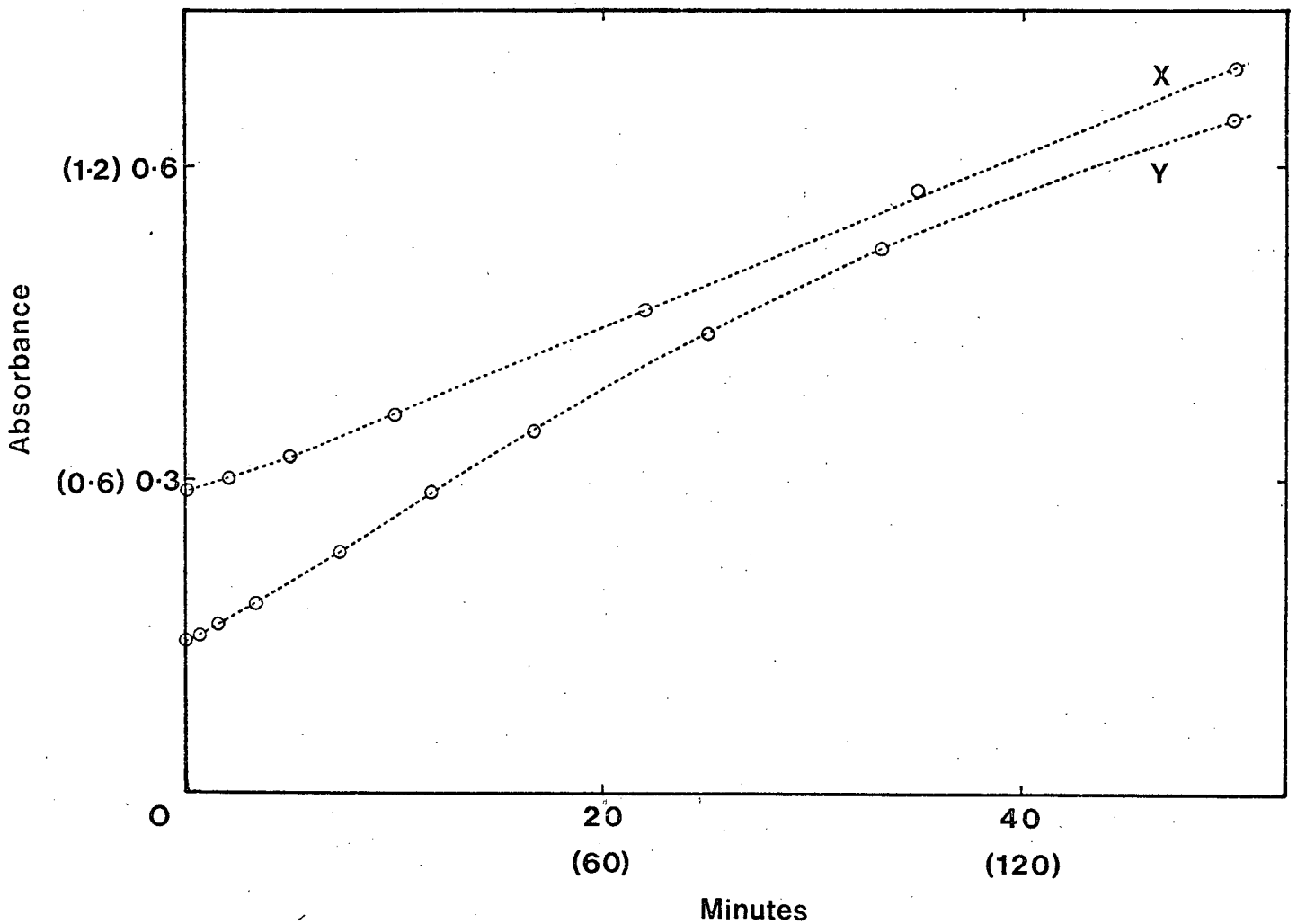
in the study of ATPase activities. An initial investigation of the inhibitory effects of AMP-PCP on an ATPase activity was carried out using the partially purified 5'-nucleotidase. In preparation for this study the hydrolysis of ATP by 5'-nucleotidase was examined to find out the conditions under which the reaction was linear. Using 0.33mM ATP as substrate the rate of release of phosphate was proportional to the reaction time until about 20% of the substrate had been hydrolyzed (Fig. 13). Keeping within a limit of 20% substrate hydrolysis, a Dixon plot (185) was made of 5'-nucleotidase activity in the presence of AMP-PCP. Two different ATP concentrations (0.5mM and 1.0mM) were used, while the AMP-PCP concentration was varied between 0 and 1.0mM (Fig. 14). AMP-PCP was a competitive inhibitor of the ATPase reaction as might be expected in view of its structural similarity to ATP. The value of K_I for AMP-PCP was found by extrapolation to be 3×10^{-4}M (Fig. 14). This is considered to be too low a value for AMP-PCP to qualify as an adsorbant in affinity chromatography without the use of an extending molecule between the matrix and the analogue (186).

Investigation of the intracellular ATPase activity

The first method that was used for the preparation of E. coli cell extracts entailed the conversion of the cells to spheroplasts and their subsequent lysis in a hypotonic medium. This method was chosen for two reasons. Firstly, it gave rise

Fig. 13. Linearity of Pi release from ATP catalyzed by 5'-nucleotidase.

The 5'-nucleotidase sample used for this experiment was the partially purified fraction from hydroxylapatite (Table X). The incubation mixture contained 5 µl of the pooled fractions from hydroxylapatite, 0.4 mmoles of glycylglycine-NaOH buffer (pH 8.0), 20 µmoles of $MgCl_2$, 400 µg of bovine serum albumin and 1.33 µmoles of ATP in a final volume of 4 ml. The assay was done at 37°. Samples (0.2 ml) were removed at time intervals and added to 5% TCA (0.1 ml). The samples were assayed for Pi by the method of Ames (166). The absorbance at 660 nm due to the phosphate is plotted against the time of sampling. Series X includes values taken over 0 - 50 min. The values for series Y were taken over 0 - 150 min (values in brackets).


X
Y
(1·2) 0·6
(0·6) 0·3
0
Absorbance
20
(60)
40
(120)
Minutes

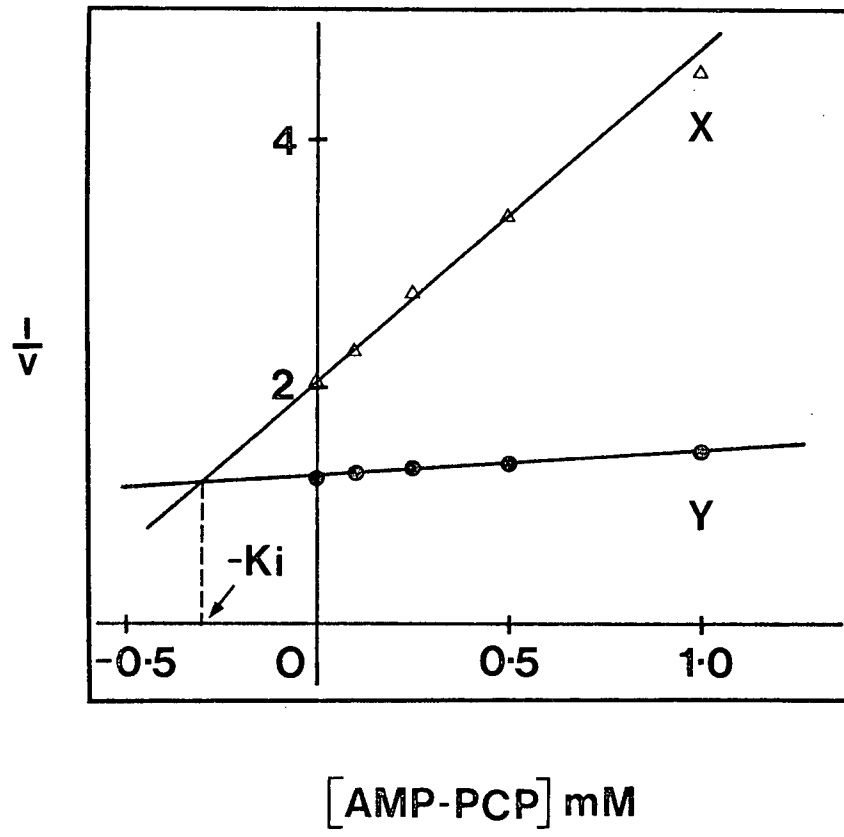


Fig. 14. Dixon plot of 5'-nucleotidase inhibition by AMP-PCP.

The 5'-nucleotidase sample was made up by mixing 30 µl of the pooled fractions from hydroxylapatite (Table X) with 170 µl of bovine serum albumin (1 mg/ml). Aliquots (5 µl) of the enzyme sample were assayed for ATPase activity in the presence of ATP (0.5mM or 1.0mM) and variable concentrations of AMP-PCP. The assays were done at 37° in the presence of 5mM $MgCl_2$ and 0.1M glycylglycine-NaOH buffer (pH 8.0) in a final volume of 0.6 ml. The Dixon plot (185) was obtained by plotting the reciprocal of the enzyme activity (units/ml enzyme sample)$^{-1}$ against AMP-PCP concentration (mM) for the substrate concentrations X, (0.5mM) and Y, (1.0mM).

to membrane fragments which were relatively uniform in size and which could easily be sedimented by low speed centrifugation (14,600 x g). Secondly, it was considered to be the most gentle method of breaking the cells, and hence the least disruptive of membrane function including the binding of ATPase.

Lysozyme-induced spheroplast formation

When spheroplasts were formed from E. coli NRC 482 by the method of Birdsell and Cota-Robles (187) there was evidence of premature lysis. During digestion of the cells with lysozyme in the presence of 1mM Na_2EDTA the suspension became increasingly viscous, even though the medium (30mM Tris-HCl, pH 8.0, containing 20% sucrose) was hypertonic. This viscosity was attributed to the release of DNA from the lysed cells since the suspension became less viscous if Mg^{2+} and DNAse were added to it. If EDTA was omitted from the digest the premature lysis was avoided. At the same time lysozyme alone was sufficient to bring about spheroplast formation. In this way E. coli NRC 482 behaved more like a Gram-positive organism than a Gram-negative one. EDTA has been used to solubilize membrane-bound ATPases in a number of bacteria (40, 70). Its omission from the lysozyme digest of E. coli was fortunate in that the chelating agent could not then be responsible for premature release of the enzyme from the membrane. For the same reason attempts were made to lyse the spheroplasts in the presence of $MgCl_2$. At concentrations of $MgCl_2$ between 1mM and 5mM lysis did not occur. However in 0.2mM $MgCl_2$ lysis was able to proceed, though

at a slower rate than in the absence of $MgCl_2$.

The ability of the cells to lyse in a hypotonic medium was taken to be a measure of spheroplast formation. This osmotic fragility was demonstrated by the fall in turbidity at 420 nm after a ten-fold dilution of the cell digest as described in METHODS AND MATERIALS. That the fall in turbidity during lysis measured the extent of cell breakage is shown in Fig. 15 where the release of the cytoplasmic enzymes catalase and NADH-menadione reductase paralleled the fall in absorbance of the system. When the turbidity was at a constant, minimum value, cell survival was only 0.04% of that measured before lysozyme digestion.

Penicillin-induced spheroplast formation

A second method for the preparation of spheroplasts is described in METHODS AND MATERIALS. In this method cell wall synthesis was disrupted by the addition of penicillin to an exponentially growing culture of E. coli. The onset of osmotic fragility occurred 20 min after the addition of penicillin (Fig. 16).

Characterization of spheroplast membranes

Samples of lysozyme-induced spheroplast membranes were prepared for electron microscopy as described in METHODS AND MATERIALS. Some of the unfragmented spheroplast membranes were of a comparable size to the bacteria (appr. 2 μ long). These

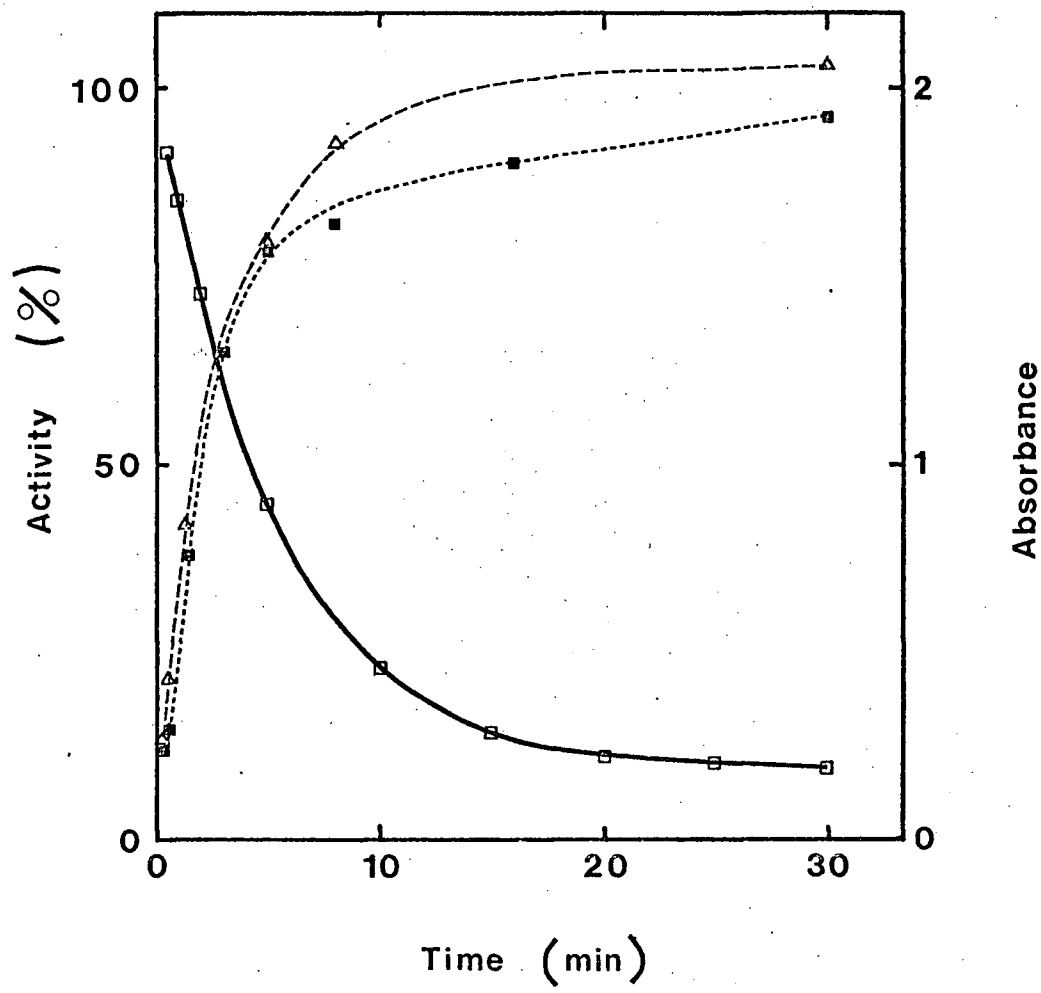


Fig. 15. Progress of osmotic lysis of E. coli spheroplasts.

Fig. 15 shows the decrease in absorbance at 420 nm and the release of soluble enzymes during lysis of E. coli lysozyme-induced spheroplasts. The preparation and lysis of spheroplasts is described in METHODS AND MATERIALS. Time was measured from the moment of dilution in 0.2mM $MgCl_2$. Absorbance (—□—) was measured at 420 nm. Activity of catalase (--△--) and NADH-K_3 reductase (····■····) are both expressed relative to the activity of a sample of the lysate which had been sonicated.

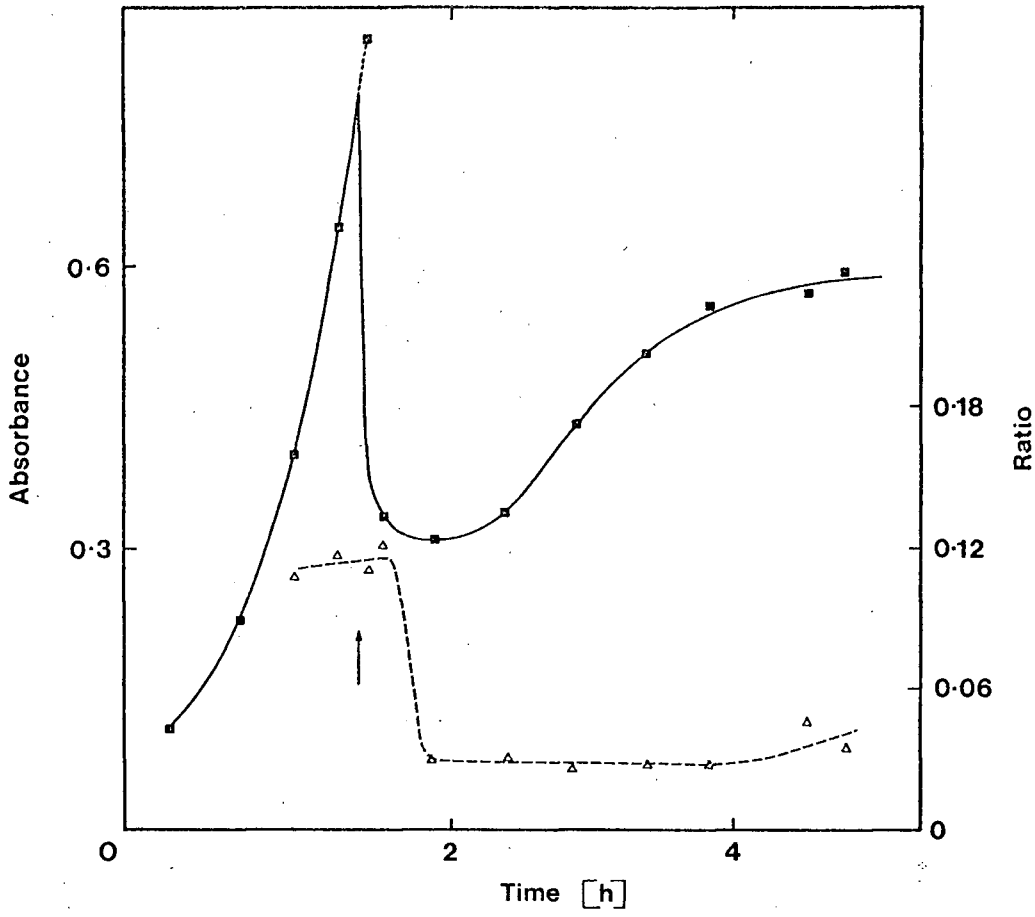


Fig. 16. Growth of penicillin-induced spheroplasts.

The growth of E. coli NRC 482 was followed before and after the addition of penicillin (↑) by measuring the absorbance of the culture at 600 nm (—■—). The osmotic fragility of the cells was monitored by diluting a sample of the culture 10-fold with water and measuring its absorbance before and after dilution. The ratio (---△---) of the absorbances of the samples (diluted/undiluted) are plotted on the same time scale as the growth curve.

membrane "ghosts" (Plate 1) are typical of membranes as they appear after negative staining. For example they look similar to the mitochondrial membranes on page 181 of reference 188.

Most of the unfragmented spheroplast membranes were smaller in size and appeared as semi-sealed vesicles. The inner surfaces seemed to be covered with spherical particles. These were most noticeable at the edges of the vesicles (Plate 2). After sonication of the membranes the vesicles were more fragmented and in several places the spherical particles could be detected on the outer surface of the fragments.

In conjunction with electron microscopy the effects of sonication on the enzyme activities of spheroplast membranes were also studied. Lysozyme-induced spheroplast membranes from 0.45 g of cells were prepared as described in METHODS AND MATERIALS. The membranes were washed by resuspension in 25 ml of 5mM Tris-HCl (pH 7.4) containing 0.2mM $MgCl_2$, and were recovered by centrifugation at 17,600 x g for 30 min at 2°- 5°. The membrane pellet was resuspended in 3.5 ml of 5mM Tris-HCl (pH 7.4). After sampling this suspension it was sonicated at 110 watts using a model W185C sonic oscillator (Heat systems - Ultrasonics Inc.). Sonication was stopped at time intervals (Fig. 17). During each pause in the sonication samples were removed and the sonicate was allowed to cool in ice for 60 seconds.

Each of the samples was assayed for ATPase, NADH-oxidase, NADH-K_3 reductase and NADPH-K_3 reductase (Fig. 17). The initial 15 second period of sonication increased all the enzyme activities

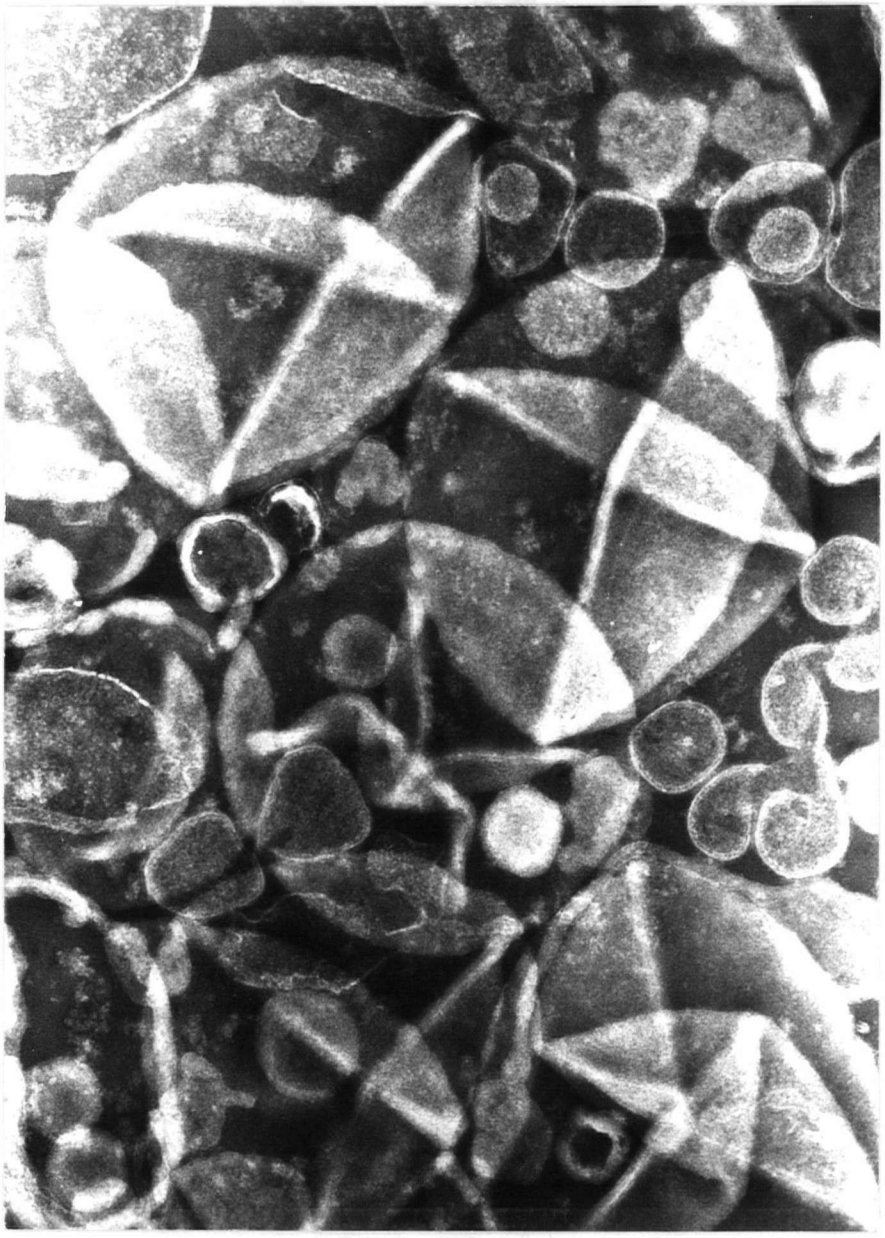


Plate 1. Spheroplast membrane "ghosts".

Lysozyme-induced spheroplasts were osmotically lysed and prepared for electron microscopy as described in METHODS AND MATERIALS. Plate 1 at a magnification of 35,000 X shows the larger membrane fragments or "ghosts".

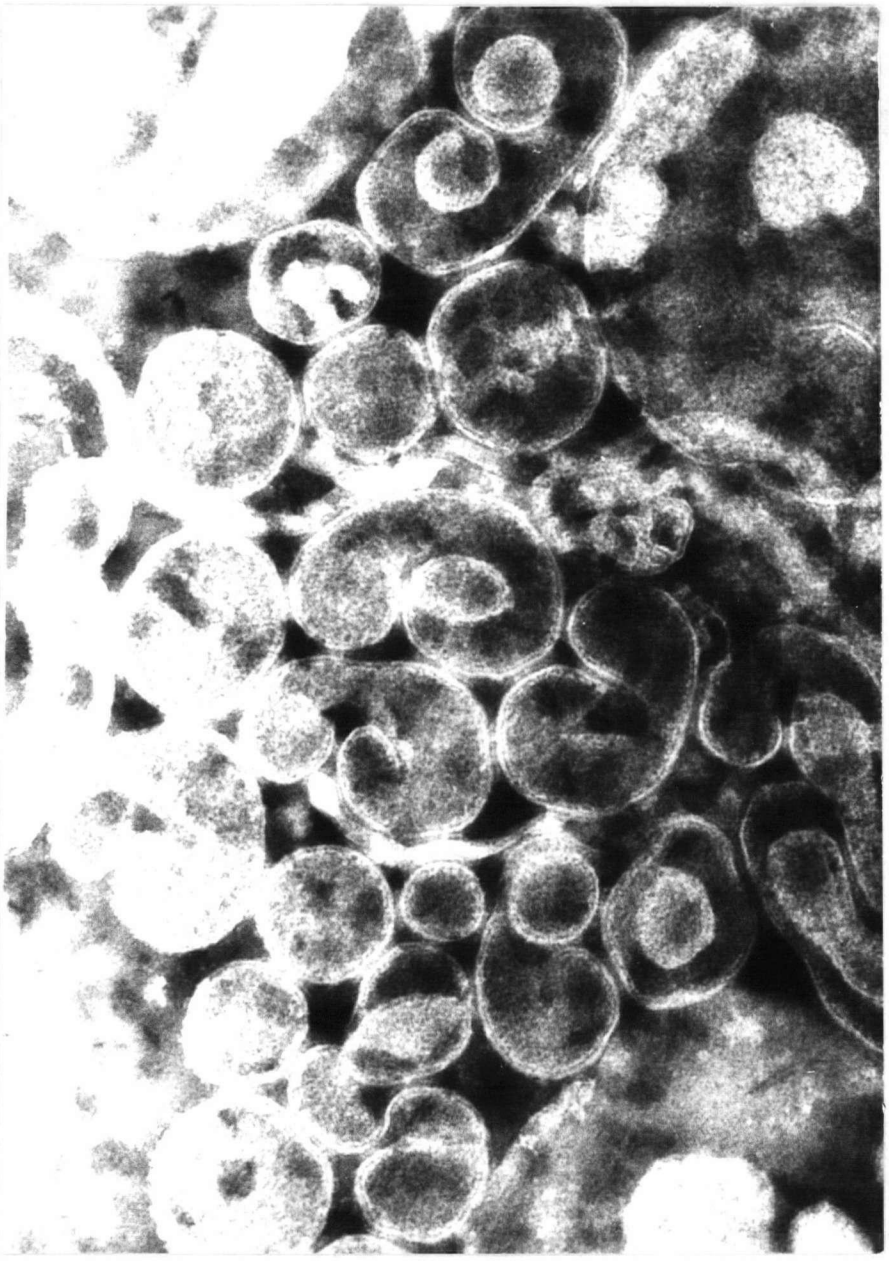
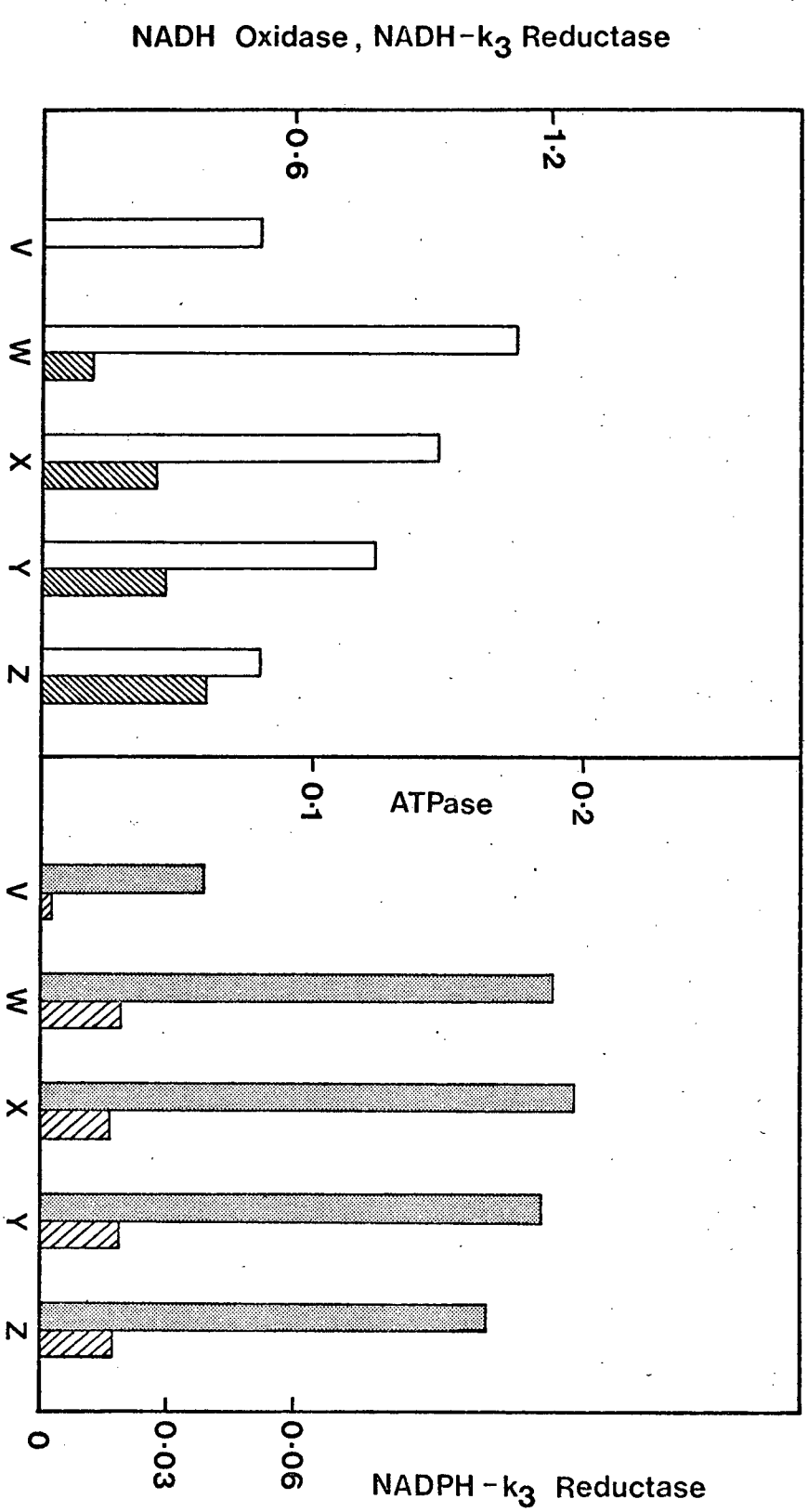


Plate 2. Spheroplast membrane vesicles.

Lysozyme-induced spheroplasts were osmotically lysed and prepared for electron microscopy as described in METHODS AND MATERIALS. Plate 2 at a magnification of 70,000 X shows the smaller membrane fragments or vesicles. The inner surfaces of the vesicles are covered with spherical particles.

Fig. 17. The effect of sonication on enzyme activities in spheroplast membranes.

The times at which samples were removed from the sonicate for analysis are designated V, W, X, Y and Z. These letters correspond to 0, 15, 45, 105 and 240 seconds respectively. Each sample was assayed for NADH oxidase (□), NADH-K_3 reductase (▨), NADPH-K_3 reductase (▧), and ATPase (▩)as described in METHODS AND MATERIALS. The oxidase and reductase activities are expressed in units/ml of sonicate. The ATPase activities are expressed in units/mg of protein.


NADH Oxidase, NADH-k3 Reductase
0·6
1·2
V
W
X
Y
Z
0·1
ATPase
0·2
V
W
X
Y
Z
0
0·03
0·06
NADPH-k3 Reductase

by 2- to 4-fold. Further sonication did not change the levels of the ATPase or NADPH-K_3 reductase activities. However the NADH-oxidase activity declined over the 4 min period while the NADH-K_3 reductase activity increased. It would appear that the NADH-oxidase activity was labile to the conditions of sonication.

Over all it would seem that the vesicles were at least partially sealed prior to sonication, with the original inner side of the membrane innermost. Sonication unsealed the vesicles and perhaps inverted some or all of them such that the membrane-bound enzymes became more exposed.

Distribution of enzymes in lysed spheroplasts

Lysozyme-induced spheroplasts were prepared from 1.02 g of freshly grown E. coli NRC 482 and lysed in the normal manner. The lysate was fractionated into supernatant and membrane fractions by centrifugation at 14,600 x g for 30 min at 2°- 5°. The sedimented membranes were resuspended in 5 ml of 100mM Na-glycylglycine (pH 8.0). The ATPase, ADPase and AMPase activities of the membrane suspension and the supernatant fraction were measured and compared (Expt. #1, Table XIII). In a second experiment (Expt. #2, Table XIII). 0.94 g of cells were treated in the same manner as above with the exception that they were osmotically shocked prior to digestion with lysozyme. The distribution of ATPase, ADPase and AMPase between the spheroplast membranes and the soluble fraction were measured in each experiment (Table XIII). In both experiments most of the ATPase activity occurred in the soluble fraction. It was

Table XIII. Distribution of enzyme activities between spheroplast membrane and supernatant fractions.

Spheroplast membranes and supernatant fraction were prepared from E. coli NRC 482 (1 g) by using lysozyme as described in METHODS AND MATERIALS. The spheroplast membranes were resuspended in 5 ml of 0.1M glycylglycine buffer (pH 8.0). The two fractions were assayed for ATPase, ADPase and AMPase activities as described in METHODS AND MATERIALS. The cells used in experiment 2 were "osmotically shocked" prior to spheroplast formation.

Expt.	Fraction	ATPase		ADPase		AMPase	
		Units	% of total	Units	% of total	Units	% of total
1	Membrane	1.01	9%	0.229	4%	0.014	11%
1	Supernatant	8.29	91%	5.65	96%	0.11	89%
2	Membrane	1.85	21%	0.55	15%	0.022	100%
2	Supernatant	7.08	79%	3.23	85%	0	0%

not clear at this time whether the soluble ATPase was the same as the membrane-bound ATPase and had been released from the membrane despite the gentle method used to break the cells, or if the soluble ATPase activity was due to another enzyme. The same was true of the ADPase activity. The AMPase activity in both fractions was extremely low in comparison with the ATPase and ADPase activities. The level of AMPase activity was even lower in those cells which had been put through the osmotic shock procedure prior to spheroplast formation.

Each of the samples in Table XIII was stored at 0° and re-assayed at intervals. At the end of the storage period a portion of each sample was heated to 55° for 10 min and then cooled in ice before being assayed in the same manner as the other samples. The collected data on the lability of the ATPase, ADPase and AMPase activities are presented in Table XIV. That the AMPase activity was lower in the cells which had been put through the osmotic shock procedure prior to spheroplast formation was confirmed after heat treatment of the extracts. The membrane and supernatant fractions in Expt. 1, Table XIV showed far more AMPase activity after heat treatment than did the corresponding fractions in Expt. 2. Clearly the AMPase activity of both membrane and supernatant fractions was due to 5'-nucleotidase and was largely masked by the heat-labile inhibitor. Furthermore the osmotic shock treatment in Expt. 2 was successful in removing much of the 5'-nucleotidase.

Both the ATPase and ADPase activities declined on storage

Table XIV. Loss of enzyme activity in spheroplast membrane and supernatant fractions on storage at 0°.

The membrane and supernatant fractions from E. coli NRC 482 were prepared as described in the legend to Table XIII. These fractions were reassayed at time intervals during storage at 0°. In experiments 1 and 2, samples that had been stored for 12 days and 9 days respectively were treated by being incubated for 10 min at 55° followed by cooling in ice. These samples were then assayed for the three enzyme activities in the usual manner. Enzyme activity is expressed as nmoles/min/mg protein.

Expt.	Fraction	Storage time (days)	Pre-heated	ATPase	ADPase	AMPase
1	Membrane	0	-	101	22.9	1.4
1	"	3	-	78.8	25.7	1.8
1	"	12	-	25.9	16.0	0.8
1	"	12	+	2.5	16.2	5.1
1	Supernatant	0	-	82.9	56.5	1.1
1	"	3	-	60.5	56.1	3.2
1	"	12	-	13.2	23.6	2.3
1	"	12	+	8.4	41.1	26.4
2	Membrane	0	-	74	21.9	0.9
2	"	4	-	46.2	12.7	0
2	"	9	-	29.5	13.0	0
2	"	9	+	11.0	13.7	1.6
2	Supernatant	0	-	117	44	0
2	"	4	-	19.4	17.4	0
2	"	9	-	15.8	20.0	0
2	"	9	+	6.0	16.8	1.0

at 0°. This was particularly true of the ATPase activity. However the rate of loss of activity was not sufficient to preclude study of these enzymes. Although the ATPase was inactivated by the heat treatment, at least part of the ADPase activity was not. This heat-stable ADPase could not be due to 5'-nucleotidase since the corresponding ATPase and AMPase activities were too low to fit the ratio observed with the whole cells in Table VII.

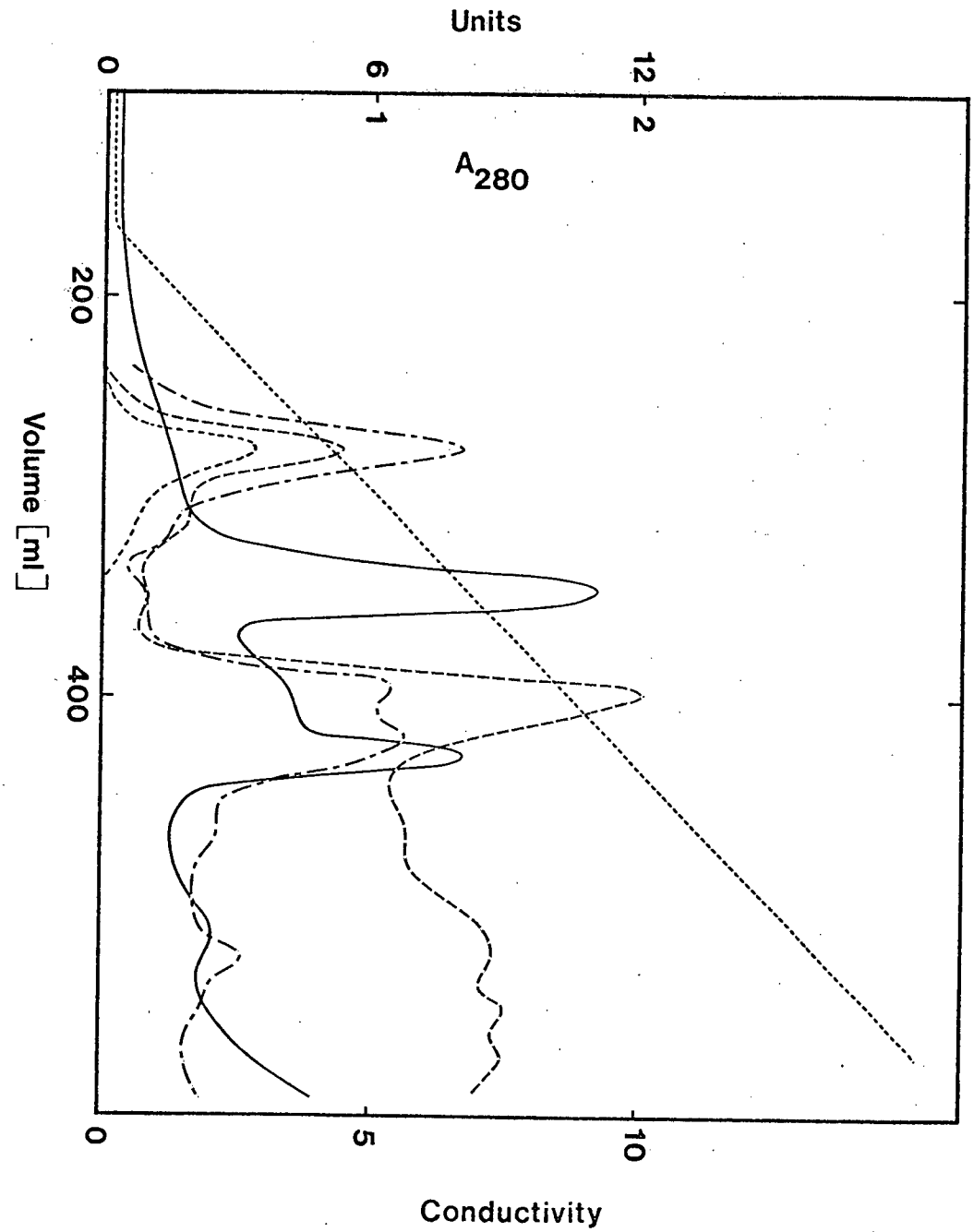
Chromatography of the supernatant fraction from lysed spheroplasts

A chromatographic separation of the supernatant fraction of lysed spheroplasts was attempted to see if the ATPase activity was due to more than one enzyme, and to see if it was related to the ADPase activity (Fig. 18). The first fractions containing ATPase activity which were eluted from DEAE-cellulose by the gradient also showed ADPase and AMPase activity. These fractions hydrolyzed ATP faster than ADP and ADP faster than AMP. The AMPase activity was increased when assayed at pH 6.0 in the presence of Co^{2+} according to the method for 5'-nucleotidase (Fig. 19). The elution profiles of all four activities were identical indicating that the enzyme was indeed 5'-nucleotidase.

A second peak of ATPase activity was eluted at a higher NaCl concentration. This peak was broader than the first one and there was a suggestion that more than one component was present. The fractions through the ATPase peak also showed ADPase activity which was twice as active as the ATPase. The fractions following the second ATPase peak contained varying amounts of these two

Fig. 18. Chromatography of supernatant from lysed E. coli on DEAE-cellulose.

The supernatant (563 ml) from 1.6 g of lysed cells was prepared as described in the text. The supernatant was passed through a column of DEAE-cellulose (25 cm x 5 sq. cm) which had been equilibrated earlier with 5mM Tris-HCl (pH 7.4). The sample was washed into the column with 170 ml of the same buffer and then eluted with a linear gradient of increasing NaCl concentration ranging from 0 to 0.5M. The gradient was made up in 5mM Tris-HCl (pH 7.4) and had a total volume of 500 ml. Fractions (4 ml) were collected and assayed for AMPase (------), ADPase (———), ATPase (—·—·—), A_{280} (———) and conductivity (·····). The entire procedure was carried out at 4°. Units of enzyme activity are expressed in nmoles/min/ml fraction.


Units
0
6
12
1
2
A_{280}
200
400
Volume [ml]
0
5
10
Conductivity

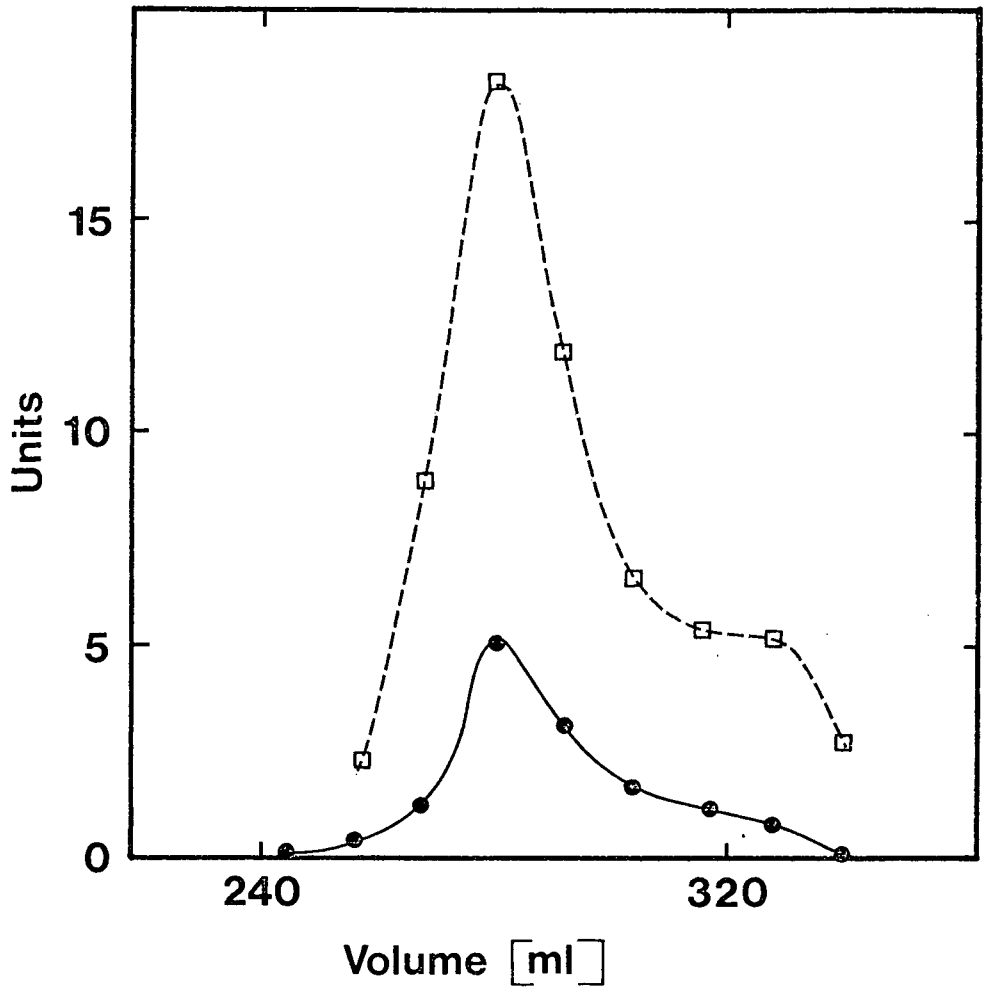


Fig. 19. Coincidence of 5'-nucleotidase and AMPase activities after DEAE-cellulose chromatography.

Fractions from the chromatography on DEAE-cellulose of supernatant from lysed cells of E. coli NRC 482 (Fig. 18) which had AMPase activity were re-assayed for Co^{2+}-stimulated 5'-nucleotidase activity. AMPase activity (—●—) was measured at pH 8.0 in the presence of 5mM $MgCl_2$. Co^{2+}-stimulated 5'-nucleotidase activity was measured at pH 6.0 in the presence of 5mM $CoCl_2$ (--□--). The units of enzyme activity are expressed in nmoles/min/ml fraction.

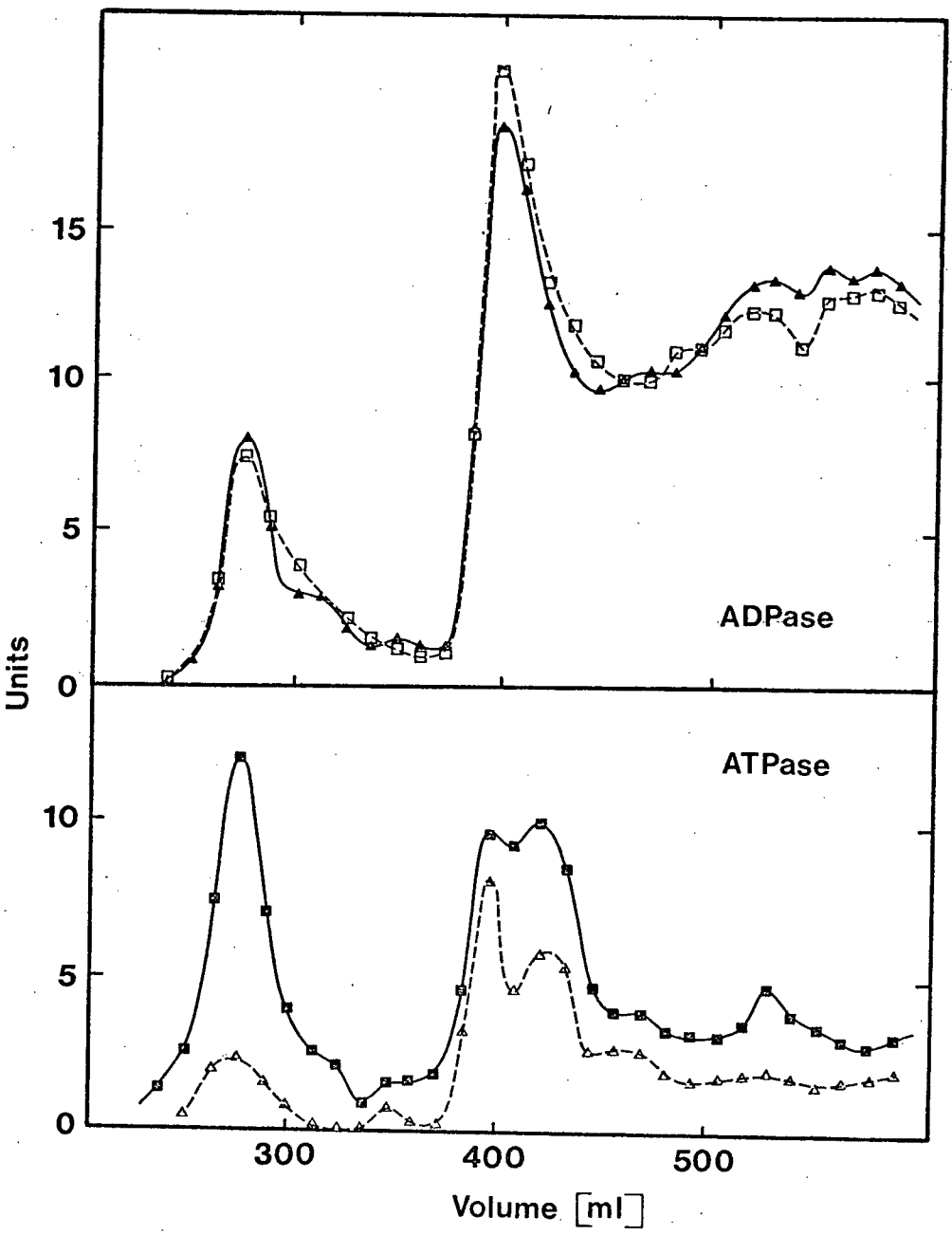
activities with ADPase always exceeding the ATPase by a factor of at least 2 to 1. This region of the gradient is where small membrane fragments would be expected to elute (65). Since the spheroplast membranes were sedimented by a low speed centrifugation (14,600 x g), it is probable that the supernatant fraction contained some membrane fragments and that the ATPase and ADPase activities in the latter fractions from the DEAE-cellulose column (Fig. 18) were in fact membrane-bound.

There was no detectable AMPase activity under the second peak of ATPase activity nor in the subsequent fractions. The ADPase activity in this region of the elution profile was stable and did not lose any activity after storage of the fractions for eleven days at 4°. However under the same circumstances the ATPase activity declined markedly (Fig. 20). The stability of these activities is in line with the results shown in Table XIV.

In view of the interference from membrane fragments it was not possible to say on the basis of this chromatographic separation if there was more than one ATPase present, and whether or not it was associated with ADPase activity. In an effort to circumvent this problem the supernatant from lysed spheroplasts was first fractionated by gel filtration on Sepharose 6B. A Sepharose 6B column (38 cm x 2.5 cm) was prepared and calibrated for molecular weight determinations as described in METHODS AND MATERIALS. The calibration curve of log molecular weight against K_{av} (Fig. 21) includes a duplicate determination for yeast alcohol dehydrogenase and two values for bovine catalase. The value for

Fig. 20. Lability of ADPase and ATPase activities after chromatography on DEAE-cellulose.

Fractions prepared by DEAE-cellulose chromatography of supernatant from lysed cells of E. coli NRC 482 (Fig. 18) were reassayed for ATPase (△) and ADPase (□) activities after 10 days and 8 days respectively at 4°. These activities are compared to the original ATPase (■) and ADPase (▲) activities. The enzyme assays were done as described in METHODS AND MATERIALS. The units of enzyme activity are expressed in nmoles/min/ml fraction.


15
10
5
0
Units
ADPase
ATPase
10
5
0
300
400
500
Volume [ml]

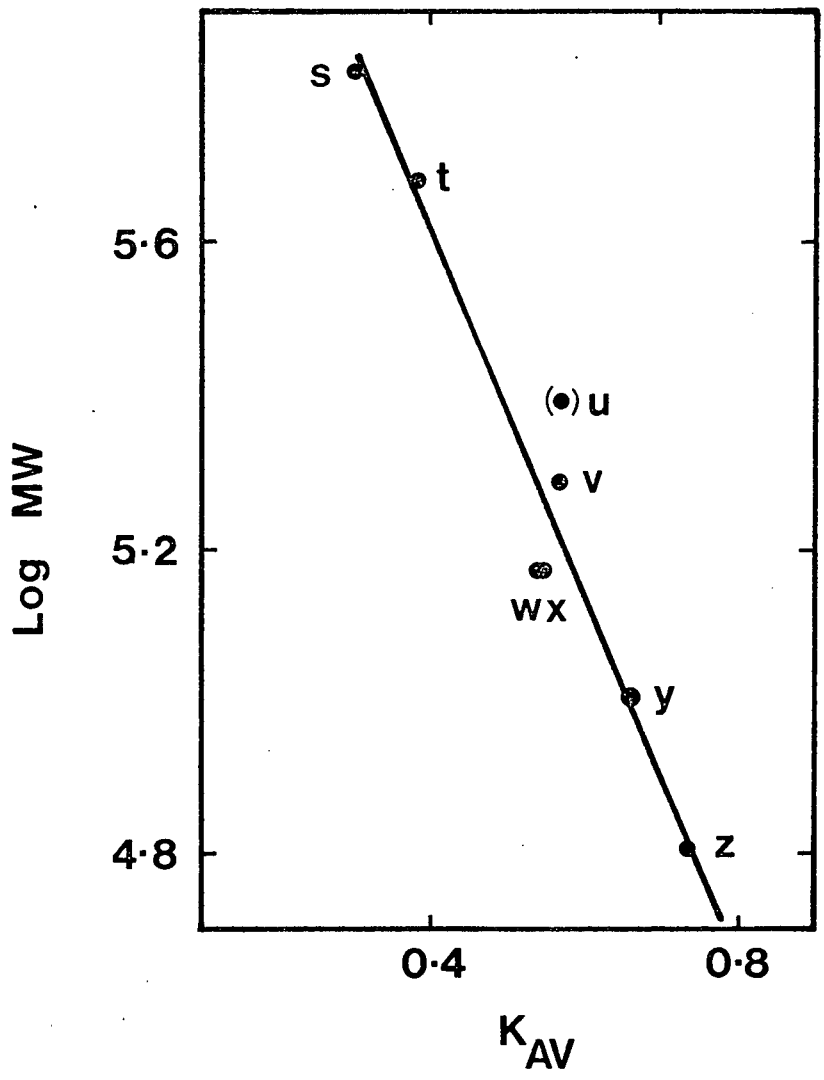


Fig. 21. Calibration curve for determining molecular weights on Sepharose 6B.

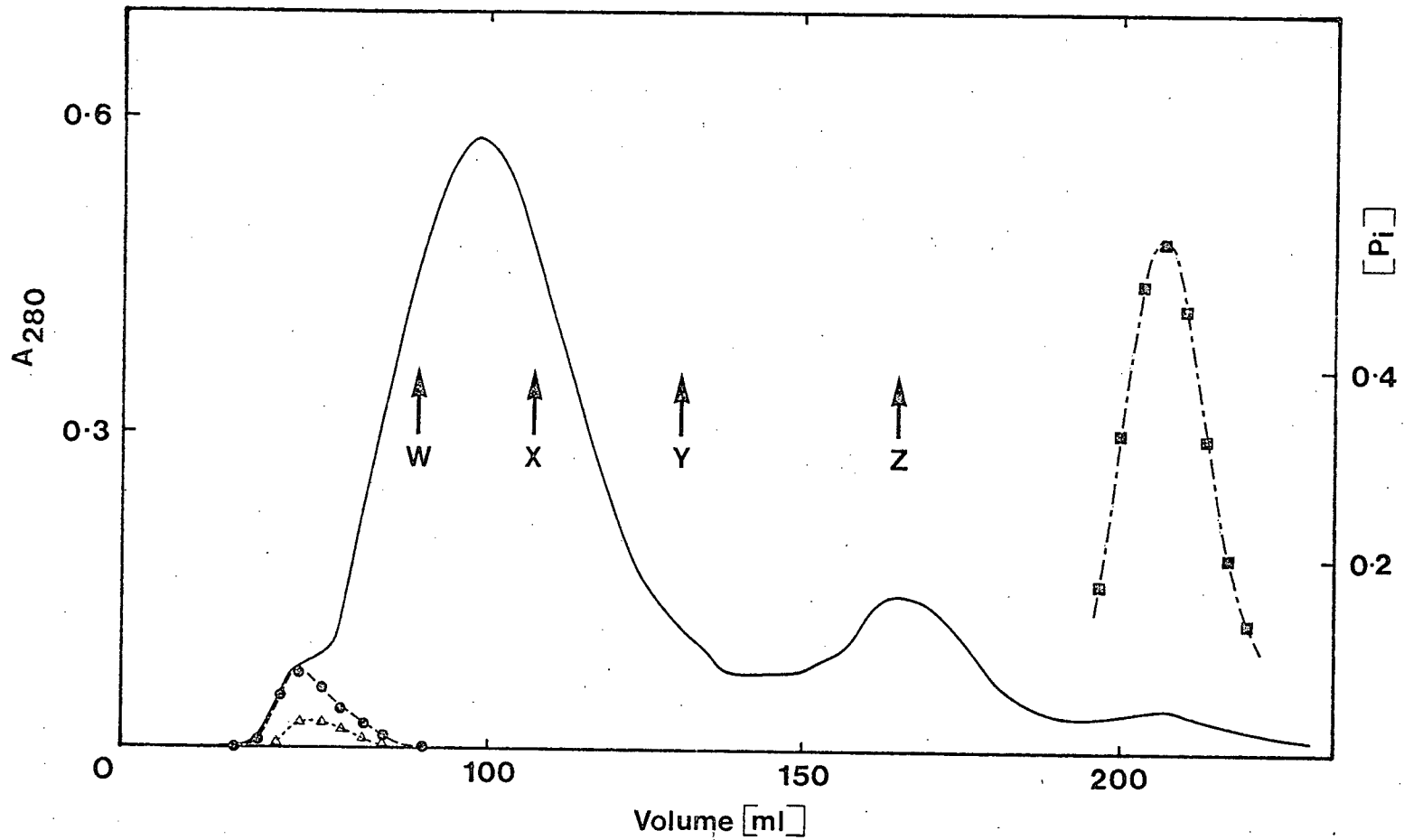
The elution volumes of the protein standards were determined on Sepharose 6B as described in METHODS AND MATERIALS. Points s, t, y, and z correspond to thyroglobulin, apoferritin, hexokinase and hemoglobin respectively. Points w and x are duplicate determinations of alcohol dehydrogenase. Point u corresponds to catalase with a molecular weight of 248,000, whereas its apparent molecular weight of 195,000 (175) would place this marker at v.

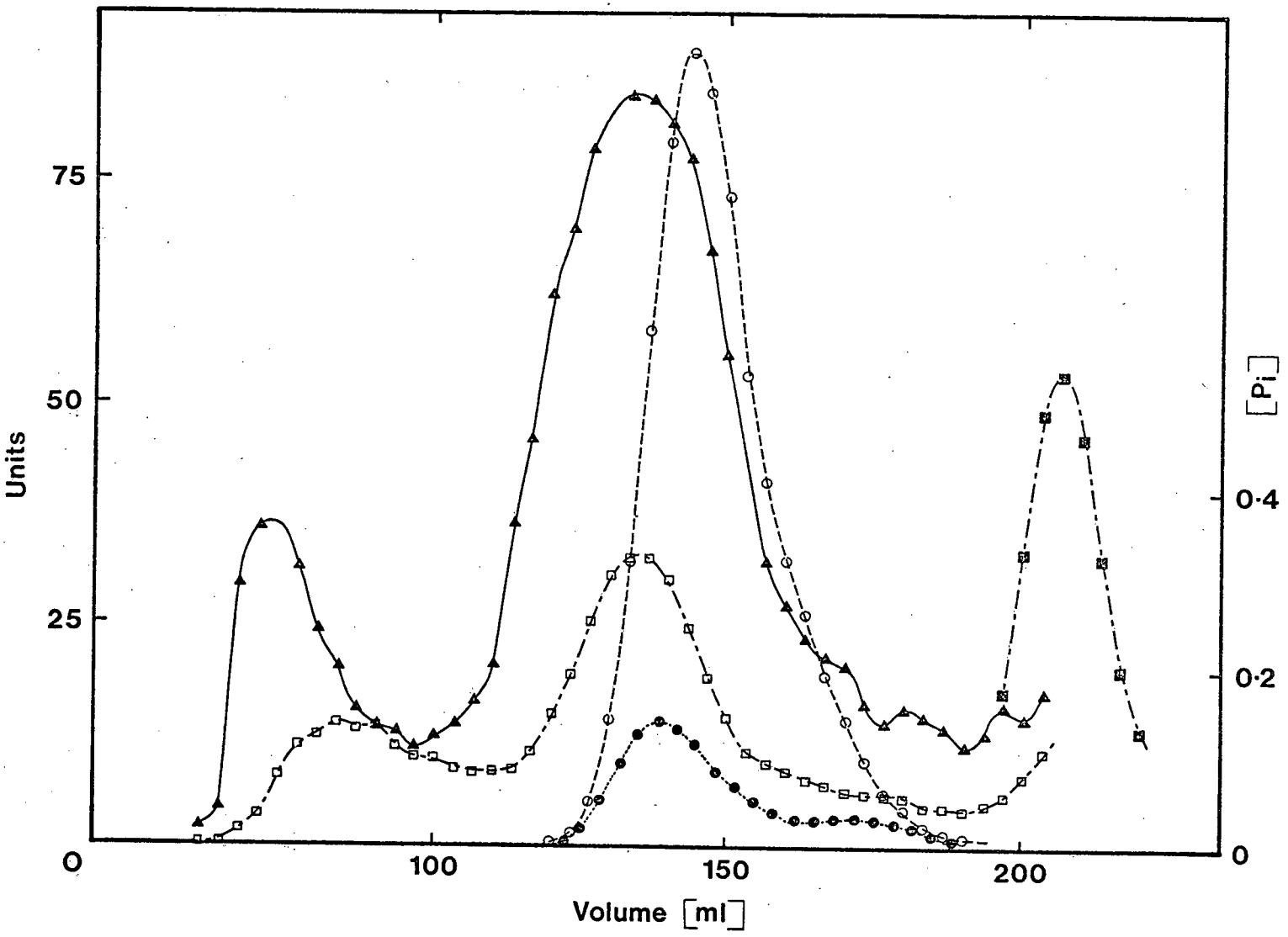
catalase that is farthest from the line corresponds to the actual molecular weight of 248,000, while the other value was calculated on the basis of the observed molecular weight of catalase (195,000) (175).

The supernatant (360 ml) from 1.2 g of lysed cells was concentrated and chromatographed as described in the legends to Figs. 22 and 23. The elution profile of the material that absorbed at 280 nm showed a major peak of optical density in the region where the 30 s and 50 s ribosomal subunits would be expected to chromatograph. This peak had a shoulder which corresponded to the void volume of the column. Under this shoulder there were peaks of NADH oxidase, menadione reductase and ATPase activity as well as some ADPase activity. The fractions in this region were turbid and obviously contained the small membrane fragments. The major peak of ATPase activity occurred in the middle of the elution profile (fractions 36 to 65). The width of this peak, when compared to the peak of phosphate at V_t, suggested that more than one component was present. The mean molecular weight of the ATPase was estimated to be 280,000. ADPase activity occurred in two places in the elution profile. Firstly there was a small peak of ADPase activity immediately following the void volume. Then there was a larger peak of ADPase activity directly under the main ATPase peak. The AMPase activity also occurred in the middle of the elution profile towards the lower molecular weight end of the major ATPase peak (fractions 45 to 65). This AMPase activity corresponded to a molecular weight estimate of $>$ 200,000

Figs. 22 and 23. Gel filtration of the supernatant fraction from E. coli NRC 482.

Lysozyme-induced spheroplasts were prepared from E. coli NRC 482 (1.14 g) in the usual manner. After lysis of the spheroplasts the supernatant fraction was obtained, concentrated and chromatographed on Sepharose 6B as described in METHODS AND MATERIALS. Fractions (1.7 ml) were assayed for Pi (—■—), endogenous catalase, NADH oxidase (--●--), NADH-K_3 reductase (---△---), and their absorbance at 280 nm (——) was recorded (Fig. 22). Assays were also done for ATPase (—▲—), ADPase (—⊟—), and AMPase (----●----) before, and after (--○--) heat treatment (Fig. 23). The heat treatment was performed on individual fractions by incubating them for 10 min at 50° followed by cooling in ice. Since NADH oxidase, NADH-K_3 reductase and catalase were used as markers their activities in the fractions were not quantitated. The units of ATPase, ADPase and AMPase activity are expressed in nmoles/min/ml fraction. Pi content is expressed in μmoles/ml fraction. W and X correspond to the calculated elution volumes of spherical molecules with molecular weights of 1.8×10^6 and 0.85×10^6 respectively. Y and Z correspond to the elution volumes of endogenous catalase and NADH-K_3 reductase respectively.


0·6
0·3
0
A_{280}
W
X
Y
Z
100
150
200
Volume [ml]
0·4
0·2
[Pi]


75
50
25
0
Units
100
150
200
Volume [ml]
0·4
0·2
0
[Pi]

and was therefore unlikely to be due to free 5'-nucleotidase which has a molecular weight of 53,000 (19). This observation was confirmed when samples of the fractions in this region were heated to 50° for 10 min, cooled, and then assayed again for AMPase activity (Fig. 23). Under these conditions the AMPase activity increased 8-fold indicating that the unactivated AMPase corresponded to the 5'-nucleotidase + inhibitor complex. Neu (25) estimated that the 5'-nucleotidase inhibitor had a molecular weight in the upper 60,000s. A 1:1 stoichiometry of 5'-nucleotidase plus inhibitor would give a complex with a molecular weight of around 120,000.

The AMPase activity which was present without heat activation could have been due to incomplete inhibition of 5'-nucleotidase in the complex as observed by Glaser *et al.* (22), or it could have been due to the gradual denaturation of the inhibitor in the collected fractions. The 5'-nucleotidase would also have an ATPase activity which would contribute to the major ATPase peak. This was adequately demonstrated (Fig. 24) by re-assaying the major ATPase peak from the Sepharose 6B column after storing the fractions for 4 days at 4°. Fractions on the lower molecular weight side of the peak showed increased ATPase activity on storage (due to the denaturation of the 5'-nucleotidase inhibitor) while those fractions on the higher molecular weight side of the peak decreased in activity. The net result was a narrowing of the width of the peak and an apparent shift in the peak towards the lower molecular weight end of the profile.

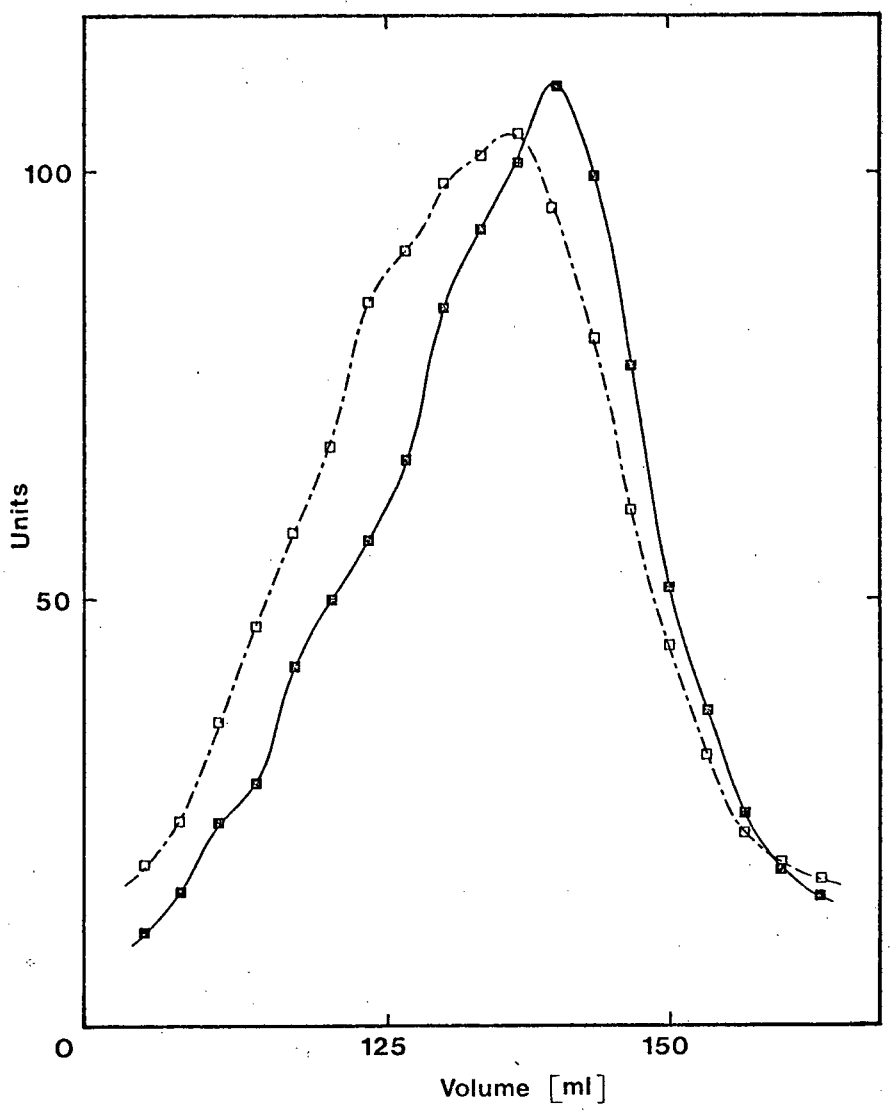


Fig. 24. Reassay of ATPase activity following chromatography on Sepharose 6B.

Fractions from the chromatography on Sepharose 6B (Figs. 22 and 23) of the supernatant fraction from lysed cells were reassayed for ATPase activity after 24 h (--⊟--) and 96 h (—■—) of storage at 4°. The ATPase activity is expressed as nmoles/min/ml fraction.

Two stage chromatography of the supernatant fraction from lysed spheroplasts

Although the largest molecular weight component of the ATPase peak from Sepharose 6B showed some lability on storage, the overall recovery of ATPase activity after gel filtration was far better than after ion exchange chromatography on DEAE-cellulose. The large molecular weight component was clearly a soluble enzyme since it was well separated from the void volume of the Sepharose 6B column. Having removed the membrane fragments from the supernatant in this way the fractions (36 to 65) comprising the major ATPase peak were then chromatographed on DEAE-cellulose to remove 5'-nucleotidase.

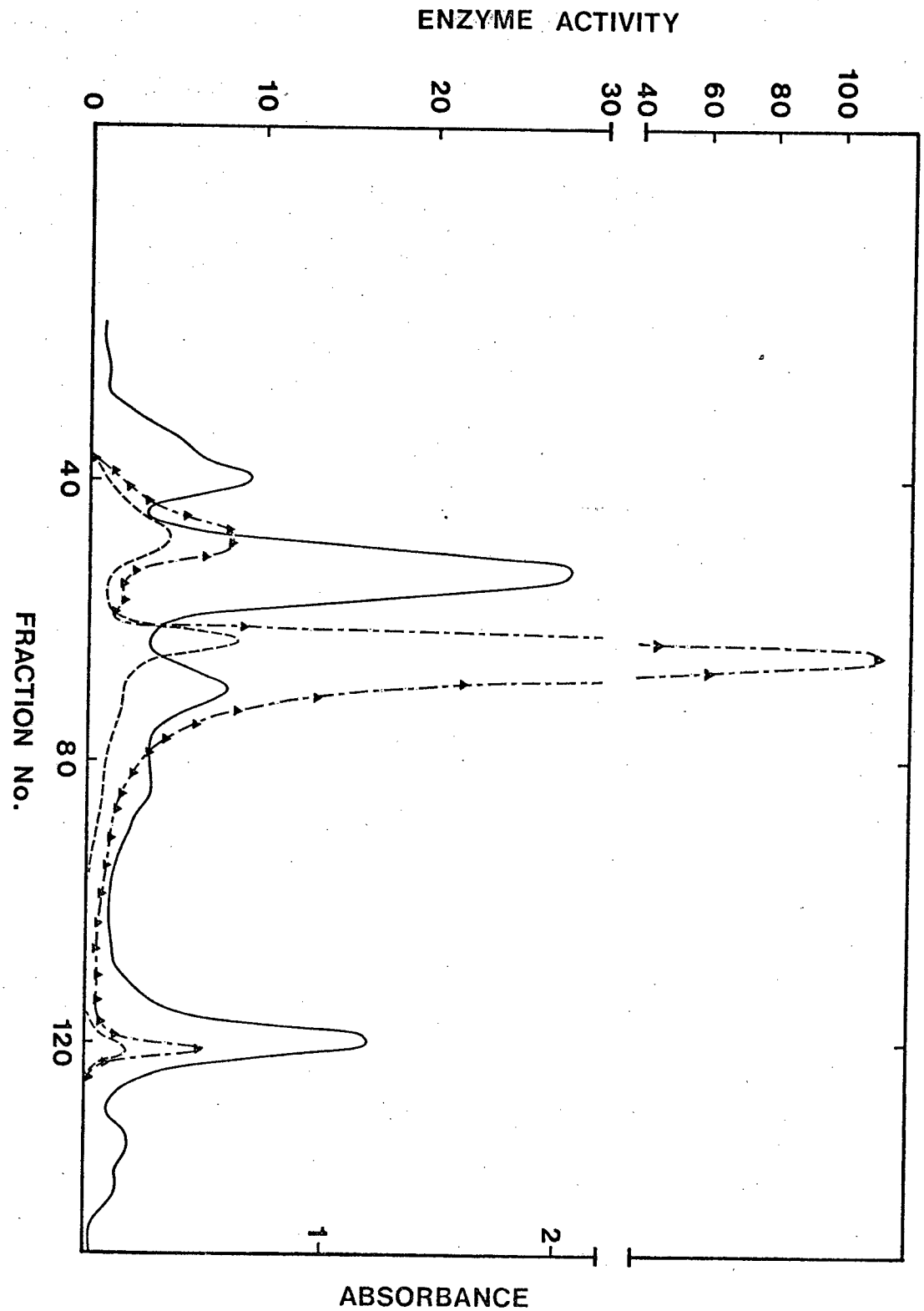
This was accomplished as shown in Figs. 25 and 26. As with the previous DEAE-cellulose chromatography the first peak of ATPase activity eluted by the gradient was due to 5'-nucleotidase. A second peak which also had AMPase, ADPase and ATPase activities was eluted soon after. This was shown by the usual heat treatment (Fig. 26) to be due to the 5'-nucleotidase/inhibitor complex.

The third, and major, peak of ATPase activity was eluted at the same point in the gradient (conductivity 9 mΩ) as the main ATPase peak from the earlier separation done on DEAE-cellulose (Fig. 18). Once again a peak of ADPase activity eluted in the same position as the ATPase, and once again the activity of the latter had decreased markedly. This major peak was very sharp and there was no evidence of there being more than one

Figs. 25 and 26. Chromatography of partially purified soluble ATPase on DEAE-cellulose.

The column was packed with DEAE-cellulose (bed volume 20 cm x 1.2 cm) and washed with 5mM Tris-HCl (pH 7.4) 400 ml. Alternate fractions through the major ATPase peak from Sepharose 6B (Fig. 23) were combined (26 ml) on day 2 of the experiment and diluted by the addition of 39 ml of water to reduced the concentration of Tris-HCl to 20mM. This sample was run into the DEAE-cellulose column followed by 100 ml of 5mM Tris-HCl (pH 7.4). The sample was eluted by a linear gradient of increasing NaCl concentration ranging from 0 to 0.6M NaCl. The gradient was made up in the above buffer and had a total volume of 250 ml. The column was finally eluted with 50 ml of buffer which contained 0.75M NaCl. Fractions (volume 2 ml) were collected and assayed for ATPase (– – –), ADPase (– · – · –), A_{280} (———), and conductivity (·····), (Fig. 25); AMPase (– – –) and heat-activated AMPase (– · ▲ · –) (Fig. 26). Units of enzyme activity are nmoles/min/ml fraction. The entire procedure was done at 4°.

A_{280}
0
1
2
10
20
Conductivity
60
120
Fraction No.
10
20
30
Activity


ENZYME ACTIVITY
0
10
20
30
40
60
80
100
40
80
120
FRACTION No.
1
2
ABSORBANCE

component present with ATPase activity. Also there was no trailing of ATPase and ADPase activities in the later fractions of the gradient since the membrane fragments had been removed earlier. A fourth, and very minor peak of ADPase with ATPase activity was observed at the end of the gradient where the 0.75M NaCl was run into the column.

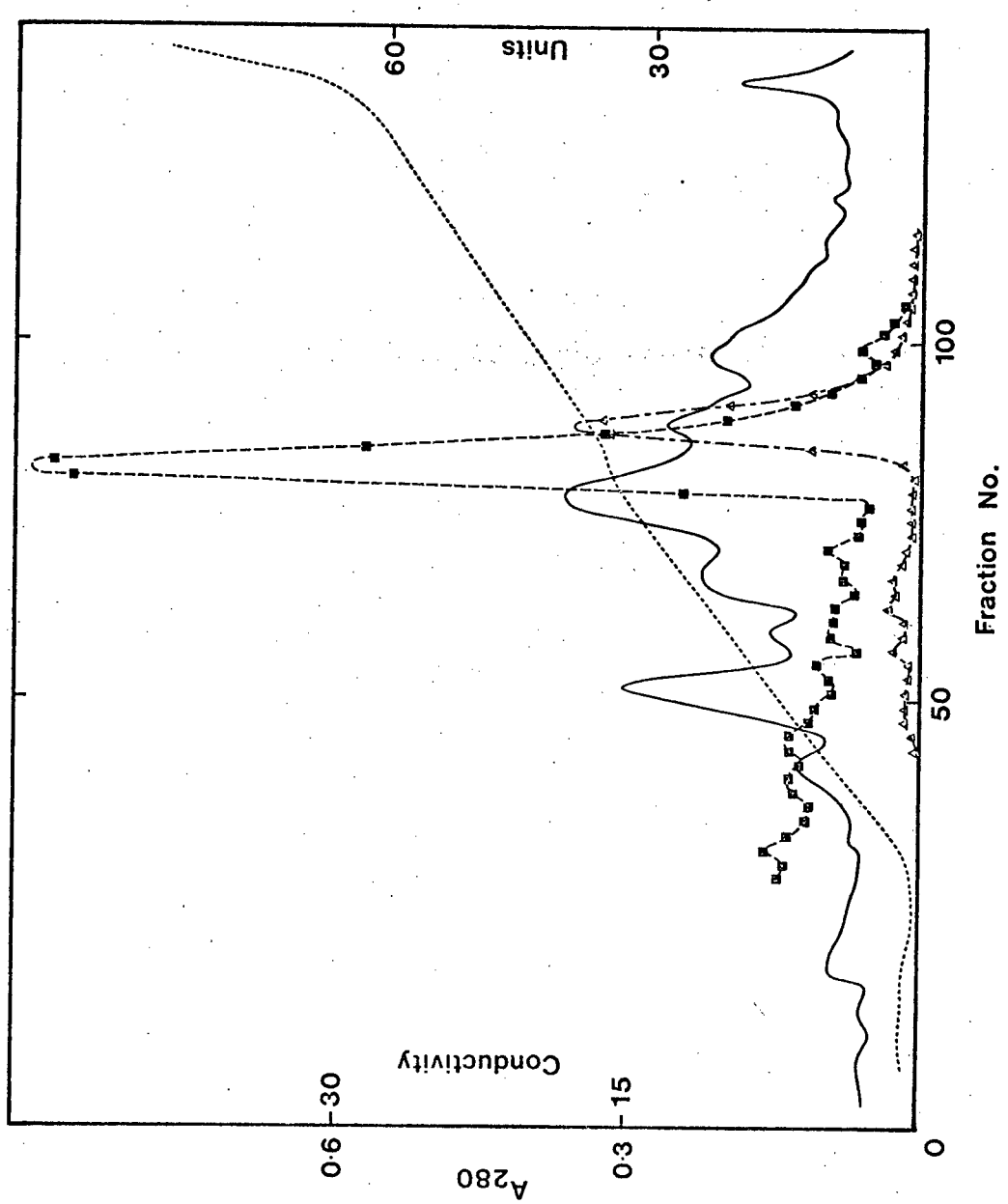
Attention was focussed on the activities in the third peak (fractions 77 - 89) in an effort to identify them. If the ADPase and ATPase activities both resided in one enzyme then it would be logical for the two activities to be catalyzed by the same active site. How then could the ATPase activity have declined while the ADPase activity remained constant? If the two activities resided in separate enzymes then these enzymes had remained together through two chromatographic procedures. When this two-stage chromatography was later repeated at 22°- 24° the ADPase and ATPase activities were not quite coincident. Their peak activities were separated by about 6 fractions. This indicated that the two activities belonged to separate enzymes (Fig. 27).

The effect of adenylate kinase acting in conjunction with ATPase

In the crude cell extracts as prepared for Table XIV it is conceivable that some of the ADPase activity might be due to a combination of adenylate kinase and ATPase. With ADP as the substrate adenylate kinase can catalyze the reaction $2\ ADP \Longleftrightarrow AMP + ATP$. In this way ATP is formed and can then be hydrolyzed by the ATPase to liberate Pi and regenerate ADP. The

Fig. 27. Chromatography of partially purified soluble ATPase on DEAE-cellulose at 22°- 24°.

This chromatographic separation was similar to that described in the legends to Figs. 25 and 26 with the following exceptions. The DEAE-cellulose column was repacked at 22°- 24° and all subsequent steps were done at this temperature. The combined fractions (25 ml) from the previous chromatography on Sepharose 6B (also done at 22°-24°) were diluted to 75 ml with water and then run into the column. The absorbance of the fractions (——) was measured at 280 nm. Fractions were assayed for ATPase (--■--), ADPase (—△--) and conductivity. Enzyme activity was measured in nmoles/min/ml fraction. Conductivity (·····) was measured in mℛ.


A280
0·6
0·3
0
Conductivity
30
15
Units
60
30
50
100
Fraction No.

net reaction being ADP ⟶ AMP + Pi. This process has been observed in mitochondria (106) and has been postulated to occur in E. coli (63).

The adenylate kinase activity in sonic extracts of E. coli NRC 482 was measured to see if it could produce ATP at a rate sufficient to account for some or all of the ADPase activity. Freshly grown cells (1.75 g) were osmotically shocked to release 5'-nucleotidase. The "shocked" cells were resuspended in 15 ml of 10mM Tris-HCl (pH 7.4), containing 10mM $MgCl_2$, and sonicated as described in METHODS AND MATERIALS. The sonicate was centrifuged at 175,000 x g for 2 h at 2°- 5°. The supernatant was removed and assayed for adenylate kinase as described in METHODS AND MATERIALS. A commercial preparation of myokinase (Rabbit muscle) was also assayed by the same method. The reported activity of the commercial preparation was 775 μmoles/min/mg of protein at 37°. This compares favorably with the measured value of 300 μmoles/min/mg of protein at 24°. The supernatant fraction of the sonicate had an activity of 1.98 μmoles/min/mg of protein at 24°. The rate of production of ATP is half of this value, i.e., 1 μmoles/min/mg of protein. In the same sample the ADPase specific activity had an average value of 0.053 μmoles/min/mg of protein at 37°. This specific activity would be somewhat less at 24°. Clearly the adenylate kinase would be able to generate ATP at least 20 times faster than the ADPase could produce Pi, and could in conjunction with the ATPase have been responsible for some of the ADPase activity of crude cell extracts.

This hypothesis cannot explain all of the ADPase activity present in the cell extracts, since in the combined reaction of adenylate kinase + ATPase the ADPase activity can never exceed the ATPase activity. However in some of the stored extracts (Table XIV), due to denaturation the level of ATPase activity fell below that of the ADPase. Similarly the major peak of ADPase activity that appeared after DEAE-cellulose chromatography (Figs. 18 and 25) could not be due to adenylate kinase for the same reason. This ADPase activity was found to be due to polynucleotide phosphorylase.

Polynucleotide phosphorylase

Polynucleotide phosphorylase catalyzes a number of related reactions including the synthesis of polyribonucleotides from nucleoside-5'-diphosphates with the elimination of Pi (189). This reaction takes place readily at pH 8 and shows a requirement for Mg^{2+}. Both these conditions occur in the ADPase assay. Since polynucleotide phosphorylase hydrolyzes UDP at a faster rate than ADP, the fractions (77 - 89) comprising the major ADPase peak from the DEAE-cellulose separation (Fig. 25) were re-assayed using UDP as the substrate in place of ADP. Each fraction was more active with UDP than ADP, while at the same time the UDPase and ADPase peaks were superimposable (Fig. 28). These fractions (77 to 89 inclusive) were combined (21 ml) and re-chromatographed on Sepharose 6B as described in the legend to Fig. 29. The peak of ADPase activity was eluted just after the peak of apoferritin and

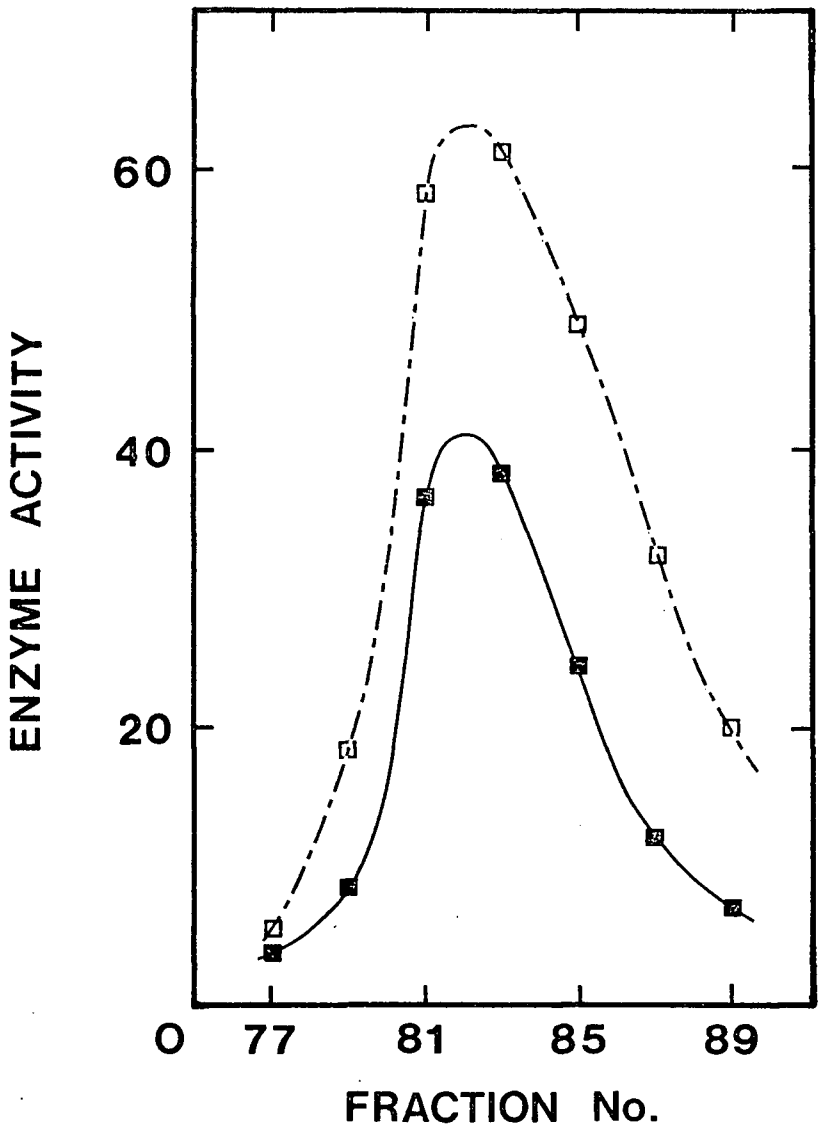


Fig. 28. Coincidence of UDPase and ADPase activities after DEAE-cellulose chromatography.

Alternate fractions (77 to 89 incl.) from the chromatography of partially purified soluble ATPase on DEAE-cellulose (Fig. 25) were assayed for the hydrolysis of UDP (UDPase). The UDPase assay was done in a similar way to the ADPase assay, with 5mM UDP replacing ADP as the substrate. The UDPase activity (–··⊡··–) was compared to the ADPase activity (—■—). Both were expressed in nmoles/min/ml fraction.

Fig. 29. Gel filtration of partially purified soluble ATPase.

Fractions (77 to 89 inclusive) from the chromatography on DEAE-cellulose (Fig. 25) were combined (21 ml) and concentrated to a volume of 1 ml by ultrafiltration using a PM10 ultrafilter in a model 8MC cell (Amicon Corporation). A solution of molecular weight markers was made up by dissolving hemoglobin (3 mg) and apoferritin (15 mg) in 1 ml of 0.9% NaCl. This solution was added to the concentrate along with 0.1 ml of 0.2M potassium phosphate buffer (pH 7.1). The combined mixture was chromatographed by gel filtration on Sepharose 6B at 4° as described in METHODS AND MATERIALS. The eluting buffer was 50mM Tris-HCl (pH 7.4) containing 0.2mM Na_2-EDTA. The absorbance of the fractions at 415 nm (—·—·—) and at 230 nm (——) was measured along with ADPase activity (--●--). Enzyme activity is measured in nmoles/min/ml fraction. The phosphate content (---△---) of the fractions (μmoles/ml fraction) was assayed only in the "total volume" region of the elution profile.

ABSORBANCE
0
0·6
1·2
40
80
120
FRACTION No.
[Pi]
0·4
0·8
0
5
10
15
ENZYME ACTIVITY

corresponded to a molecular weight of 400,000 (Fig. 29). This is twice as large as the molecular weight reported for polynucleotide phosphorylase in E. coli (190). However when the peak fractions were assayed for polynucleotide phosphorylase as described in METHODS AND MATERIALS the profiles were almost superimposable (Fig. 30).

The ATPase activity continued to decrease such that after this third chromatographic procedure there was no detectable hydrolysis of ATP under the peak of ADPase activity, in contrast to the results shown in Fig. 23.

Suggestion of cold-lability

The preliminary work on the soluble ATPase activity of E. coli indicated that the enzyme hydrolyzed ATP specifically among the adenine ribonucleotides, and that it had a large molecular weight in the range 300,000 to 400,000. In these respects the enzyme was similar to the ATPases that were solubilized from protoplast membranes of S. faecalis, B. megaterium and M. lysodeikticus (1-3, 56, 72-74). Since all these enzymes showed some evidence of cold-lability, this property was looked for in the ATPase from E. coli. It became apparent that this ATPase was indeed cold-labile (cf. Figs. 25 and 27 where the separations on DEAE-cellulose were carried out at 4° and 22°- 24° respectively). The cold-lability of the ATPase was not excessive in crude extracts but it increased during the partial purification of the enzyme (cf. Table XIV and Figs. 23, 25 and 30). A similar observation

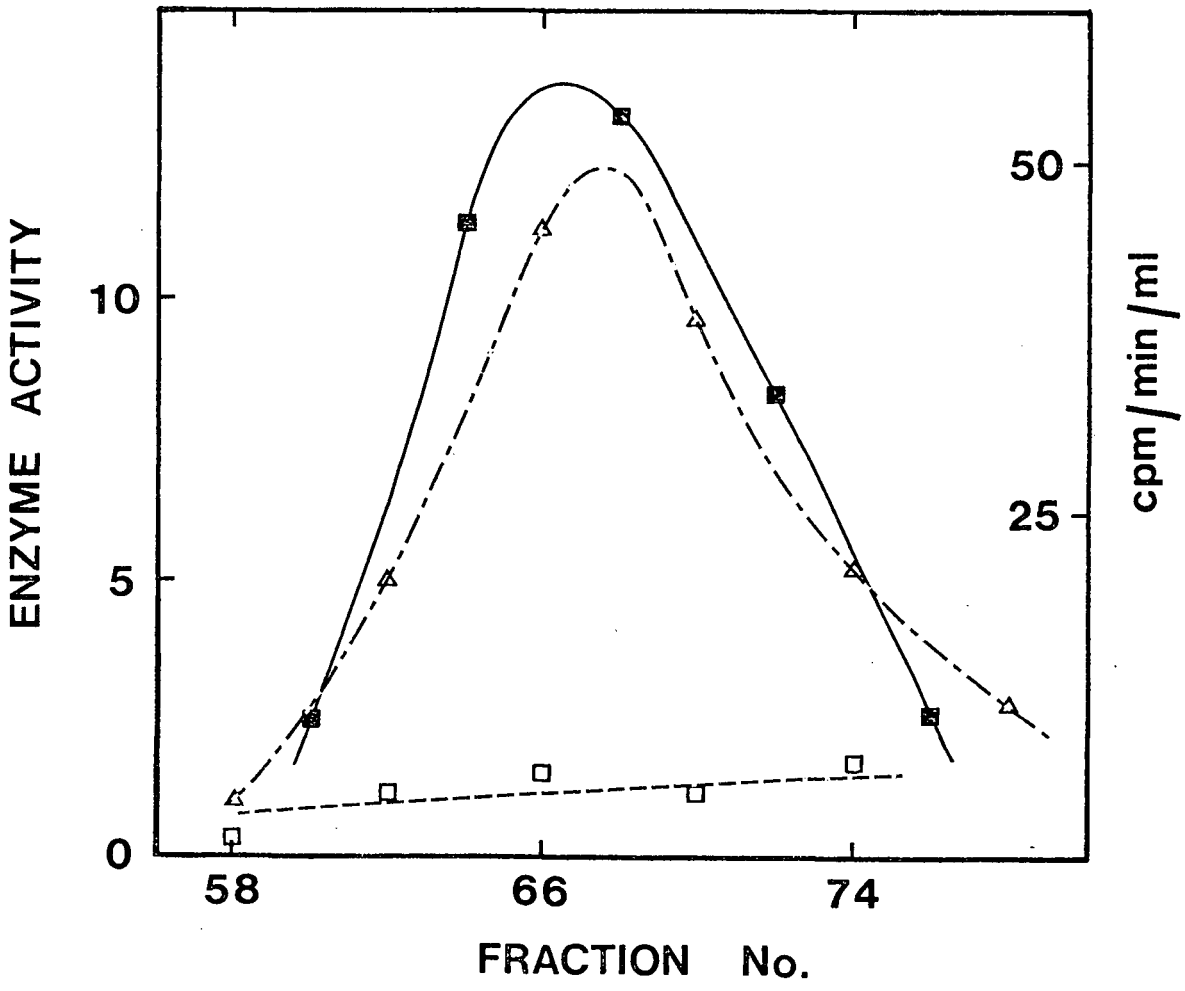


Fig. 30. Coincidence of ADPase and polynucleotidase phosphorylase activities after partial purification of ADPase.

Fractions (between 58 and 78 inclusive) from chromatography on Sepharose 6B (Fig. 29) were assayed for polynucleotide phosphorylase as described in METHODS AND MATERIALS. This activity is expressed in cpm/min/ml fraction (at 37°). The polynucleotide phosphorylase activity (—■—) is compared to the ADPase (—·△·—) and ATPase (—□—) activities expressed as nmoles/min/ml fraction.

has been made for the ATPases of M. lysodeikticus (2) and the mitochondrion (191). For this reason subsequent experimental work was carried out at room temperature.

Membrane-bound ATPase

The membrane-bound ATPase of E. coli has been well characterized by Evans (57). He has also solubilized this ATPase activity with the aid of sodium dodecyl sulphate. The molecular weight of the solubilized ATPase (100,000) (71) was very different from the molecular weights of the solubilized ATPases from other bacterial strains (2, 4, 72, 80). It also differed from the molecular weight estimate of the soluble ATPase from E. coli NRC 482. In order to clarify the situation an attempt was made to solubilize the ATPase from spheroplast membranes of E. coli by a modification of the method of Abrams (26). The solubilization of this enzyme would also enable the membrane-bound ATPase to be compared with the soluble ATPase.

The modified washing procedure was carried out on spheroplast membranes (170 mg protein) from 10 g of cells as described in METHODS AND MATERIALS. The supernatant fractions from each washing step were assayed by the regular ATPase method (pH 8.0, Mg^{2+}) and by the assay method for Ca^{2+}- or Mg^{2+}-activated ATPase. Both methods showed that there was a sudden release of ATPase from the membranes during the 5th washing step (Fig. 31 and Table XV). This coincided with a change in the suspending medium from 50mM Tris-HCl (pH 7.4) to 1mM Tris-HCl (pH 7.4). The release of the

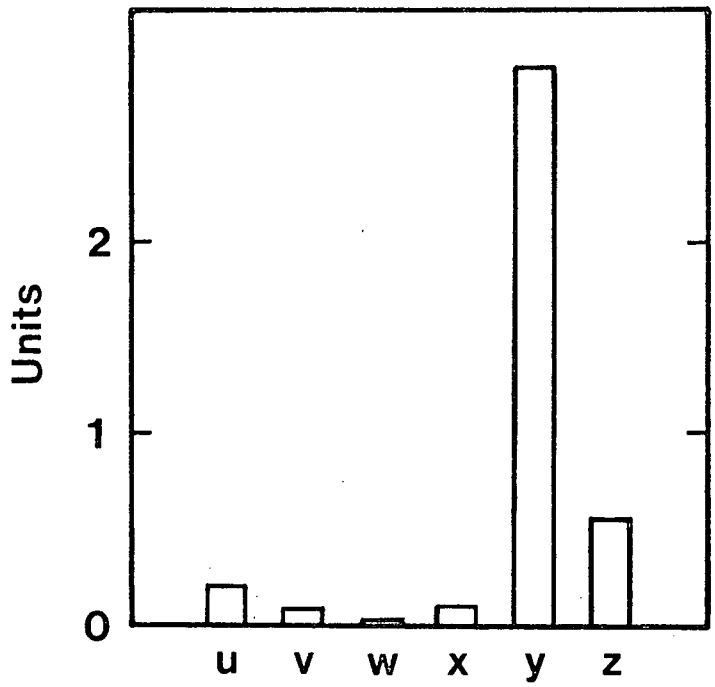


Fig. 31. Release of Ca^{2+}-activated ATPase from spheroplast membranes.

Fig. 31 is a diagrammatic representation of one of the experiments described in Table XV starting with spheroplast membranes (170 mg protein) from E. coli NRC 482 (10 g). Fractions u to z represent the supernatants from the six washing steps. The total units of Ca^{2+}-activated ATPase activity in each of the supernatants is recorded.

Table XV. Distribution of ATPase during washing of E. coli spheroplast membranes.

The experimental procedure is described in METHODS AND MATERIALS. All resuspensions were done to a volume of 250 ml at pH 7.4. ATPase was measured in the presence of 5mM $CaCl_2$ at pH 9.0. These results represent the average of two experiments starting with spheroplast membranes (170 mg protein) from 10 g of freshly grown E. coli NRC 482.

Washing step	Fraction	Total protein (mg)	Total activity (units)	Specific activity x 10^{-2} (units/mg protein)
	Initial membrane suspension in 10mM Tris-HCl - 0.5mM $MgCl_2$	170	17.8	10.5
1	Supernatant from the initial membrane suspension	7.8	1.51	19.4
2	Supernatant from 2nd membrane suspension in 2M LiCl-50mM Tris-HCl	9.4	0.04	0.4
3	Supernatant from 3rd membrane suspension in 2M LiCl-50mM Tris-HCl	6.6	0.02	0.3
4	Supernatant from 4th membrane suspension in 50mM Tris-HCl	6.4	0.14	2.2
5	Supernatant from 5th membrane suspension in 1mM Tris-HCl	11.7	3.36	28.7
6	Supernatant from 6th membrane suspension in 1mM Tris-HCl	7.7	1.12	14.1
	Final membrane suspension in 1mM Tris-HCl	121	11.8	9.8
	Recovery	170.6	18	

ATPase at this point is similar to the results obtained with S. faecalis (26) and M. lysodeikticus (69) membranes. It suggests that the nature of the binding of the ATPase to the membrane is similar in these bacteria. The overall amount of ATPase released by the washing steps varied between 20% and 67% of the total membrane-bound ATPase. The averaged figures for two such experiments are presented in Table XV. Not only did the supernatant from the 5th washing step contain the most units of ATPase but it also had the highest specific activity. Overall however, the yield of solubilized ATPase and its increase in specific activity both fell well below the values obtained with S. faecalis (26).

In the second method of solubilizing the membrane-bound ATPase, spheroplast membranes were dialyzed against 0.5mM EDTA at pH 9.0. This technique, as described in METHODS AND MATERIALS superceded the washing procedure. By this method up to 85% of the membrane-bound ATPase was solubilized with a 10-fold increase in specific activity.

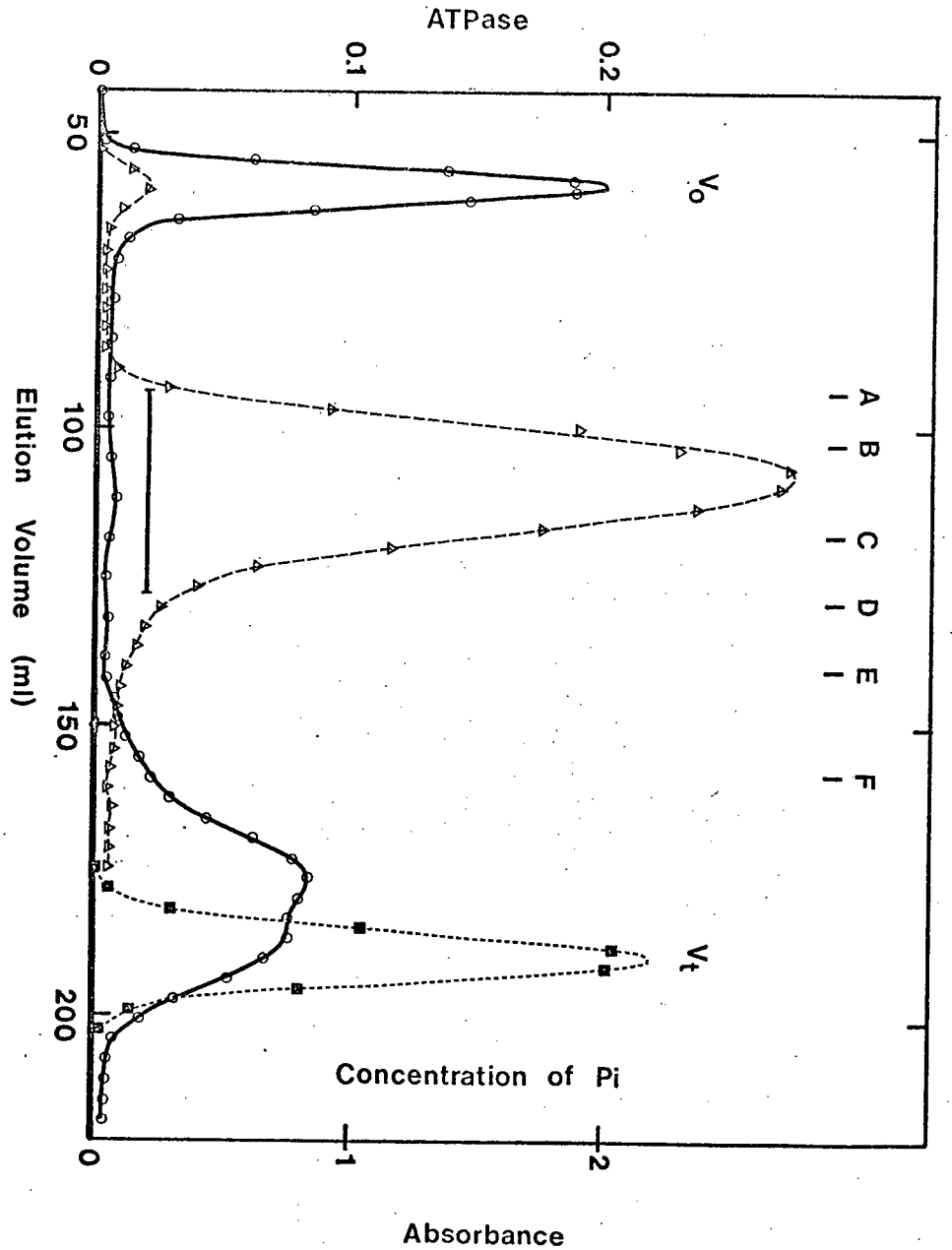
Partial purification of the solubilized ATPase

The supernatant from the 5th washing step (Table XV) was prepared as described earlier. In order to further purify the solubilized ATPase, to prove that it was indeed soluble, and to compare this enzyme with the ATPase of the cytoplasm, it was chromatographed on Sepharose 6B as described in the legend to Fig. 32.

The ATPase activity eluted from the column as a single

Fig. 32. Gel filtration of solubilized ATPase.

Elution pattern from Sepharose 6B column of the supernatant from the 5th washing of E. coli spheroplast membranes. Chromatography was done as described in METHODS AND MATERIALS. Absorbance (—o—) was measured at 280 nm. Ca^{2+}-activated ATPase activity (-△-) is expressed as units/ml fraction, and concentration of phosphate (---■---) as μmoles/ml fraction. Markers A - F indicate the elution positions of thyroglobulin, apoferritin, glucose-6-phosphate dehydrogenase, alcohol dehydrogenase, hexokinase and hemoglobin, respectively. Fractions covered by the bar constitute the pooled enzyme used in later experiments.


ATPase
0
0.1
0.2
50
100
150
200
Elution Volume (ml)
V_o
V_t
A
B
C
D
E
F
Concentration of Pi
0
1
2
Absorbance

peak which was well separated from the two major peaks of material that absorbed at 280 nm. The small amount of ATPase activity that occurred in the turbid, void fractions could have been membrane-bound. Clearly most of the ATPase had been solubilized. Two separate experiments with independently calibrated columns gave molecular weight values of 390,000 and 365,000 for the ATPase, provided that the enzyme is a spherical molecule. The gel filtration step gave a 20-fold purification of the ATPase as well as 100% recovery of activity.

The fractions covered by the bar in Fig. 32 were pooled and used for further experiments. The overall purification of the enzyme is set out in Table XVI.

Influence of pH on the solubilized ATPase activity

The variation of Ca^{2+}- or Mg^{2+}-activated ATPase activity with pH (Fig. 33) was similar to that of the membrane-bound enzyme (57). Optimal activity occurred between pH 8.5 and pH 9.5 in the presence of 5mM divalent cation. For this reason the pH of the ATPase assay was changed from pH 8.0 to pH 9.0 by substituting 100mM Tris-HCl buffer (pH 9.0) for the 100mM glycylglycine-NaOH buffer (pH 8.0).

The pH-activity profile was noticeably sharper when Ca^{2+} was the activating cation.

Table XVI. Partial purification of solubilized ATPase of E. coli.

ATPase activity was measured in the presence of 5mM $CaCl_2$. These results are the average of two experiments starting with spheroplast membranes (170 mg protein) from 10 g of freshly grown E. coli NRC 482.

Fraction	Total ATPase activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification	Recovery (%)
Initial membrane suspension	17.8	170	0.105	1	100
Supernatant from 5th washing step	3.36	11.7	0.287	2.75	19
Pooled fractions from Sepharose 6B	3.82	0.66	5.80	55	21

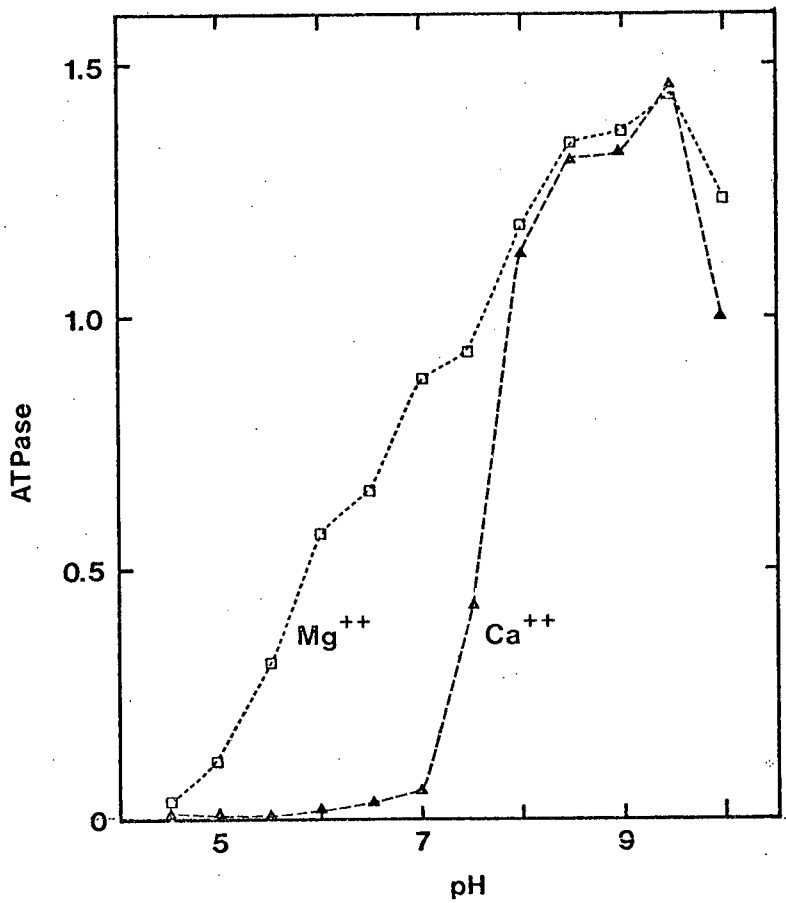


Fig. 33. Effect of pH on the specific activity of the solubilized ATPase.

ATPase activity is expressed as units/mg protein. Values were obtained in the presence of 5mM $MgCl_2$ (□) or 5mM $CaCl_2$ (▲). The Tris-HCl buffer (pH 9.0) of the regular assay was replaced by 0.1M Tris-acetate buffers of pH values ranging from 4.5 to 9.0. Solutions of pH 9.5 and 10.0 were obtained by using 0.1M glycine adjusted to the required pH with NaOH.

Optimum divalent cation : substrate ratios for solubilized ATPase activity

In the absence of divalent metal ions there was essentially no ATPase activity (Fig. 34). The optimum ion to substrate ratio for Mg^{2+}-activated ATPase activity was 0.4 : 1 while for Ca^{2+}-activated ATPase activity it was 0.66 : 1. Higher ratios of metal ions to ATP resulted in inhibition.

Modification of the ATPase assay

Clearly the ATPase assay in which Mg^{2+} was used in a 1 : 1 ratio with ATP only gave 40% of the optimum activity (Fig. 34). Consequently Ca^{2+} was used in preference to Mg^{2+} since its ion to substrate ratio was not so critical. Alternatively the Mg^{2+} ion concentration in the assay was lowered to 2mM while maintaining the ATP concentration at 5mM, to attain the optimal ion : substrate ratio of 0.4 : 1. This alteration and the previous change of pH form the basis of the (Ca^{2+}-Mg^{2+})-ATPase assay as opposed to the ATPase assay.

Substrate specificity of solubilized ATPase

There was no detectable hydrolysis of AMP with either of the divalent ions: thus, 5'-nucleotidase and non-specific phosphatases were absent from the preparation (Table XVII). Hydrolysis of ADP occurred only in the presence of Mg^{2+} to the extent of 19% of the value for ATP hydrolysis. Since hydrolysis

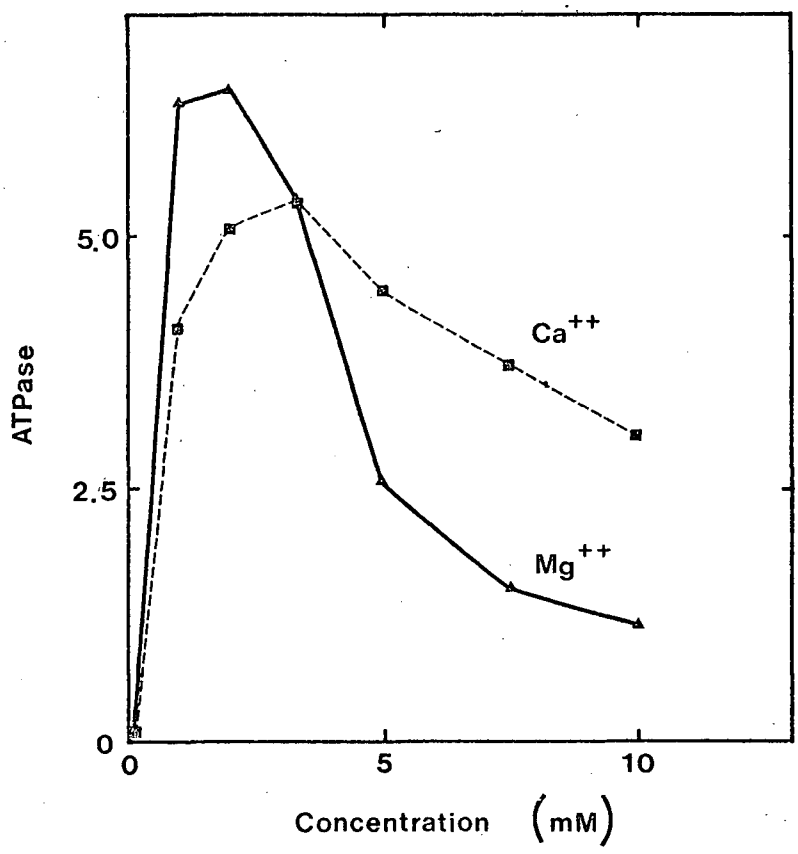


Fig. 34. Effect of divalent metal ion concentration on solubilized ATPase activity.

The ATP concentration was kept constant at 5mM while the Mg^{2+} and Ca^{2+} concentrations were varied from 0 to 10mM. ATPase activity is expressed in units/mg protein. For this experiment the ATPase preparation from the Sepharose 6B column (Fig. 32) was dialysed against 5mM Tris-HCl (pH 7.4) to remove the 0.2mM EDTA which was present in the enzyme fractions. Mg^{2+} (—▲—), Ca^{2+} (--■--).

Table XVII. Substrate specificity of solubilized ATPase of *E. coli*.

Enzyme activity is expressed as a percentage relative to the amount of hydrolysis of ATP. Specific activities of the ATPase were 4.57 units/mg protein in the presence of Mg^{2+} and 3.19 units/mg protein with Ca^{2+}. The concentrations of $MgCl_2$ and $CaCl_2$ were 2mM and 5mM respectively. Substrate concentration was 5mM in each assay. Each assay contained 2.2 µg protein. These results represent the average of two experiments.

Substrate	Activity in the presence of Ca^{2+} (%)	Activity in the presence of Mg^{2+} (%)
ATP	100	100
dATP	55	131
GTP	22	62
ITP	26	41
UTP	9	27
CTP	1	19
ADP	0	19
AMP	0	0

of ADP did not occur with Ca^{2+} this effect may have been due to a contaminating activity like polynucleotide phosphorylase or adenylate kinase in conjunction with the ATPase. This was another reason why Ca^{2+} was preferred as the activating ion in the Ca^{2+}-Mg^{2+}-activated ATPase assay.

Of the other substrates tested dATP was by far the most active especially with Mg^{2+}. The purine nucleoside triphosphates, ITP and GTP, were more readily attacked than the pyrimidine nucleoside triphosphates, UTP and CTP. This is in agreement with the specificities of other bacterial ATPases (Tables I and II, INTRODUCTION). With each of these substrates, as with the adenosine derivatives, Ca^{2+} gave a higher degree of specificity than Mg^{2+}.

Effect of monovalent cations on solubilized ATPase

At a concentration of 50mM, Li^+, Na^+, K^+, Cs^+ and Na^+ plus K^+ inhibited the enzyme between 8% and 28% (Table XVIII). With these ions there was no differential effect between the Ca^{2+}-activated and the Mg^{2+}-activated ATPase activities. However NH_4^+ gave more inhibition with Ca^{2+} (55%) than with Mg^{2+} (26%). No (Na^+ + K^+)-activated ATPase similar to that described by Hafkenscheid and Bonting (63, 66) for lyophilized cells tested in the presence of urea was detected.

Table XVIII. Effect of monovalent cations on solubilized ATPase of *E. coli*.

The specific activities of the Mg^{2+}- and Ca^{2+}-activated ATPases were 7.89 units/mg protein and 6.06 units/mg protein respectively. The concentrations of $MgCl_2$ and $CaCl_2$ were 2mM and 5mM respectively. Both enzyme activities were tested in the presence of 50mM monovalent cations, all of which were added as the chloride. When Na^+ and K^+ were tested together their individual concentrations were 25mM. The inhibition of the ATPase is expressed relative to its activity in the absence of monovalent cations.

Cation	Inhibition of Mg^{2+}-activated ATPase activity (%)	Inhibition of Ca^{2+}-activated ATPase activity (%)
NH_4^+	26	55
Li^+	28	23
Na^+	11	12
K^+	9	17
Cs^+	12	8
$Na^+ + K^+$	13	12

Inhibition of solubilized ATPase by ADP

The inhibition of the Ca^{2+}-activated ATPase as a function of ADP concentration is shown in Fig. 35. The affinity of the enzyme for ATP and ADP appeared to be of the same order since at equal concentrations of the two nucleotides there was 72% inhibition of ATPase activity. The effect of ADP on the Mg^{2+}-activated ATPase was not investigated since ADP was attacked in the presence of Mg^{2+} (Table XVII).

Cold-lability of solubilized ATPase

The soluble ATPase was very labile when stored at 0° (Fig. 36). The half-life of its activity was 4 h at this temperature compared to a half-life of 6 days for the membrane-bound enzyme. The soluble enzyme was much more stable at 22°- 24° and less than 40% of the activity was lost after 80 h. In the presence of 20% glycerol there was no apparent loss of ATPase activity under these conditions. Glycerol (20%) also stabilized the enzyme kept at 0°. There was no significant difference in the behaviour of the Ca^{2+}-activated and Mg^{2+}-activated enzyme in this respect.

Other temperature effects on the solubilized ATPase

The solubilized ATPase showed no sign of heat activation. On the contrary it was rapidly inactivated at 55°, and lost 50% of its activity after 7 min at this temperature (Fig. 37).

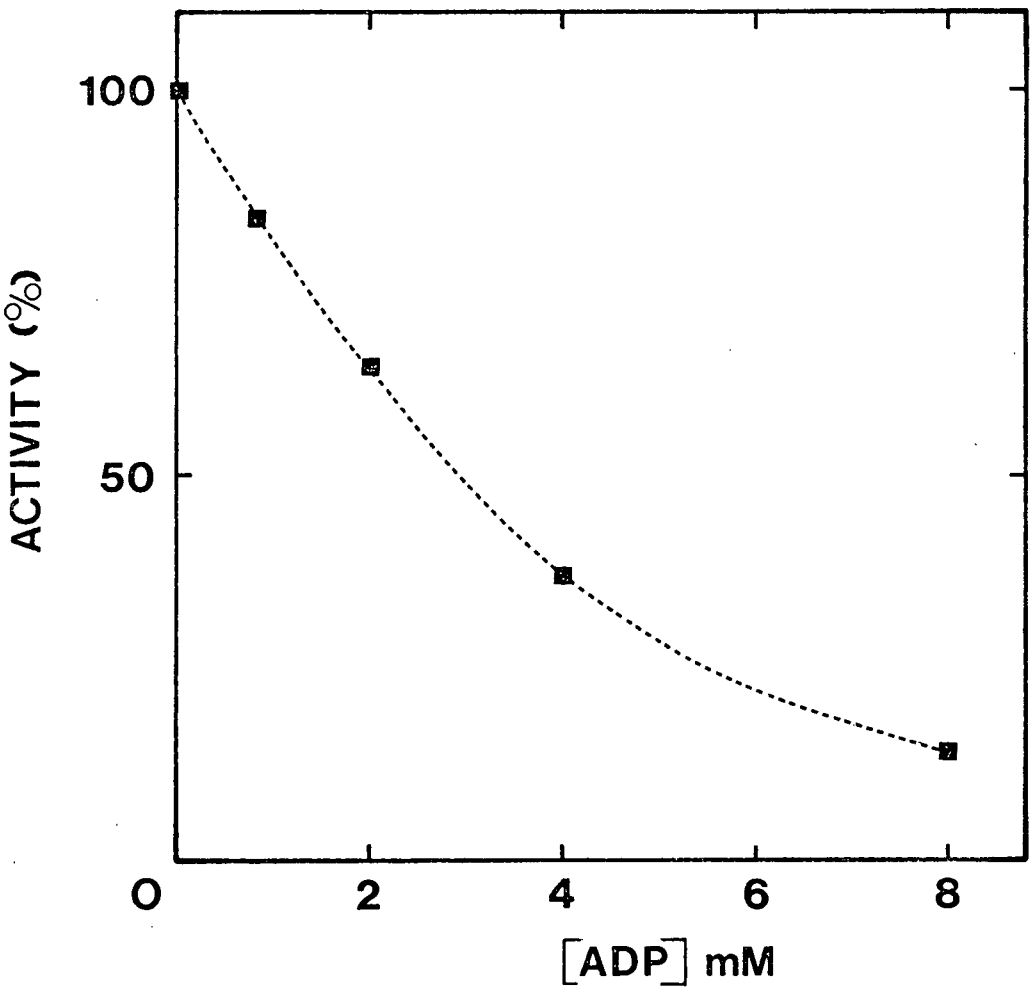
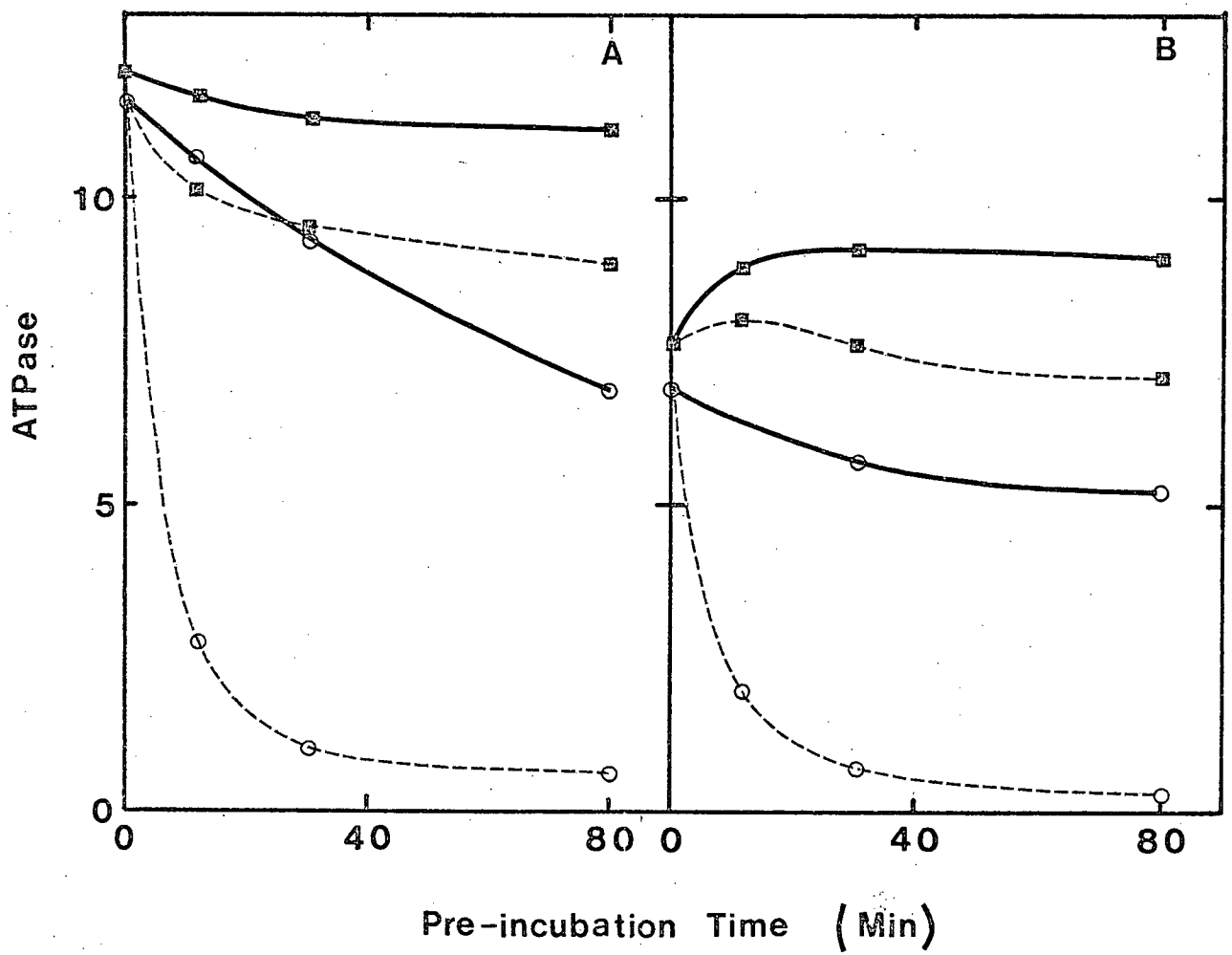


Fig. 35. Inhibition by ADP of solubilized Ca^{2+}-activated ATPase.

The specific activity of the Ca^{2+}-activated ATPase was 4.90 units/mg protein. Activities in the presence of 0 to 8mM ADP (Na salt) are expressed as a percentage relative to this value. No corrections were made for the presence of small amounts of ADP (about 2%) in the ATP substrate.

Fig. 36. Cold-lability of solubilized ATPase.

Four enzyme solutions were prepared. Two were kept in 20% glycerol by the addition of 1 vol of glycerol to 4 vol of the pooled enzyme-containing fractions from Sepharose 6B. In the other two preparations distilled water replaced the glycerol to act as a control. One control tube and one with added glycerol were kept at 0° (-----), and the other two were kept between 22°- 24° (———). After preincubation at these temperatures in the presence (■) or absence (O) of 20% glycerol, samples were removed at time intervals and assayed for ATPase activity. The activity of ATPase is expressed in units/mg protein. Determination of ATPase activities in A and in B were done in the presence of 2mM $MgCl_2$ and 5mM $CaCl_2$, respectively.


A
B
ATPase
10
5
0
0
40
80
0
40
80
Pre-incubation Time (Min)

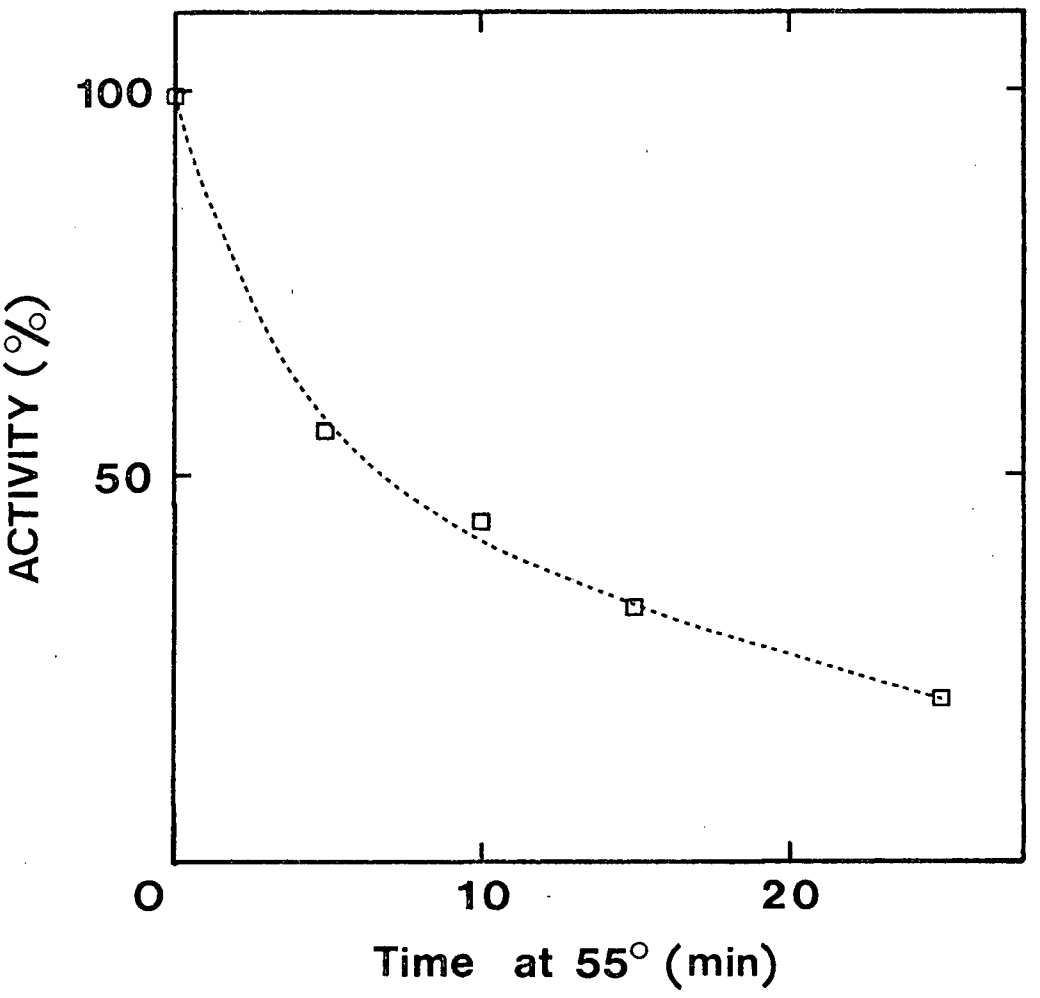


Fig. 37. Inactivation of solubilized Ca^{2+}-activated ATPase after heating to 55°.

The specific activity of the Ca^{2+}-activated ATPase was 2.69 units/mg protein. Prior to the assay samples of the enzyme were heated for various time periods (0 to 25 min) at 55°. Activities after the heat-treatment are expressed as a percentage relative to this value.

Sweetman and Griffiths (192) observed that in the reaction catalyzed by a membrane-bound ATPase of E. coli there was a sharp break at 18.8° in the Arrhenius plot. This transition temperature is thought to indicate the point at which the lipid of the membrane undergoes a phase change (193). If the solubilized enzyme contained any lipid it might well show such a break in the Arrhenius plot. The effect of temperature on the ATPase reaction was examined over the range 4°- 36°, and as can be seen in Fig. 38 all but one of the points fell close to a straight line with no indication of a transition point. The anomalous activity at 4.3° may have been due to the cold-lability of the enzyme. The activation energy for the reaction was 20.7 Kcal/mole.

Rebinding of solubilized ATPase to depleted membranes

Mirsky and Barlow (3) working with B. megaterium reported rebinding solubilized ATPase to membranes depleted of ATPase, in the presence of 10mM $CaCl_2$. A similar experiment was attempted with E. coli NRC 482 (Table XIX). The membranes were depleted of ATPase by dialyzing them against EDTA as described in METHODS AND MATERIALS. After centrifugation of the dialysate the supernatant fraction was added back to a suspension of the depleted membranes in the presence and absence of Ca^{2+}. The two fractions were incubated together at 22°- 24° for 2 h. At the end of this time the mixture was re-centrifuged at 23,700 x g for 20 min at 18°. When Ca^{2+} had been present in the mixture, ATPase activity was

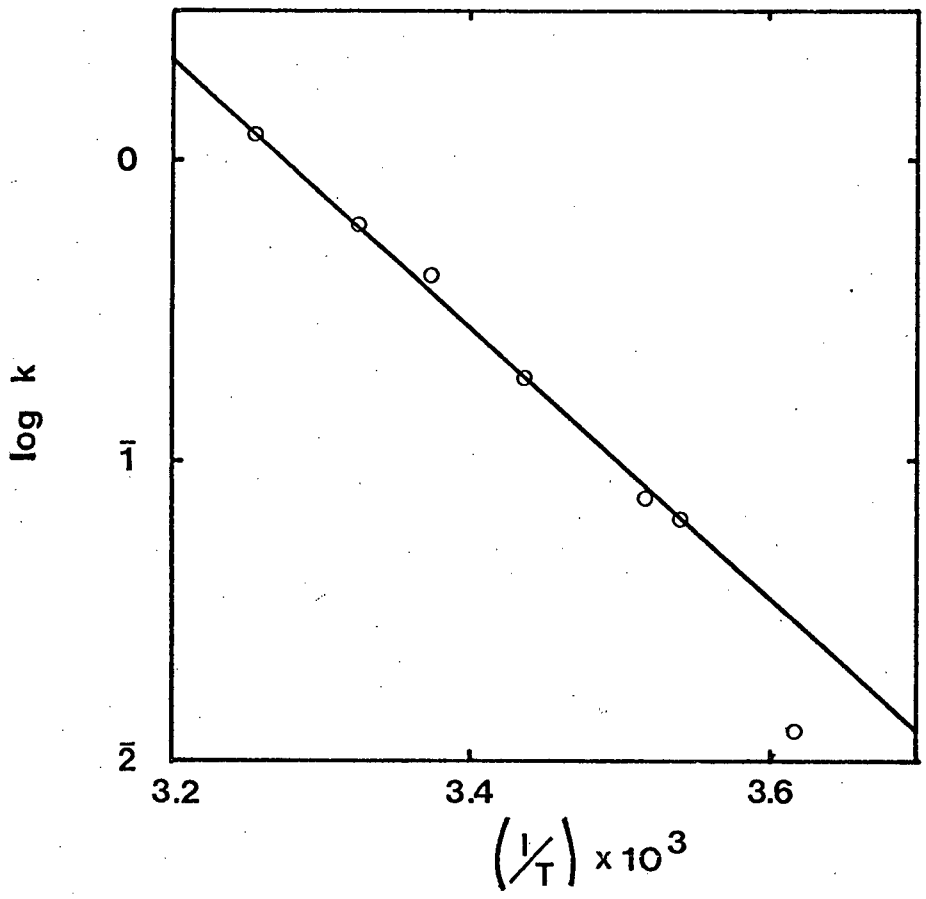


Fig. 38. Arrhenius plot for hydrolysis of ATP by solubilized Ca^{2+}-activated ATPase of E. coli.

The enzyme tested was the pooled enzyme-containing fractions from Sepharose 6B. Each assay mixture contained 4.4 µg protein and 5mM Ca^{2+}. k, rate of hydrolysis of ATP (µmoles/h); T, incubation temperature (°K). The incubation time was extemded beyond 60 min for assays done at low temperature so that the extent of hydrolysis of ATP was approximately the same at each temperature.

Table XIX. Rebinding studies with depleted membranes and solubilized ATPase.

This binding experiment was done as described in RESULTS. The buffer solution was 0.2M Tris-HCl (pH 7.4) and the $CaCl_2$ solution was 100mM. The membrane suspension (8.33 mg protein/ml) was prepared as described in the legend to Table XXI. The supernate added to the depleted membranes contained 0.13 mg protein/ml. The specific activities of these fractions measured in the presence of 5mM $CaCl_2$ at pH 9.0 were 0.0015 and 1.83 units/mg protein respectively.

After preincubation of the mixtures 1 - 4 the membranes were sedimented and the Ca^{2+}-activated ATPase activity remaining in the supernatant fractions was determined.

Tube #	Contents					Activity remaining in the supernatant (nmoles/min/ml)
	Buffer (ml)	Membrane suspension (ml)	$CaCl_2$ (ml)	H_2O (ml)	Supernatant (ml)	
1	0.5	0.5	0.2	-	0.8	23.9
2	0.5	0.5	-	0.2	0.8	41.8
3	0.5	0.5	0.5	-	0.5	11.4
4	0.5	0.5	-	0.5	0.5	58.2

lost from the supernatant. When the concentration of Ca^{2+} was increased from 10mM to 25mM the ATPase activity in the supernatant decreased further.

It appeared that Ca^{2+} was promoting the binding of solubilized ATPase back to the depleted membranes. However when the supernatant fraction alone was centrifuged at 23,700 x g in the presence of 25mM $CaCl_2$ its activity declined somewhat. This result threw doubt on the validity of the rebinding. Consequently the experiment shown in Fig. 39 was done. In this experiment the ratio of supernatant to depleted membrane was varied by increasing the amount of depleted membrane in the system while keeping the amount of supernatant constant. The $CaCl_2$ concentration was maintained throughout at 25mM. After incubating the fractions together for 1 h at 22°, the membranes were re-sedimented and the supernatant fraction was analyzed.

Since the amount of ATPase remaining in the supernatant fraction did not vary with the amount of depleted membrane present there was no indication of rebinding in this system (Fig. 39).

Effect of trypsin on membrane-bound ATPase

In view of the reported trypsin-activation of membrane-bound ATPase in M. lysodeikticus (2, 79), this property was looked for in the membrane-bound ATPase of E. coli NRC 482. To test for trypsin-activation aliquots of a suspension of spheroplast membranes were treated at 22°- 24° for different time periods

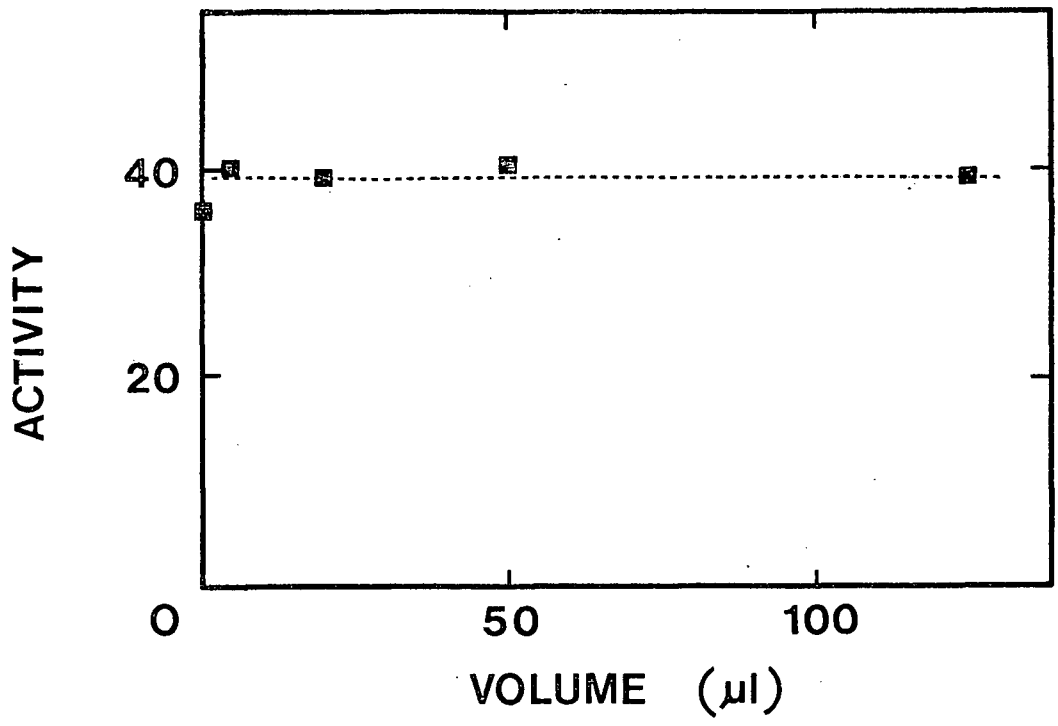


Fig. 39. Rebinding studies with depleted membranes and solubilized ATPase.

This binding study is described in RESULTS. The samples (final volume 0.5 ml) each contained 12.5 µmoles of $CaCl_2$, 25 µmoles Tris-HCl (pH 7.4), 125 µl of supernatant (0.28 mg protein/ml) from membranes which had been dialyzed against EDTA as described in METHODS AND MATERIALS, and a variable amount (0 - 125 µl) of depleted membrane suspension (1.99 mg protein/ml). The supernatant and depleted membrane fractions had Ca^{2+}-activated ATPase activities with specific activities of 3.74 units/mg protein and 0, respectively.

The samples were incubated together for 60 min at 22°- 24° before being recentrifuged at 23,700 x g for 20 min at 18°. The supernatant fractions from these samples were assayed for Ca^{2+}-activated ATPase activity in the usual manner with the exception that the $CaCl_2$ concentration was 7.5mM rather than 5mM. The activities of these supernatant fractions are expressed in nmoles/min/ml fraction (Fig. 39). The volume (µl) refers to the volume of depleted membrane suspension that was present in the original sample.

with a fixed amount of trypsin. At the end of the incubation period the trypsin was inhibited by the addition of trypsin inhibitor, and the membranes were assayed for ATPase activity in the usual way.

There was no sign of trypsin-activated ATPase activity when undepleted spheroplast membranes were digested. In fact there was slight decrease in ATPase activity as the digestion period approached 60 min (Table XX). However this did not rule out the possibility of there being two ATPase activities associated with the membrane, one sensitive to trypsin, the other activated by trypsin. To eliminate this possibility spheroplast membranes were depleted of ATPase by dialysis against EDTA and then digested with trypsin. This was thought to be the best way of revealing a latent membrane-bound ATPase activity which might otherwise be overshadowed by the other membrane-bound ATPase activity. A 10 min digestion of the depleted membranes with trypsin increased the ATPase activity by only 20% (Table XXI). This increase in activity is insignificant (less than 0.2%) when compared to the activity of the undepleted spheroplast membranes. From these results there is no evidence of a latent membrane-bound ATPase in E. coli which can be activated by trypsin.

Common identity of the soluble ATPase and the ATPase solubilized from the membrane

The soluble ATPase which occurred in the supernatant fraction after cell breakage resembled in every way the ATPase

Table XX. Effect of trypsin on Mg^{2+}-activated ATPase of spheroplast membranes.

Lysozyme-induced spheroplast membranes were prepared from E. coli NRC 482 (1 g) in the usual manner and were resuspended in 5 ml of 10mM Tris-HCl (pH 7.4) containing 0.5mM $MgCl_2$. Aliquots (0.5 ml) of the membrane suspension (3.53 mg protein/ml) were dispensed into 7 tubes and equilibrated at 30°. Each aliquot was treated with 10 µl of trypsin solution (2 mg/ml). At the end of the preincubation time (0 - 60 min) the digestion was stopped by the addition of trypsin-inhibitor (10 µl) (4 mg/ml) with rapid mixing. The trypsin-treated membrane suspensions were assayed for Mg^{2+}-activated ATPase activity which is expressed as nmoles/min/mg protein.

Time of preincubation (min)	Mg^{2+}-ATPase (nmoles/min/mg protein)
0	236
5	236
10	230
20	227
40	228
60	220

Table XXI. Effect of trypsin on ATPase-depleted membranes.

Lysozyme-induced spheroplast membranes were prepared and depleted of ATPase as described in METHODS AND MATERIALS. A suspension was made in 5mM Tris-HCl (pH 7.4) (8.33 mg protein/ml). Aliquots (0.2 ml) of the membrane suspension were treated with 0.4 ml trypsin (2.5 mg/ml) at pH 8.0 in the presence of 30mM Tris-HCl (pH 8.0) for 0, 5 or 10 min at 22°- 24°. The digestion was stopped by the addition of 0.4 ml trypsin inhibitor (2.5 mg/ml). The trypsin-treated membrane suspensions were assayed for Mg^{2+}-activated ATPase activity which is expressed as nmoles/min/mg protein.

Time of preincubation (min)	Mg^{2+}-ATPase (nmoles/min/mg protein)
0	1.50
5	1.38
10	1.81

which could be solubilized from the membranes. These two enzymes chromatographed alike on DEAE-cellulose (cf. Figs. 18 and 25 to Fig. 27). After gel filtration through Sepharose 6B the two enzymes had similar K_{av} values and hence similar molecular weights (cf. Figs. 23 and 32). Both enzymes showed cold-lability which became more acute as the enzymes were further purified. Like the solubilized ATPase the soluble enzyme was more active under the Ca^{2+}- or Mg^{2+}-activated ATPase assay conditions than under the standard ATPase assay conditions.

The evidence pointing the the common identity of the two enzymes was strengthened when the pH profile and the substrate specificity of the soluble enzyme were shown to be identical to those of the solubilized enzyme. If the enzymes are indeed identical then it is not immediately apparent why the enzyme should exist in these two forms.

Large-scale isolation of ATPase

In order to obtain a homogeneous preparation of the Ca^{2+}- or Mg^{2+}-activated ATPase it was felt necessary to increase the amount of starting material. Many different separation techniques were tried and the most successful combination for the large-scale isolation of the ATPase is described in METHODS AND MATERIALS.

Although most of the ATPase activity in E. coli NRC 482 occurred in the soluble fraction, the membrane fraction was chosen as the starting material in order to make use of the extra purification that occurs during the release of the ATPase from the membrane.

This extra step, the dialysis of the membrane fraction against Na_2EDTA, solubilized 30% of the membrane-bound ATPase but was accompanied by some loss of enzyme activity (Table XXII). Consequently there was a net decrease in the specific activity of the ATPase compared to the activity in the original extract.

The second purification step, that of chromatography on DEAE-cellulose by step-wise elution with Tris-HCl, was based on the method used by Kobayashi and Anraku (194). However, glycerol (10%) was used to stabilize the enzyme instead of methanol (20%). Most of the ATPase was eluted by 0.2M and 0.25M Tris-HCl (Fig. 40) between fractions 65 to 95. In these fractions there was very little material that absorbed at 280 nm. The specific activity of the ATPase in the combined fractions 84 to 89 inclusive was increased 16-fold by the ion exchange chromatography (Table XXII).

The next step in the purification procedure (gel filtration on Sepharose 6B) removed some contaminating proteins (Fig. 41) but did not increase the specific activity of the ATPase owing to some loss of enzyme activity (Table XXII). The elution profile from Sepharose 6B suggested that the enzyme was not homogeneous, since the ATPase activity coincided with the leading edge of a peak of material that absorbed at 280 nm. This heterogeneity was confirmed when fractions 27 to 31 inclusive were combined, concentrated and examined by disc gel electrophoresis as described in METHODS AND MATERIALS. Two major bands of protein were detected on the gels as well as several minor bands (Plate 3). The major protein band which had migrated the least distance from the origin corres-

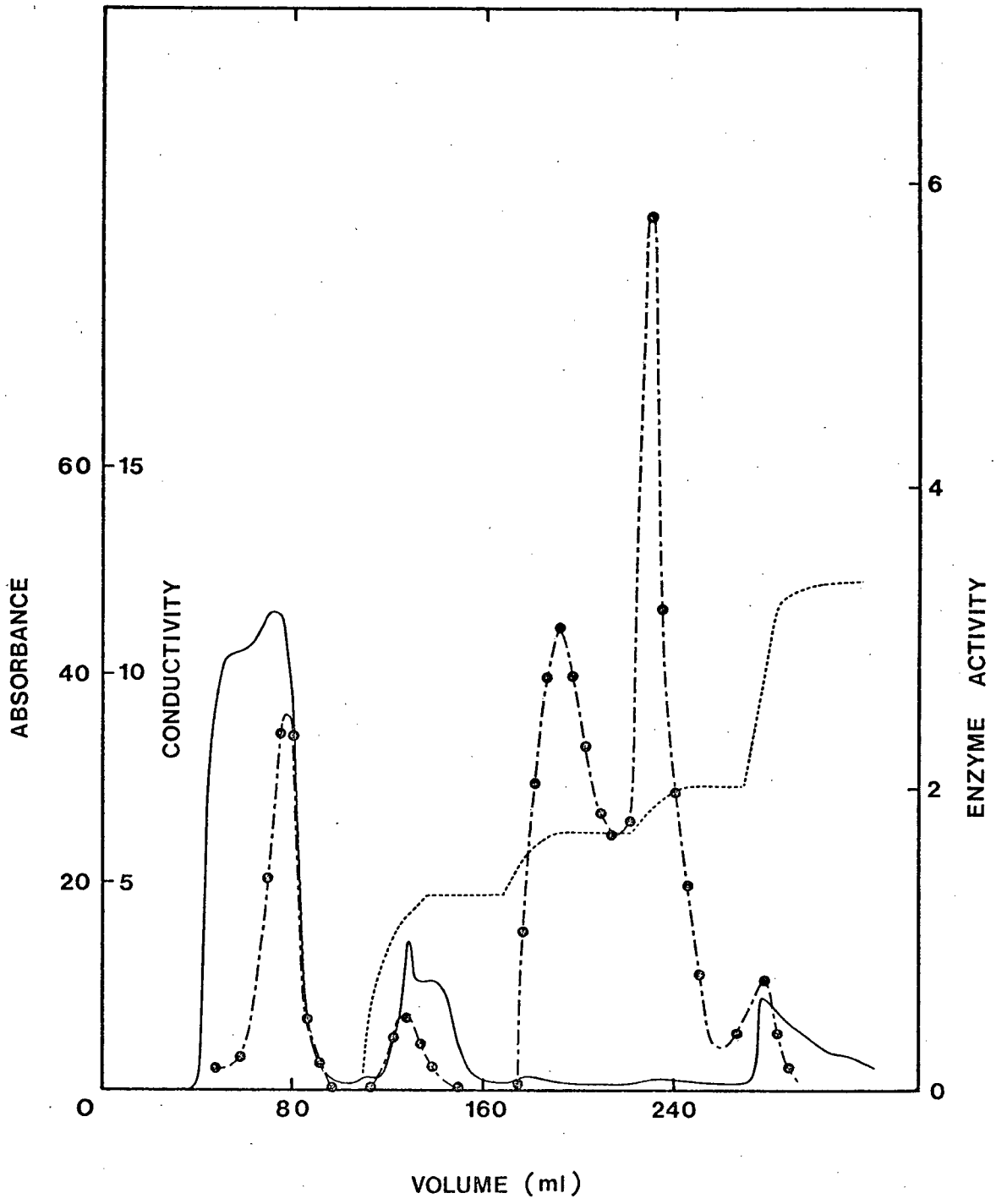
Table XXII. Large-scale purification of solubilized ATPase.

The ATPase was purified as described in METHODS AND MATERIALS starting with an extract of E. coli NRC 482 prepared by breaking the cells in a French press. ATPase activity was determined in the presence of 5mM $CaCl_2$ at pH 9.0.

Step	Fraction	Activity (units/mg protein)	Total (units)	Yield (%)
	Cell extract	0.742	1590	100
1	Membrane suspension before dialysis	0.776	695	43.7
	Membrane suspension after dialysis	0.471	556	35.6
2	Dialysate supernatant	0.615	166	10.4
3	Pooled fractions from DEAE-cellulose	10.5	64.1	4.0
4	Pooled fractions from Sepharose 6B	10.1	17.9	1.1

Fig. 40. Chromatography of solubilized ATPase on DEAE-cellulose using step-wise elution.

This chromatographic separation was done as described in METHODS AND MATERIALS. The absorbance of the fractions (——) was measured at 280 nm. The fractions were assayed for conductivity (·····) and for Ca^{2+}-activated ATPase activity (-·-●-·-) which was measured in units/ml fraction.


ABSORBANCE
CONDUCTIVITY
ENZYME ACTIVITY
60
40
20
15
10
5
6
4
2
0
0
80
160
240
VOLUME (ml)

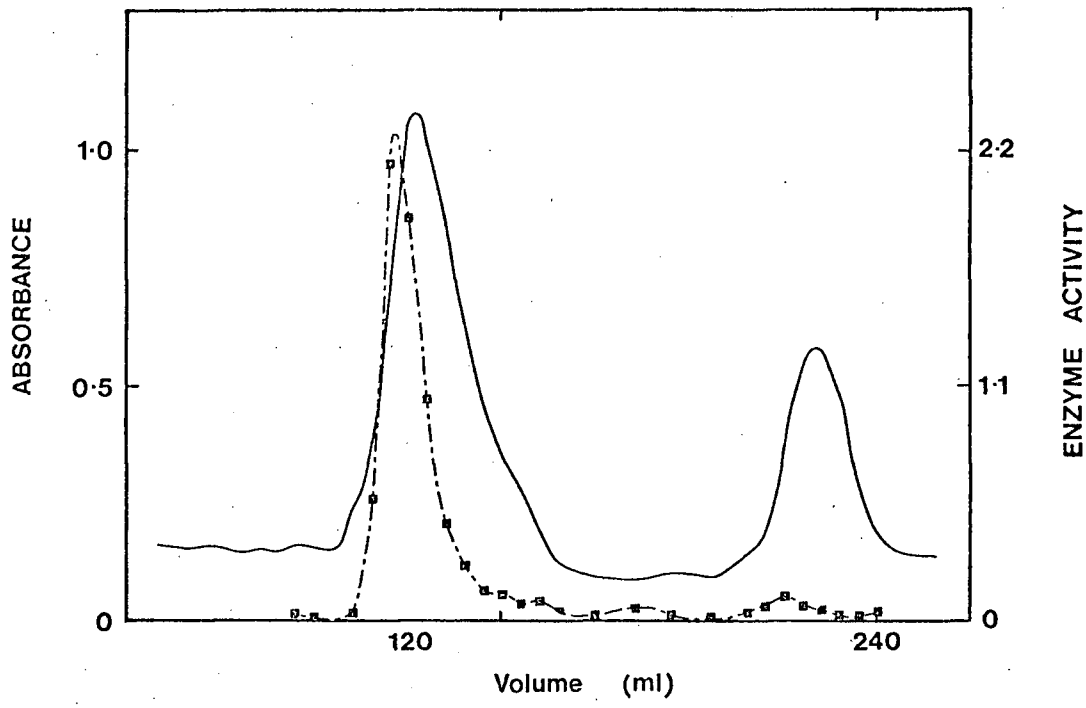


Fig. 41. Chromatography of partially purified solubilized ATPase on Sepharose 6B.

Combined fractions from the previous DEAE-cellulose chromatography of solubilized ATPase were chromatographed on Sepharose 6B at 22°- 24° as described in METHODS AND MATERIALS. The absorbance of the fractions was measured at 230 nm (——). The fractions were assayed for Ca^{2+}-activated ATPase activity (—■—). The enzyme activity is expressed as units/ml fraction.

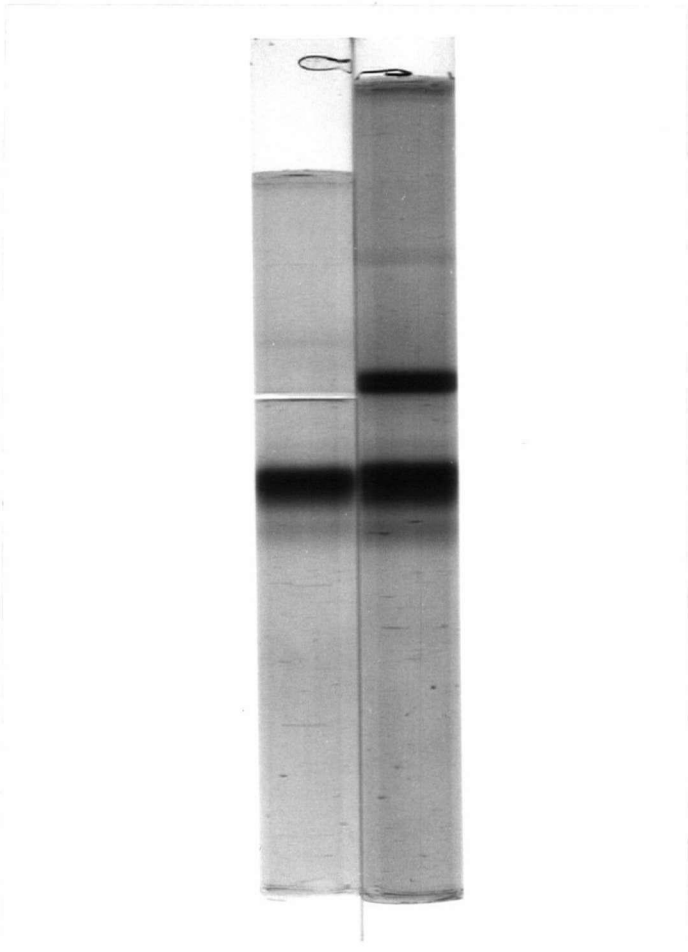


Plate 3. Gel electrophoresis of partially purified Ca^{2+}- or Mg^{2+}-activated ATPase.

The ATPase sample (180 µg protein) from the pooled fractions from Sepharose 6B (Table XXII) was prepared and resolved by electrophoresis as described in METHODS AND MATERIALS ("Large-scale isolation of ATPase"). The protein band corresponding to the ATPase was cut out from the left hand gel prior to staining with Coomassie Blue.

ponded to the ATPase. This was shown by staining for ATPase activity *in situ* (Plate 4) and after sectioning the gel and assaying the individual pieces for activity (Fig. 42).

Once the position of the ATPase had been determined it was possible to cut out the corresponding section from other gels prepared in the same manner. In order to show that only the ATPase band had been excised, the remaining gel segments were stained with Coomassie blue. The segments contained all the protein bands except that of the ATPase (Plate 3). The ATPase activity resided in a single protein band.

ATPase levels in *E. coli* NRC 482 relative to growth phase

Ca^{2+}-activated ATPase activity was measured in cell-free extracts of *E. coli* NRC 482 prepared at different phases of growth on glucose. In each instance the cell extracts were prepared by using the French press as described in METHODS AND MATERIALS, after the cells had been harvested and washed in the usual manner. The total ATPase activities of the unfractionated extracts were expressed relative to total protein and to the cytochrome b_1 contents of the extracts (Table XXIII). The three phases of growth chosen for the assays were mid-log, late-log and stationary phase. These phases were identified from the growth curve of the bacteria (Fig. 43). During the growth of the bacteria the amount of glucose remaining in the growth medium was determined at time intervals. The late-log phase cells were harvested at the point of glucose depletion in the medium. The stationary phase cells were harvested 140 min

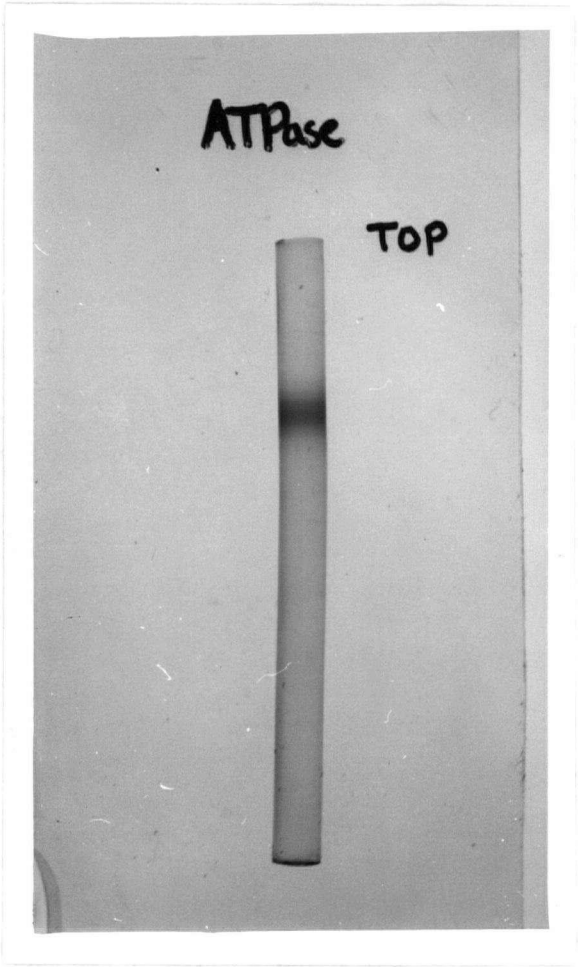


Plate 4. Gel electrophoresis of partially purified Ca^{2+}- or Mg^{2+}-activated ATPase: staining for ATPase activity.

The sample of ATPase was prepared for electrophoresis as described in METHODS AND MATERIALS. After resolution of the sample the entire gel was stained for ATPase activity as described in METHODS AND MATERIALS. The transient, blue band of reduced phospho-molybdate was photographed immediately.

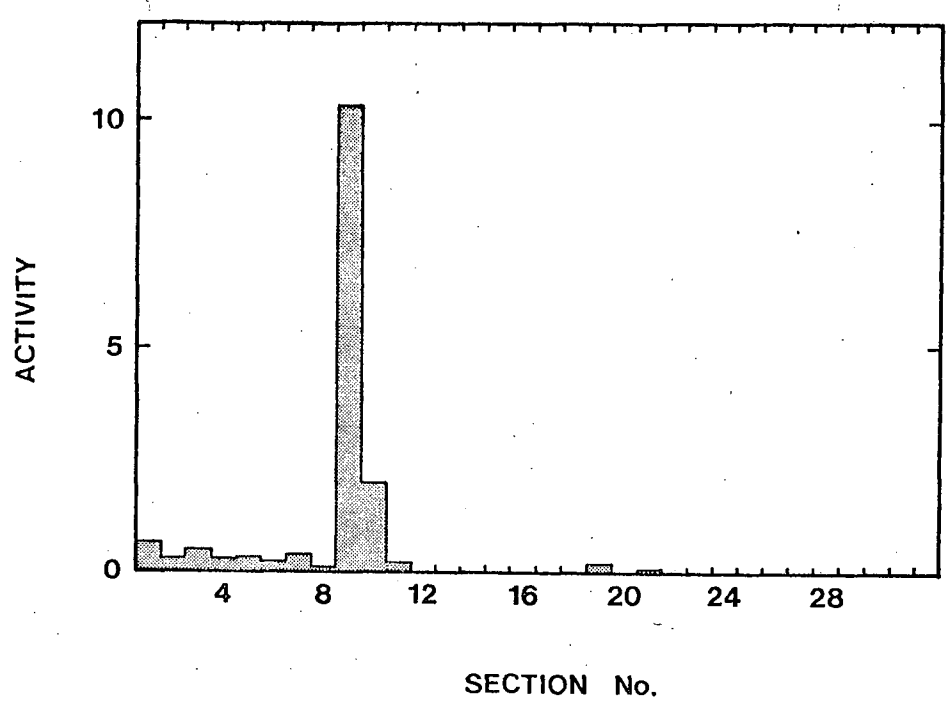


Fig. 42. Assay of gel sections for ATPase activity.

Samples of ATPase were prepared for electrophoresis as described in METHODS AND MATERIALS. After electrophoresis of the samples on polyacrylamide disc gels as described in METHODS AND MATERIALS the gels were sectioned into 32 approximately equal pieces. Each piece was assayed for Ca^{2+}-activated ATPase activity. Sections 1 to 32 ran from top to bottom in the gel. Activity was measured in nmoles/min/gel section.

Table XXIII. The effect of growth phase on the levels of Ca^{2+}-activated ATPase in E. coli.

Samples of E. coli NRC 482 were harvested at different phases of growth as indicated in the legend to Fig. 43. These cell samples were sonicated in the usual manner and the sonicates were assayed for cytochrome b_1 content and Ca^{2+}-activated ATPase activity.

Sample	Expt.	ATPase (units/mg protein)	cyt b_1 (nmoles/mg protein)	Ratio of ATPase to cyt b_1
mid-log	(1)	0.414	0.226	1.83 : 1
late-log	(1)	0.450	0.283	1.59 : 1
stationary	(1)	0.423	0.246	1.72 : 1
mid-log	(2)	0.558	0.123	4.52 : 1
late-log	(2)	0.529	0.148	3.58 : 1
stationary	(2)	0.508	0.146	3.48 : 1

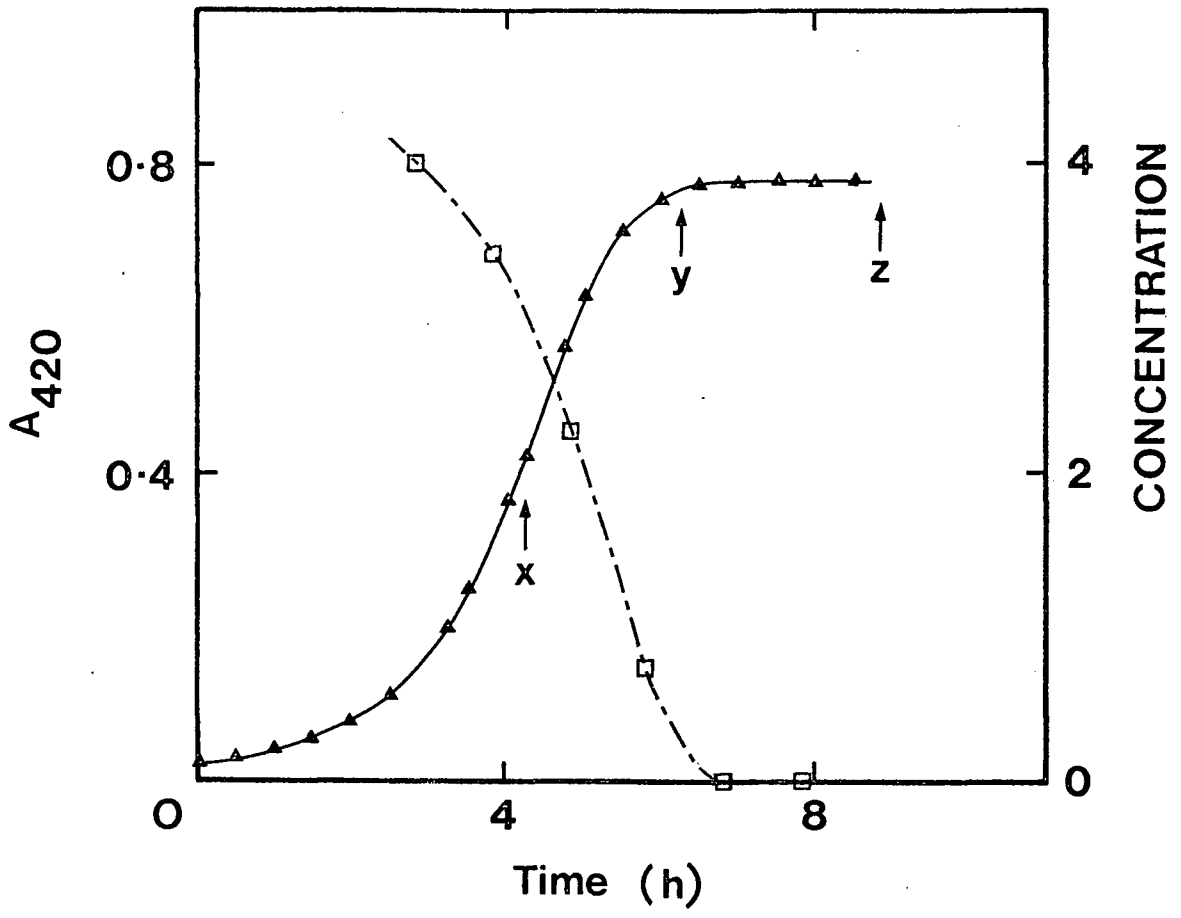


Fig. 43. Depletion of glucose during growth of E. coli NRC 482.

The growth of the culture was monitored by measuring its absorbance at 420 nm (—▲—). Glucose concentration (—·□·—) is expressed in mg/ml of culture medium. The times at which mid-log, late-log and stationary phase cells were harvested are marked by X, Y and Z respectively.

after glucose depletion.

The results of the assays (Table XXIII) show that there was no variation in ATPase activity with growth phase, either relative to protein or to cytochrome b_1. The cytochrome b_1 content of the cell extracts in experiment 1 was twice as great as in experiment 2. This affected the ATPase:cytochrome b_1 ratio accordingly. However within each experiment the ratio was very constant.

ATPase levels in E. coli NRC 482 relative to carbon source

A similar set of experiments were done in which cells were harvested in the late-log phase after growth on different carbon sources. Glucose (0.4%) was replaced in turn by glycerol (0.4%), lactic acid (0.8%), diNa succinate (0.8%) and acetic acid (0.8%). The lactic and acetic acids were adjusted to pH 7.0 by the addition of KOH prior to mixing the carbon source with the salts medium. In this series of experiments sodium citrate was omitted from the growth medium.

After growth on these different carbon sources the Ca^{2+}-activated ATPase activity of the unfractionated, cell-free extracts was remarkably constant (Table XXIV). The greatest variation in specific activity (13%) occurred between glucose-grown and glycerol-grown cells. The ATPase:cytochrome b_1 ratio was more varied owing to the different cytochrome b_1 concentrations.

Table XXIV. The effect of carbon source on the levels of Ca^{2+}-activated ATPase in E. coli.

Samples of E. coli NRC 482 were harvested in the late-log phase after growth on different carbon sources. These substrates were glucose (0.4%), glycerol (0.4%), Na_2 succinate (0.8%), lactic acid (0.8%) and acetic acid (0.8%). They were used in conjunction with the usual salts medium. Lactic acid and acetic acid were adjusted to pH 7.4 by the addition of KOH prior to the addition of the salts medium. The cell samples were treated as described in the legend to Table XXIII.

Substrate	Growth phase	ATPase (units /mg protein)	[cyt b_1] (nmoles/mg protein)	Ratio of ATPase to cyt b_1
glucose	late-log	0.525	0.101	5.90 : 1
glycerol	"	0.596	0.154	4.25 : 1
succinate	"	0.551	0.140	4.30 : 1
lactate	"	0.560	0.146	3.84 : 1
acetate	"	0.541	0.110	4.94 : 1

ATPase levels in K^+ mutants

Two mutant strains of E. coli K12 (FRAG 1 and FRAG 5) and their parent type 2K401M were kindly supplied by W. Epstein (Univ. of Chicago). In order to grow successfully, these mutants required an elevated concentration of K^+ in the growth medium. The mutants did not appear to have a primary alteration in K^+ transport and are referred to as K^+-dependent (195). Since the bacterial ATPases have been implicated in active transport processes it was thought that the K^+ mutants might have levels of ATPase activity that were either above or below normal, just as S. faecalis responded to a growth medium deficient in K^+ by increasing the amount of ATPase in the cell (160).

After the three strains had been grown as described in reference 195 they were harvested in the late-log phase of growth. Cell extracts were prepared by sonication in the usual manner. The Ca^{2+}-activated ATPase activities of the unfractionated cell extracts were determined. The ATPase levels in the two mutant strains were compared to the level in the parent strain and were found to be very similar (Table XXV).

Variability in the specific activity of membrane-bound ATPase

During the course of other investigations membrane particles were obtained from cells grown under a variety of conditions, such as anaerobic growth on glucose or growth on glucose in the presence of 3'-5'-cyclic AMP. These included membrane

Table XXV. Ca^{2+}-activated ATPase levels in extracts of potassium mutant strains.

The strains were grown and extracts prepared as described in RESULTS. The specific activity of the Ca^{2+}-activated ATPase was determined for each extract.

Strain	ATPase (units/mg protein)
2K401M	0.380
FRAG 5	0.400
FRAG 1	0.387

Table XXVI. Ca^{2+}-activated ATPase activity of membrane particles prepared from miscellaneous strains of *E. coli*.

E. coli NRC 482 were grown on Na_2 succinate (0.8%) with or without a supplement of ferric citrate (final concentration 12 μM). This strain was also grown on the usual glucose-salts medium anaerobically or aerobically when supplemented with 3'5'-cyclic AMP (final concentration 2.5mM). The catabolite derepressed strains CAT 5,6 and 11 and their parent strain were grown on glucose-salts medium. Each batch of cells were grown to the late-log phase and membrane particles were prepared as described in RESULTS. The pellet of membrane particles obtained after centrifugation at 100,000 x g was resuspended in 50mM Tris-H_2SO_4 (pH 7.8) containing 10mM $MgCl_2$. Two ml of buffer were used for every g wet wt. of cells processed. The specific activity of the Ca^{2+}-activated ATPase was determined for each of the membrane particle suspensions.

Strain	Aeration	Carbon source	Supplements	ATPase (units/mg protein)
E. coli NRC 482				
WT	aerobic	succinate	Fe	0.773
WT	"	"	-	0.839
WT	"	glucose	cAMP	0.783
WT	anaerobic	"	-	0.686
E. coli B				
WT	aerobic	glucose	-	0.522
CAT 5	"	"	-	0.567
CAT 6	"	"	-	0.768
CAT 11	"	"	-	0.775

particles obtained from mutants of E. coli B which were incapable of catabolite repression (strains kindly supplied by Dr. A. Kropinski, University of B. C.).

In each instance the particles were prepared from cells which had been broken in the French press. The particles were obtained by centrifugation at 100,000 x g for 2 h at 2°- 5° to remove unbroken cells and the larger membrane fragments. Other manipulations prior to the ATPase assay were done at 0°- 4°. The extracts were prepared in 50mM Tris-H_2SO_4 (pH 7.8) buffer containing 10mM $MgCl_2$.

The ATPase activities of these membrane particles (Table XXVI) ranged from 0.522 µmoles/min/mg of protein to 0.839 µmoles/min/mg of protein. The significance of these values will be discussed in the DISCUSSION section.

DISCUSSION

Comparison of the solubilized ATPases from E. coli NRC 482 and E. coli W3092

We have published most of the results concerning the partial purification and properties of the solubilized ATPase of E. coli NRC 482 (68). At the time this publication (68) was in press, Kobayashi and Anraku reported on the membrane-bound ATPase of E. coli W3092, a derivative of E. coli K12 (194). A comparison of the properties of the ATPases from these two strains of E. coli shows that the enzymes are almost identical in their behaviour.

Both enzymes are solubilized from the membane under conditions of low ionic strength in the absence of divalent cations. Both enzymes are cold-labile but can be stabilized against cold-lability by glycerol. They have optimal activity at pH 9.5 and are activated by Mg^{2+} with the optimal molar ratio of Mg^{2+}:ATP being 2:5. Other divalent metal ions like Ca^{2+} can replace Mg^{2+}. Neither of the ATPases are activated by heat-treatment and both are inhibited by ADP.

Kobayashi and Anraku reported (194) that the activity of the solubilized ATPase was stimulated by 10% in the presence of 15mM Na^{+}. However when the Na^{+} concentration was increased above 40mM this cation proved to be inhibitory. This is in

agreement with the finding that the ATPase from E. coli NRC 482 is inhibited by 50mM Na^+. Both ATPases are inhibited by a combination of Na^+ and K^+.

The substrate specificity of the two ATPases is similar, since the extent of hydrolysis of the ribonucleotide triphosphates is in the order ATP > GTP > UTP > CTP. However in the presence of Mg^{2+} the ATPase from E. coli W3092 had little activity with ADP (less than 1% hydrolysis relative to ATP), compared with 19% hydrolysis of ADP using the ATPase preparation from E. coli NRC 482.

This difference almost certainly reflects the greater purity of the preparation from E. coli W3092 compared to the NRC 482 strain (specific activity 58.4 and 10.6 - 5.8 units/mg protein, respectively). The ADPase activity of the ATPase preparation from E. coli NRC 482 is probably due to contamination with polynucleotide phosphorylase. Evidence to support this claim appears in Figs. 20, 27, 28, 30 and Table XVII. In summary, this evidence includes the observations that the ADPase and ATPase activities have different divalent metal ion requirements (Table XVII) and can be partially resolved by DEAE-cellulose chromatography (Fig. 27). The ADPase activity co-chromatographed on DEAE-cellulose with UDPase activity (Fig. 28), and with polynucleotide phosphorylase after subsequent chromatography on Sepharose 6B (Fig. 30), and unlike the ATPase activity showed no sign of cold-lability (Fig. 20).

In E. coli W3092 30% - 40% of the total ATPase activity was membrane-bound compared to 10% - 20% in the NRC 482 strain. This difference is reflected in the higher specific activity

of the membrane-bound ATPase in the former strain and was undoubtably an asset in obtaining a homogeneous preparation of ATPase after only two more purification procedures.

The stepwise elution of solubilized ATPase from DEAE-cellulose gave a 5-fold increase in specific activity in the purification scheme of Kobayashi and Anraku. Since gradient elution of ATPase from DEAE-cellulose had failed to yield an increase in specific activity owing to the large loss of enzyme activity, the stepwise method of Kobayashi and Anraku was adopted in the large scale isolation of ATPase (as described in METHODS AND MATERIALS). This method gave a 16-fold purification of the ATPase (Table XXII).

The ATPase from E. coli W 3092 had a specific activity of 58.4 units/mg protein when homogeneous. Bragg and Hou (196) have subsequently purified ATPase from E. coli NRC 482 to homogeneity at which point its specific activity was 35.9 units/mg protein. The purest preparation of ATPase described in the work reported here had a specific activity of 10.1 units/mg protein. This preparation was shown by polyacrylamide gel electrophoresis to contain one other major and several minor protein contaminants.

Finally, the molecular weight estimate obtained here for the solubilized enzyme (365,000 - 390,000) is probably a more accurate value than that quoted by Kobayashi and Anraku (400,000 - 600,000). Although their determination was also done by gel filtration they used only two molecular weight markers besides blue dextran for determining the void volume. One of

these markers was catalase from bovine liver. This enzyme has been shown to migrate atypically during gel filtration such that its apparent molecular weight is 195,000 (175) as compared to its true molecular weight of 248,000. Since Kobayashi and Anraku used a value of 250,000 for the molecular weight of catalase their estimate of the molecular weight of ATPase (400,000 - 600,000) is probably too high. Another estimate of the molecular weight of the ATPase from E. coli can be obtained by summing the average molecular weights of the individual subunits (Table XXVII) determined by Bragg and Hou (196). There is a great similarity between the solubilized mitochondrial ATPase (F_1) and the solubilized ATPase of E. coli NRC 482 in terms of the pattern of protein bands obtained after gel electrophoresis of the depolymerized enzymes (197). The values obtained for the individual subunits compare favorably, such that if one assumes that there are three each of the two major polypeptides and one each of the three minor polypeptides (this was the subunit stoichiometry determined by Senior and Brooks (197) for beef heart mitochondrial ATPase), the average molecular weight of the E. coli ATPase would be 388,000.

In conclusion the two ATPases are very similar in their properties as would be expected since both came from different strains of E. coli. Thus the results of Kobayashi and Anraku substantiate many of the findings described here.

Table XXVII. Comparison of the properties of ATPases from *E. coli* NRC 482 and mitochondria (beef heart). (Activities measured in the presence of Mg^{2+}.)

Source	*E. coli*	Mitochondria (Beef heart)
References	this thesis, (196)	(104, 105, 197, 198)
Activation by Ca^{2+} and by Mg^{2+}	present	present
Optimum Mg^{2+}:ATP ratio	2:5	2:3
pH optimum	pH 9.5	pH (8.5 - 9.3)
Substrate specificity ATP:ADP:AMP	100:0:0 (with Ca^{2+})	100:0:0
Hydrolysis of other substrates relative to ATP (%)	GTP > UTP > CTP 62%: 27%: 19%	GTP > UTP > CTP 75%: 63%: 0%
Effect of Na^{+} and K^{+}	slight inhibition	none
Molecular weight	375,000	360,000
Cold-lability	labile when soluble	labile when soluble
Subunit molecular weights	56,750 51,750 30,450 20,950 11,500	53,000; 54,000 50,000; 50,000 25,000; 33,000 12,500; 17,300 7,500; 5,700 (197) (198)

Comparison of the solubilized ATPase from E. coli NRC 482 with the membrane-bound enzyme from E. coli B and K12

The membrane-bound ATPase of E. coli B has been well characterized by Evans (57). At the time that this work (57) was published the membrane-bound ATPase of E. coli NRC 482 was being investigated. However in the light of Evans' work, this investigation was not continued. More recently the membrane-bound ATPase of E. coli K12 has been reported on by Roisin and Kepes (58). A comparison of those properties which are shared by both membrane-bound and solubilized ATPases shows that many similarities exist between the two forms of the enzyme. This is particularly true between the B and NRC 482 strains when comparing the pH optima (Fig. 33) or the optimal ion to substrate ratio (Fig. 34). The similarity extends even to contaminants since the membrane-bound ATPase also shows an ADPase activity to be present which can be activated by Mg^{2+} but not by Ca^{2+}.

Conservation of these fundamental properties between the membrane-bound and solubilized states of the ATPase has been observed in nearly all the bacteria studied (see INTRODUCTION). An exception must of course be made for allotopic properties such as cold-lability. In these respects E. coli is similar to B. megaterium, B. sterothermophilus or S. faecalis and none of the properties of the solubilized ATPase as described here are unreasonable in the light of the properties previously reported for the membrane-bound ATPase.

Similarity of the ATPase from E. coli to other bacterial ATPases and to the mitochondrial ATPase

A brief summary of the properties of the solubilized ATPase of E. coli is presented in Table XXVII. The ATPases of various bacteria show many similarities in their properties (Tables I - III, INTRODUCTION). When the data in Table XXVII are compared to that in Table III it can be seen that the ATPase of E. coli is no exception. At the same time many similarities are seen on comparing the bacterial ATPases with the mitochondrial ATPase. This is true too for the ATPase of E. coli as shown in Table XXVII; indeed in this comparison the similarities extend as far as the number and size of the individual subunits that make up the ATPase molecules. With such a close physical relationship existing between the two enzymes it is tempting to postulate that they fulfil identical roles in their respective organisms.

Function of the ATPase in E. coli

To postulate a functional relationship between these two enzymes based solely on their physical characteristics is mere conjecture. However evidence gleaned in the last two years from work done on mutant E. coli deficient in energy conservation indicates that their functions are essentially the same (see INTRODUCTION). The ATPase can participate in the reaction by which "high-energy intermediates" (or a "proton motive force") from the electron transport chain can generate ATP, or the reverse

of this reaction in which ATP from the cytoplasm can generate "high-energy intermediates" (or a "proton motive force"). This reaction scheme is outlined in Fig. 44.

ATPase levels in E. coli

Before this reaction scheme (Fig. 44) was established in which the membrane functions of active transport, oxidative phosphorylation and transhydrogenase are unified through the utilization of the same high-energy source, it was felt that an ATPase might be involved in just one of these functions and that there might be different ATPases for each function. To investigate this possibility the levels of ATPase in E. coli NRC 482 were measured in cells grown under a variety of conditions. This was done to see if there was any induction or repression of this enzyme which could be correlated with the state of the bacteria, such as aerobiosis or efficiency of transport or oxidative phosphorylation.

ATPase levels and oxidative phosphorylation in intact E. coli

Hempfing has measured P/O ratios in intact E. coli (199, 200). He found that during logarithmic growth of the cells on glucose the P/O ratios were low (≈0.1), whereas during the stationary phase of growth on glucose the ratios approached 3, the theoretical maximum. These findings can be explained on the basis of the bacteria metabolizing glucose by glycolysis during the logarithmic

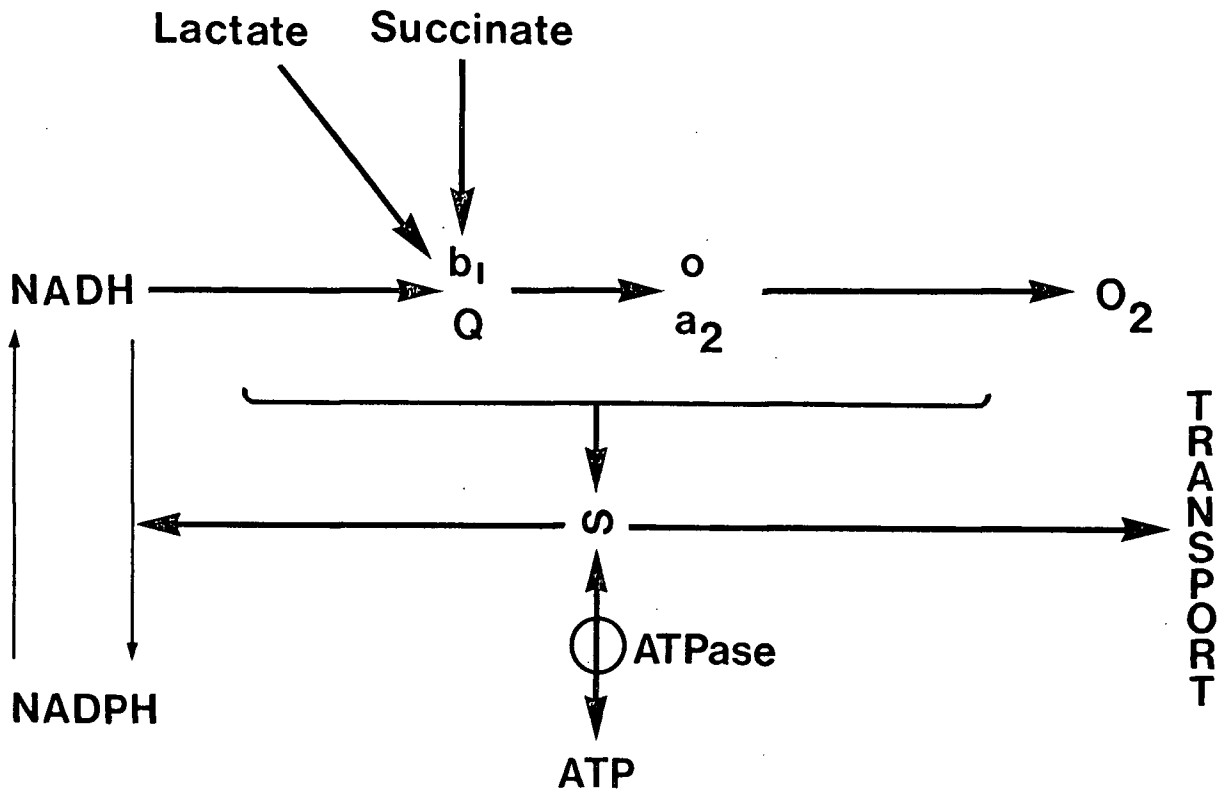


Fig. 44. Schematic representation of the role played by ATPase in energy conservation in E. coli.

A rough outline of the electron transport chain is presented here through which the oxidation of NADH, lactate or succinate can generate "high energy intermediates" or a "proton motive force" as denoted by ∽. The energy of ∽ can be utilized in a number of ways as shown. The postulated position of the ATPase is such that it can act reversibly, either using ∽ to generate ATP by oxidative phosphorylation or producing ∽ from ATP to power the other membrane functions.

phase of growth. In this way ATP is produced by substrate-level phosphorylation. In line with this reasoning cells grown on succinate had a P/O ratio of about 2. In later work (201) Hempfling and Beeman showed that the transition from low to high efficiency of oxidative phosphorylation could be brought about by including cAMP in the growth medium.

If the ATPase of E. coli were involved solely in oxidative phosphorylation it would be conceivable that the level of this enzyme activity would fluctuate with the P/O ratio of the intact cell. From the results in Tables XXII and XXIII it is clear that the growth conditions which produced such a large variation in the P/O ratios as determined by Hempfling and co-workers did not affect the ATPase levels. Indeed the levels of cytochrome b_1 were more variable than the levels of ATPase.

Significance of the constant ATPase levels in E. coli

ATPase levels were not different in the K^+ ion mutants from their parent strain (Table XXV) nor did they vary significantly under conditions of catabolite de-repression or anaerobiosis (Table XXVI). Clearly then the ATPase is a constitutive enzyme.

This conclusion strongly supports the reaction scheme outlined in Fig. 44, for in this scheme there is always a requirement for the ATPase to be present. During glycolysis or anaerobiosis ATP produced by substrate level phosphorylation can be hydrolyzed by the ATPase to generate "high-energy intermediates"

(or a "proton motive force") which in turn can be used for active transport, NAD^+ reduction by reverse electron transfer or transhydrogenation. During the oxidation of TCA cycle intermediates the ATPase would act in the opposite direction to synthesize ATP from ADP and inorganic phosphate.

Conclusion

The findings presented in this thesis indicate that there is only one major ATPase activity in E. coli; that this enzyme activity is very similar to activities found in many other unrelated bacteria; and that these similarities extend to the mitochondrial and chloroplast ATPases. Furthermore these findings are in line with the postulate that ATPase fulfils a major role in energy conservation in E. coli analogous to that held by the mitochondrial ATPase in the mitochondrion.

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