In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Microbiology

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date 30/11/86
Evidence is presented for the existence of an ATPase activity in D. discoideum plasma membranes. This activity was distinct from the mitochondrial ATPase in that it was insensitive to azide and oligomycin. The ATPase was stimulated by Mg$^{+2}$ and to a lesser extent by Ca$^{+2}$ and was not affected by equimolar Na$^+$/$K^+$ or ouabain. Vanadate, DES, thimerosal and DCCD all proved to be partially inhibitory.

Enzyme activity was solubilized with a wide variety of detergents, with lysolecithin giving the best results. Concomitant with solubilization was a partial loss of DES sensitivity which was shown to be due to the presence of a labile DES sensitive ATPase in addition to a stable DES insensitive activity. The DES sensitive ATPase was stimulated by Mg$^{+2}$ but only somewhat by Ca$^{+2}$ whereas the DES insensitive enzyme was stimulated equally by either. The DES sensitive enzyme also displayed Michaelis-Menten kinetics when enzyme activity was measured as a function of the ATP concentration while the DES insensitive ATPase displayed kinetics which were indicative of a sigmoidal relationship between substrate concentration and enzyme activity.

Fractionation of solubilized plasma membranes by ion exchange chromatography resolved two DES insensitive ATPase activities, designated peaks I and II. Peak I was insensitive to vanadate and expressed optimum activity with pyrophosphate. Optimal activity was at alkaline pH values.
Peak II was purified by two different procedures. The first involved an initial separation on DEAE-Sephacel followed by centrifugation through a linear glycerol gradient. The second involved an initial chromatographic fractionation by Sephacryl S-300 gel filtration followed by DEAE-Sephacel ion exchange chromatography of the ATPase containing fractions. Both procedures resulted in preparations that contained a single major component of apparent molecular weight 64 kDa, as assessed by SDS-polyacrylamide gel electrophoresis.

The purified ATPase was sensitive to vanadate and fluoride but insensitive to DCCD, thimerosal and N-ethylmaleimide. The enzyme was activated equally by either Mg\(^{2+}\) or Ca\(^{2+}\) in millimolar concentrations. ATP hydrolysis was also stimulated by millimolar concentrations of Mn\(^{2+}\), Zn\(^{2+}\) or Cu\(^{2+}\). The ATPase displayed sigmoidal kinetics when assayed as a function of ATP concentration in the absence of any divalent cation. Addition of 1 or 10 mM Mg\(^{2+}\) or Ca\(^{2+}\) increased the substrate affinity of the enzyme, while 100 mM divalent cation proved inhibitory. The enzyme was not stimulated by low concentrations of Ca\(^{2+}\) or by Ca\(^{2+}\)-calmodulin, suggesting that it was probably not a Ca\(^{2+}\) pump.
TABLE OF CONTENTS

Abstract ................................................................. ii
Table of Contents ......................................................... iv
List of Tables ............................................................ vi
List of Figures ............................................................ viii
List of Abbreviations ....................................................... xiii
Acknowledgments ........................................................... xi

INTRODUCTION ............................................................. 1

MATERIALS AND METHODS ................................................ 16
I. Materials .................................................................. 16

II. Methods
A. Organisms and Culture Conditions ................................. 16
B. Plasma Membrane Preparation .................................. 17
C. Solubilization of Plasma Membranes ............................ 19
D. Protein Determination .............................................. 20
E. Preparation of Phosphatidylcholine ............................... 20
F. Enzyme Assays ......................................................... 20
G. Polyacrylamide Gel Electrophoresis ............................... 22
H. Purification of ATPase
   i. Sephacryl S-300 Gel Filtration .................................. 23
   ii. Ion Exchange Chromatography on DEAE-Sephacel .......... 23
   iii. Glycerol Gradient Density Centrifugation .................... 24
   iv. Phenyl-Sepharose, Reactive Red-Agarose and ATP-Agarose Chromatography ........................................ 24
I. ³²P Labeling Experiments ............................................ 25
RESULTS

SECTION I. IDENTIFICATION OF ATPASE ACTIVITY IN PURIFIED D. DISCOIDEUM PLASMA MEMBRANES

a. Plasma Membrane Isolation ........................................... 26

b. Characterization of the Plasma Membrane ATPase ............................ 27

c. Solubilization of the Plasma Membrane ATPase .............................. 39

d. Evidence for a Labile DES Sensitive Plasma Membrane ATPase ............... 43

e. Comparison of the Properties of the DES Sensitive and Insensitive ATPase Activities in Plasma Membrane ................................. 50

SECTION II. PURIFICATION OF THE PLASMA MEMBRANE ATPASE ACTIVITY

a. Fractionation of Solubilized ATPase Activity by Ion Exchange Chromatography .... 60

b. Purification of ATPase Activity by Density Gradient Centrifugation ............ 60

c. Affinity and Hydrophobic Chromatography .................................. 69

d. Purification by Gel Filtration .............................................. 69

SECTION III. PROPERTIES OF THE FRACTIONATED DES INSENSITIVE ATPASES

a. Peak I from DEAE-Sephacel ................................................. 78

b. Properties of Purified Peak II .............................................. 78

c. Phosphorylation of the ATPase .............................................. 93

d. Effect of DIF on ATPase Activity ........................................... 97

DISCUSSION ................................................................ 99

REFERENCES ....................................................................... 121
### LIST OF TABLES

| I. | Distribution of enzyme markers during plasma membrane purification | 28 |
| II. | Distribution of hydrolyzed products resulting from incubation of plasma membranes with $[^3H]$-ATP | 30 |
| III. | Effect of ouabain on plasma membrane ATPase activity | 34 |
| IV. | Utilization of other nucleotides by the plasma membrane Mg$^{2+}$-ATPase | 37 |
| V. | Effect of 100 mM KCl on plasma membrane ATPase activity | 40 |
| VI. | Effect of exogenous phospholipid on solubilized DES sensitive ATPase activity | 53 |
| VII. | Effect of DES on peaks I and II ATPase activity | 62 |
| VIII. | ATPase activity from peaks I and II from DEAE-Sephacel | 63 |
| IX. | Purification of the DES-insensitive plasma membrane ATPase | 68 |
| X. | Affinity chromatography of peak II ATPase | 70 |
| XI. | Phenyl-Sepharose chromatography of peak II ATPase activity | 71 |
| XII. | Purification of the plasma membrane ATPase | 77 |
| XIII. | Substrate specificity of peak I | 80 |
| XIV. | Effect of divalent cations on peak I enzyme activity | 81 |
| XV. | Effect of various cations on purified ATPase activity | 88 |
| XVI. | Effect of monovalent cations on Mg$^{2+}$ or Ca$^{2+}$ stimulated ATPase activity | 89 |
XVII. Substrate specificity of the purified ATPase.........................91
XVIII. Effect of calmodulin on purified ATPase activity................94
XIX. Effect of DIF on ATPase activity..............98
XX. Comparison of the purified D. discoideum plasma membrane ATPase with several other membrane bound phosphatases......110
LIST OF FIGURES

Figure 1. Life cycle of Dictyostelium discoideum..........................2

Figure 2. Effect of azide and oligomycin on plasma membrane and mitochondrial ATPase activity.....................29

Figure 3. Stimulation of plasma membrane ATPase activity by Mg\(^+\)\(^2\) and Ca\(^+\)\(^2\)........32

Figure 4. The effect of various cations on plasma membrane ATPase activity..........................33

Figure 5. The effects of various inhibitors on plasma membrane ATPase activity.....35

Figure 6. Inhibition of plasma membrane ATPase activity by N-ethylmaleimide....36

Figure 7. Developmental changes in plasma membrane ATPase activity..............38

Figure 8. Solubilization of plasma membrane ATPase activity with lysolecithin......41

Figure 9. Detergent solubilization of plasma membrane ATPase activity.............44

Figure 10. Effect of exogenous phosphatidylcholine on deoxycholate and Z-14 extracted ATPase..........................46

Figure 11. Effect of DES on the plasma membrane bound and solubilized ATPase activity.......................................47

Figure 12. Inactivation of the DES sensitive ATPase upon solubilization with several detergents...............48

Figure 13. Effect of lysolecithin, Z-14 and deoxycholate solubilization on DES sensitive and insensitive ATPase activity........................................51

Figure 14. Effect of Ca\(^+\)\(^2\) and Mg\(^+\)\(^2\) on DES sensitive and insensitive ATPase activity.........................54
Effect of ATP concentration on DES sensitive and insensitive ATPase activity ........................................ 56
The effect of Mg$^+$ and Ca$^+$ on ATP hydrolysis kinetics of the DES sensitive ATPase ...................... 57
The effect of Mg$^+$ and Ca$^+$ on ATP hydrolysis kinetics of the DES insensitive ATPase ............... 58
The effect of pH on the DES sensitive and insensitive ATPase .............................................................. 59
DEAE-Sephacel chromatography of detergent solubilized plasma membranes ..................................... 61
SDS-polyacrylamide gel electrophoresis of various fractions obtained during the purification of the DES insensitive ATPase .......................................................... 64
Glycerol gradient centrifugation of peak II ATPase ......................................................... 66
Calibration curve of protein markers on glycerol density gradients .............................................. 67
Gel filtration chromatography in the presence of 1% (w/v) BRIJ-35 of detergent solubilized plasma membranes ................................................................. 72
Gel filtration chromatography in the presence of 0.5% (w/v) CHAPS of detergent solubilized plasma membranes ................................................................. 73
SDS-polyacrylamide gel electrophoresis of fractions obtained during the purification of the DES insensitive ATPase .......................................................... 74
DEAE-Sephacel chromatography of ATPase fraction eluted from the gel filtration column ............. 75
Effect of pH on peak I ATPase activity .................................................................................. 79
Effect of pH on the purified ATPase activity ................................................................................ 82
Figure 29. Effect of various inhibitors on purified ATPase activity ........... 84

Figure 30. Effect of N-ethylmaleimide on purified ATPase activity ........... 85

Figure 31. Inhibition of purified ATPase activity by high concentrations of Ca$^{+2}$ and Mg$^{+2}$ ..................... 86

Figure 32. Effect of Mg$^{+2}$ or Ca$^{+2}$ on purified ATPase activity ........... 87

Figure 33. Effect of divalent cations on purified ATPase activity ............. 90

Figure 34. Effect of Mg$^{+2}$ or Ca$^{+2}$ on ATPase activity determined as a function of the ATP concentration ............. 92

Figure 35. Autoradiography of $^{32}$P-labeled detergent solubilized plasma membranes .................................. 95

Figure 36. Autoradiography of $^{32}$P-labeled crude membranes ............. 96
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>adenylyl 5'-yl imidodiphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine 5'-triphosphatase</td>
</tr>
<tr>
<td>C$_{12}$$^E_9$</td>
<td>polyoxyethylene 9-lauryl ether</td>
</tr>
<tr>
<td>CHAPS</td>
<td>(3-[3-cholamidopropyl]dimethylammonio)1-propanesulfonate</td>
</tr>
<tr>
<td>CTP</td>
<td>cytidine 5'-triphosphate</td>
</tr>
<tr>
<td>DCCD</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCPIP</td>
<td>dichlorophenol indophenol</td>
</tr>
<tr>
<td>DES</td>
<td>diethylstilbestrol</td>
</tr>
<tr>
<td>DIF</td>
<td>differentiation inducing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol bis(α-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>MES</td>
<td>2[N-Morpholine]ethanesulfonic acid</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>Pi</td>
<td>orthophosphate</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-nitrophenylphosphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PPi</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Tris</td>
<td>tri(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TMB-8</td>
<td>(8-diethylamino)octyl-3,4,5-trimethoxybenzoate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5′-triphosphate</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

I would like to express my appreciation to Dr. G. Weeks for his patient guidance and helpful suggestions. I would also like to thank all my friends and lab mates for putting up with my bad temper and generally miserable disposition. In addition, I thank Dr. P. Bhanot for the many stimulating conversations over coffee, and the University of British Columbia for financial assistance in the form of a Frank Wesbrook Fellowship.

Finally, I want to thank my wife Kathy for typing this thesis and for being a never ending source of encouragement.
**INTRODUCTION**

*Dictyostelium discoideum* is a eukaryotic organism that exhibits a very distinctive developmental cycle (Loomis, 1975). The myxamoeboid form of the organism feeds on bacteria and divides by binary fission, but when the food supply is exhausted the cells initiate a complex sequence of morphological changes which ultimately results in the formation of a multicellular structure composed of two cell types, stalks and spores (Loomis, 1975). These stages are detailed in Figure 1. Upon cessation of growth the cells aggregate in response to pulses of cAMP (Gerisch et al., 1975; Darmon et al., 1975) to form a migratory slug or pseudoplasmodium. At this stage the two cell types become distinguishable for the first time as distinct pre-stalk and pre-spore cells. These cell types have a definite spatial orientation, with pre-stalk cells in the anterior and pre-spore cells in the posterior portion of the pseudoplasmodium (Loomis, 1975).

Following a period of pseudoplasmodial migration, the terminal stage of differentiation, known as culmination, begins. This process resembles a reverse fountain flow (Farnsworth, 1973) in which the pre-stalk cells move down toward the substratum, maturing into stalk cells and forming a tube with a rigid cellulosic wall. As the stalk is formed the pre-spore cells are raised from the substrate to the top of the column where they mature to spore cells. (Farnsworth,
Figure 1
Life cycle of *Dictyostelium discoideum*
1973). Under laboratory conditions this process is complete in approximately 24 hours. The relative simplicity of this developmental cycle makes *D. discoideum* a model system for studies on cell differentiation.

There have been reports that a variety of ions are involved at various stages of the developmental cycle. Mason *et al.* (1971) observed that aggregation was effectively halted when the external Ca$^{+2}$ concentration was dropped below 10$^{-6}$ M. In addition, Brachet and Klein (1977) reported that aggregation was greatly accelerated when amoebae were exposed to the Ca$^{+2}$ ionophore A23187. In contrast, Saito (1979) reported that, while the number of mature fruiting bodies was dependent on the Ca$^{+2}$ concentration, amoebae responded to extracellular cAMP even when the external Ca$^{+2}$ concentration was below 10$^{-8}$ M. Saito concluded that aggregation was a Ca$^{+2}$ independent phenomenon, although the cation was necessary for normal morphogenesis (Saito, 1979). Recently, Europe-Finner *et al.* (1984) showed that chemotaxis was strongly inhibited by EGTA induced Ca$^{+2}$ depletion, indicating an active role for the cation during aggregation.

Calcium has also been shown to inhibit adenylate cyclase in vitro (Loomis *et al.*, 1978) and to increase the number of cell surface cAMP binding sites without altering their affinity constants (Juliani and Klein, 1977). It has also been proposed that Ca$^{+2}$ regulates the polymerization of
actin and its association with myosin (Mokrin and Spudich, 1976; Spudich and Spudich, 1982).

Evidence for Ca$^{+2}$ flux during aggregation has also been obtained. Wick et al. (1978) observed a transient, rapid influx of $^{45}$Ca$^{+2}$ into starved amoebae upon excitation with cAMP. This initial influx was followed by a reappearance of the sequestered $^{45}$Ca$^{+2}$ into the medium several minutes later (Wick et al., 1978). Bumann et al. (1984) extended this work to show that Ca$^{+2}$ uptake occurred in an oscillatory fashion with a period of roughly 8 minutes and an amplitude of 3.5x10$^6$ ions per cell.

There have been some reports regarding the mechanism of Ca$^{+2}$ movement across the plasma membrane in *D. discoideum*. Working with intact cells, Parish and Weibel (1980) presented evidence for an ecto-ATPase in that extracellular [$^{14}$C]-ATP was hydrolyzed and $^{45}$Ca$^{+2}$ uptake was reduced by the uncouplers KCN, CCCP and dinitrophenol and by non-hydrolysable ATP analogues. In most instances this reduction was less than 50% and Ca$^{+2}$ uptake was generally very slow, reaching saturation after 20 minutes. Europe-Finner and Newell (1985a), on the other hand, reported Ca$^{+2}$ uptake to be extremely rapid and unaffected by pre-treatment of cells with azide, suggesting that Ca$^{+2}$ moved across the membrane via passive facilitated diffusion.

Recently, Ca$^{+2}$ has been implicated in the cAMP or folate induced formation of intracellular cGMP (Small et al., 1986). The accumulated evidence suggested that this
phenomenon was due to mobilization of intracellular Ca$^{+2}$ rather than transport across the membrane, since the effect of cAMP could be mimicked by inositol 1,4,5-triphosphate (Europe-Finner and Newell, 1985b), which has subsequently been shown to cause Ca$^{+2}$ release from non-mitochondrial pools in *Dictyostelium* amoebae (Europe-Finner and Newell, 1986). Furthermore, cGMP formation was inhibited by the intracellular Ca$^{+2}$ antagonist (8-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) (Europe-Finner and Newell, 1984; Europe-Finner et al., 1985).

Proton flux has also been implicated in *D. discoideum* development. It was proposed that changes in intracellular pH play an important role in differentiation with acid pH favoring stalk cell formation and alkaline pH favoring spore cell formation (Gross et al., 1983). Furthermore, the stalk cell differentiation inducing factor (DIF) was hypothesized to inhibit a putative cell surface proton pump, thus effecting a decrease in the intracellular pH and diverting cells from the spore to the stalk pathway (Gross et al., 1983).

Subsequent work has failed to detect any sustained change in the intracellular pH during differentiation. Jentoft and Town (1985) detected two pH peaks using $^{31}$P-NMR spectroscopy, the mitochondrial peak at pH 7.16 ± 0.03 and the cytosolic peak at pH 6.48 ± 0.02. No change in the pH occurred up to 13.5 hours of development (Jentoft and Town, 1985). A similar result was obtained by Kay et al. (1986)
although they obtained pH measurements which were somewhat higher than those seen by Jentoft and Town (1985). In another study, Ratner (1986) found the intracellular pH values of pre-spore and pre-stalk cells to be indistinguishable. The only report of a developmental change in intracellular pH is that of Jameison et al. (1984), who observed a shift from pH 6.3 to pH 7.13 after about two hours of development. This shift, however, was extremely short lived (approximately 15 minutes) and the intracellular pH quickly returned to the original value.

Proton flux has been observed during the early stages of *D. discoideum* development. Malchow et al. (1978a) reported transient decreases in extracellular pH when unbuffered cell suspensions were exposed to cAMP. Subsequent work revealed the extracellular pH to oscillate in conjunction with the pulsatile release of cAMP (Malchow et al., 1978b) suggesting that the two phenomena were linked. Recently Gottmann and Weijer (1986) have shown these proton oscillations to be inhibited by caffeine, indicating that they are coupled to cAMP oscillations. Caffeine has been shown to inhibit aggregation by interfering with adenlyate cyclase activation (Brenner and Thoms, 1984). In addition to these observations it has been reported that the Na\(^+\)/H\(^+\) antiport inhibitor amiloride disrupts normal development (Jamieson et al., 1984). These data suggest that proton fluxes may play an active role in differentiation, but there is as yet no direct evidence to
indicate that changes in internal pH are involved in the regulation of stalk and spore cell development.

Other ions have also been implicated in the differentiation process. Town (1984) observed that both the Na\(^+\),K\(^+\)-ATPase inhibitor scillaren and the K\(^+\) ionophore valinomycin inhibited spore cell development without affecting stalks when cells were differentiating in submerged monolayers. These data suggested a role for K\(^+\) in spore cell development since, under the experimental conditions used, the primary effect of both of these compounds would be to reduce the intracellular K\(^+\) concentration. Other ionophores such as the Na\(^+\)-specific ionophore monensin and the K\(^+\)/H\(^+\) exchanger nigericin inhibited the differentiation of both cell types (Town, 1984).

In view of the apparent importance of ions and ion flux in *D. discoideum* development, it would be advantageous to understand the underlying mechanism of ion transport in more detail. In many cases the transport of ions across the membrane is facilitated by ATP driven pumps. The best characterized of these enzymes are the Na\(^+\),K\(^+\)-ATPases responsible for Na\(^+\)/K\(^+\) transport, the Ca\(^{2+}\)-ATPases which translocate Ca\(^{2+}\) ions and the proton pumping Mg\(^{2+}\)-ATPases.

The Na\(^+\),K\(^+\)-ATPase has been purified from a number of sources including shark rectal gland (Hokin et al., 1973; Dixon and Hokin, 1978; Hastings and Reynolds, 1979), electroplax tissue from electric eels (Dixon and Hokin,
Dixon and Hokin, 1974) and kidney medulla from dogs (Kyte, 1971) and pigs (Jorgensen, 1974). This enzyme catalyzes the exchange of three Na\(^+\) for two K\(^+\) (Scarborough, 1982) and is generally characterized by its sensitivity to ouabain (Lane et al., 1973; Kyte, 1974; Wallick et al., 1978; Schoner et al., 1979).

The purified Na\(^+\),K\(^+\)-ATPase contains two polypeptides, a large, relatively hydrophobic \(\alpha\) subunit and a small glycosylated \(\beta\) subunit (Lane et al., 1973; Dixon and Hokin, 1974; Kyte, 1974; Perrone et al., 1975). The \(\alpha\) subunit has been shown to be phosphorylated (Kyte, 1971; Lane et al., 1973; Wallick et al., 1978; Bonting et al., 1979) and to contain the ouabain binding site (Ruoho and Kyte, 1974; Rogers et al., 1979; Charlemagne et al., 1986). The function of the \(\beta\) subunit is not known. Reported values for the molecular weight of the \(\alpha\) chain vary between 85 to 140 kDa (Hastings and Reynolds, 1979; Esmann et al., 1979; Winter and Moss, 1979; Lane et al., 1979; Craig and Kyte, 1980) while those for the \(\beta\) chain vary between 35 to 70 kDa (Lane et al., 1973; Hastings and Reynolds, 1979; Esmann et al., 1979; Winter and Moss, 1979; Lane et al., 1979; Craig and Kyte, 1980).

Much work has focussed on the stoichiometry of the two subunits. Perrone et al. (1975) proposed a stoichiometry of \(\alpha_2 \beta\) based on a minimum molecular weight of active ATPase of 250 kDa, as determined by radiation inactivation. Hastings and Reynolds (1979) used equilibrium analytical
centrifugation to determine a minimum molecular weight of 379.9 kDa, with the protein composed of two α and four β subunits. Esmann et al. (1979) estimated a 1:1 stoichiometry and determined the enzyme structure to be $\alpha_2\beta_2$. Craig and Kyte (1980), in a comprehensive crosslinking study, arrived at a molecular weight of 177 kDa and the subunit composition $\alpha/\beta$. The findings of Craig and Kyte (1980) have recently been confirmed by Jorgensen and Andersen (1986) who used sedimentation equilibrium centrifugation to show active ATPase with a quaternary structure of $\alpha/\beta$.

The proton translocating Mg$^{2+}$-ATPases that have been best characterized are those from the filamentous fungus Neurospora crassa (Scarborough, 1980; Bowman et al., 1981a; Addison and Scarborough, 1981) and the fission yeast Schizosaccharomyces pombe (Dufour and Goffeau, 1978; Villalobo et al., 1981). These proteins are major membrane constituents and relatively little purification has been required to produce homogeneous enzyme, which migrates during SDS-PAGE as a single polypeptide of 104 kDa (Dufour and Goffeau, 1978; Addison and Scarborough, 1981; Bowman et al., 1981a; Scarborough and Dame, 1984).

ATP driven proton translocation by these enzymes has been shown to be electrogenic in that it occurs concurrently with the formation of a membrane potential (Scarborough, 1980; Villalobo et al., 1981; Dufour et al., 1982; Perlin et al., 1984). It is generally believed that these enzymes
catalyze only the movement of protons across the membrane, although Villalobo (1982, 1984) has presented evidence for an energy dependent, vanadate sensitive $\text{H}^+\text{-K}^+$ exchange. It was proposed that the $\text{K}^+$ gate opened only upon the imposition of a membrane potential (Villalobo, 1984). The stoichiometry of the $\text{H}^+,\text{K}^+$ exchange was not determined.

Proton translocation and ATPase activity are sensitive to vanadate, diethylstilbestrol (DES), dicyclohexylcarbodiimide (DCCD) and mercurials such as thimerosal and $p$-chloro-mercuriphenyl sulfonate (Bowman and Slayman, 1979; Scarborough, 1980. Bowman et al.; 1981; Amory and Goffeau, 1982; Dufour et al., 1982; Perlin et al., 1984). The *Neurospora* enzyme is also strongly inhibited by N-ethylmaleimide (Brooker and Slayman, 1982, 1983a).

Like the $\text{Na}^+,\text{K}^+$-ATPase the proton ATPases of yeast and *Neurospora* form phosphorylated intermediates as part of the reaction cycle (Dame and Scarborough, 1980; Amory et al., 1980). This intermediate was characterized as being an acid-stable $\beta$-aspartyl phosphate (Dame and Scarborough, 1981; Amory and Goffeau, 1982). Similarly, like the $\text{Na}^+\text{-K}^+$-ATPase, the *Neurospora* enzyme has been shown to undergo a series of conformational changes during the catalytic cycle (Addison and Scarborough, 1982).

Plasma membrane proton translocating ATPases have also been characterized from a variety of plant species (Vara and Serrano, 1982; Sze, 1983; O'Neill and Spanswick, 1984a, 1984b, Vara and Serano, 1982). These enzymes are very
similar to those of *Neurospora* and yeast in that they are inhibited by vanadate, DCCD, DES and mercurials. However, the plant ATPases are stimulated considerably by monovalent cations, particularly K\(^+\) (Vara and Serrano, 1982; O'Neil and Spanswick, 1984a, 1984b, Brisken and Poole, 1984; Imbrie and Murphy, 1984), so in this respect they differ from the *Neurospora* and yeast enzymes which are only marginally stimulated by these cations (Bowman *et al.*, 1981).

The third major class of cell surface ATPases are the Ca\(^{2+}\)-ATPases. Initial work on rat kidney (Parkinson and Radde, 1971), guinea pig placenta (Shami and Radde, 1971) and mouse liver (Garnett and Kemp, 1975), revealed an ATPase which was stimulated by millimolar concentrations of either Ca\(^{2+}\) or Mg\(^{2+}\). Subsequently, in addition to this low affinity ATPase activity, a high affinity activity, stimulated by submicromolar concentrations of Ca\(^{2+}\) in the presence of low (5 µM) concentrations of Mg\(^{2+}\), has also been identified (Lynch and Cheung, 1979; Penniston, 1983; Carafoli *et al.*, 1984). That the low and high affinity activities are attributable to different enzymes has been shown by the fact that they can be separated by gel filtration and ion exchange chromatography (Lotersztajn *et al.*, 1981; Niggli *et al.*, 1981). The high affinity enzyme is generally referred to as the (Ca\(^{2+}\):Mg\(^{2+}\))-ATPase while the low affinity enzyme is the Ca\(^{2+}\) or Mg\(^{2+}\)-ATPase. Many high affinity (Ca\(^{2+}\):Mg\(^{2+}\))-ATPases are stimulated by calmodulin (Lynch and Cheung, 1979; Niggli *et al.*, 1981;
Kotagal et al., 1983; Caroni et al., 1983; Oliva et al., 1983; Ansah et al., 1984) which acts by increasing the velocity of ATP hydrolysis and decreasing the $K_m$ of the enzyme for Ca$^{2+}$. In some cases, addition of exogenous calmodulin to plasma membranes had no effect on ATPase activity (Verma and Penniston, 1981; Ochs and Reed, 1984; Debetto and Cantley, 1984; Tsukamoto et al., 1986), although this may have been due to the difficulty encountered in removing endogenous calmodulin from the membranes (Verma and Penniston, 1981; Ochs and Reed, 1984; Debetto and Cantley, 1984). Lotersztajn et al. (1981) have reported the purification of a (Ca$^{2+}$:Mg$^{2+}$)-ATPase from rat liver plasma membranes which was activated by an endogenous protein distinct from calmodulin and acidic phospholipids have been observed to substantially activate the (Ca$^{2+}$:Mg$^{2+}$)-ATPases from erythrocytic and pancreatic acinar plasma membranes (Niggli et al., 1981; Ansah et al., 1984).

The plasma membrane (Ca$^{2+}$:Mg$^{2+}$)-ATPases are resistant to the mitochondrial inhibitors azide, oligomycin and DCCD and to ouabain (Verma and Penniston, 1981; Ochs and Reed, 1984; Debetto and Cantley, 1984). Many of these enzymes are, however, sensitive to vanadate (Niggli et al., 1981; Gmaj et al., 1982; Debetto and Cantley, 1984), although Tsukamoto et al. (1986) have reported the existence of a vanadate resistant, Mg$^{2+}$-independent Ca$^{2+}$-ATPase from renal basolateral plasma membranes. Debetto and Cantley (1984)
have also reported that the \((\text{Ca}^{2+}:\text{Mg}^{2+})\)-ATPase from murine erythroleukemia membranes is sensitive to lanthanum ions.

The \((\text{Ca}^{2+}:\text{Mg}^{2+})\)-ATPases are believed to play a role in the maintenance of intracellular \(\text{Ca}^{2+}\) by means of energy dependent extrusion. Many such enzymes have been shown to transport \(\text{Ca}^{2+}\) across the membrane (Niggli et al., 1981; Caroni et al., 1983; Kotagal et al., 1983; Breitbart et al., 1983, Ochs and Reed, 1984) however the transmembrane orientation of the enzyme in intact cells was not determined. Evidence has been presented which suggests that the high affinity \(\text{Ca}^{2+}\)-ATPases from pancreatic acinar and renal basolateral plasma membranes are ecto-ATPases (Ansah et al., 1984; Tsukamoto et al., 1986). However, neither of these reports contained any data on \(\text{Ca}^{2+}\) pumping.

The \((\text{Ca}^{2+}:\text{Mg}^{2+})\)-ATPases form phosphorylated intermediates of molecular weight 130 to 140 kDa during the catalytic cycle (De Smedt et al., 1983; Debetto and Cantley, 1984; Ansah et al., 1984). This intermediate is similar to that seen for the \(\text{H}^{+}\)-ATPases in that it is stable at acid pH but is rapidly hydrolyzed at mildly alkaline pH (Debetto and Cantley, 1984; De Smedt et al., 1983).

There appears to be a certain amount of structural homology between the various types of membrane transport ATPases. Walderhaug et al. (1985) have demonstrated active site sequence homology between the \(\text{Na}^{+},\text{K}^{+}\)-ATPase from dog kidney, \(\text{Na}^{+},\text{H}^{+}\)-ATPase from gastric mucosa and the \(\text{H}^{+}\)-ATPase from corn roots. The common sequence around the active site
was found to be cys-(ser/thr)-asp-lys and in all cases asp was the target of phosphorylation. Serrano et al. (1986) have determined the DNA sequence for the gene encoding the yeast H^+-ATPase. Comparison between this sequence and those of the Na^+,K^+-ATPase from kidney (Shull et al., 1985) and the sarcoplasmic reticulum Ca^{2+}-ATPase (McLennan et al., 1985) revealed nine areas of extensive homology (Serrano et al., 1986). Interestingly, very little sequence homology was seen when these enzymes were compared with F_o-F_1 ATPases of bacteria, chloroplasts and mitochondria (Serrano et al., 1986).

At the outset of the work presented in this thesis only two reports existed concerning plasma membrane ATPases in D. discoideum. Parish and Weibel (1980) had presented evidence suggesting the existence of a cell surface ecto ATPase and Blanco (1982) had reported a Na^+, K^+ stimulated, azide sensitive ATPase. In addition, Parish and Weibel (1980) also suggested a link between ectopic ATPase activity and Ca^{2+} transport in that ^{45}Ca^{2+} transport was depressed by agents which served to decrease ATPase activity.

In light of these results, and in consideration of the apparent role of ions and ion transport in D. discoideum it would seem advantageous to understand the underlying mechanism which facilitates these phenomena. Plasma membrane ATPases have been well characterized in many other systems and have been shown to perform an important role in the regulation of intracellular ionic homeostasis. It is
possible that such enzymes play an equally important role in

\textit{D. discoideum} development by regulating the transmembranous

movement of ions described earlier in this introduction.

The primary goal of this study was to systematically

identify and characterize plasma membrane ATPase activity in

\textit{D. discoideum} and to attempt to define a role for the

putative ATPase in developmental regulation. Evidence is

presented for the existence of three, separate plasma

membrane ATPases. In addition, one of these enzymes was

purified and some of its properties were explored. A

preliminary description of this work has been published

(MacDonald and Weeks, 1984).

While this work was in progress, Pogge-von Strandmann

e\textit{al.} (1984) and Serrano \textit{et al.} (1985) published work on

the existence of a vanadate and DES sensitive ATPase in

purified plasma membranes. Pogge-von Strandmann \textit{et al.}

(1984) also demonstrated ATP dependent proton translocation

in reconstituted phosphatidylcholine vesicles.
MATERIALS AND METHODS

I. MATERIALS

Materials. Bacteriological peptone and yeast extract were from Oxoid and Bacto-Agar was from Difco. Tris base, sodium orthovanadate and sodium azide were from Fisher Scientific. Sucrose was of special enzyme grade and was obtained from Schwarz-Mann. Triton X-100 was from Amersham. Lysolecithin and Z-14 were from Calbiochem and sodium dodecyl sulfate (specially pure grade) was from BDH chemicals. Acrylamide and bis-acrylamide, ammonium persulfate, glycine, TEMED and the silver staining kit were from Bio-Rad. [2,8-\textsuperscript{3}H]-ATP was from ICN and [\gamma\textsuperscript{32}P]-ATP and Liquifluor were from New England Nuclear. Sephacryl S-300, DEAE-Sephacel and Phenyl-Sepharose were purchased as pre-swollen beads from Pharmacia. Reactive Red-Agarose and ATP-Agarose were from Sigma. All other chemicals were of reagent grade from Fisher Scientific or Sigma Chemical Co.

II. METHODS

A) Organisms and Culture Conditions

\textit{D. discoideum} wild type strain $V_{12}M_2$ and the axenic mutant Ax-2, obtained originally from Drs. G. Gerisch and J.M. Ashworth, were used throughout.

Strain $V_{12}M_2$ was grown in association with \textit{Enterobacter aerogenes} on nutrient agar (Sussman, 1966) plates until clearing of the bacterial lawn was discernible. The cells
were harvested from growth plates using Bonner's salts (Bonner, 1947) and washed free of residual bacteria by repeated differential centrifugation at 700 x g. Routinely four centrifugations were used.

The axenic mutant Ax-2 was grown in HL-5 media (Weeks and Weeks, 1975) at 22°C on a gyratory shaker, to a density of $5 \times 10^6$ to $10^7$ cells/ml. The cells were harvested by centrifugation at 700 x g and washed twice with Bonner's salts (Bonner, 1947).

B) Plasma Membrane Preparation

Plasma membranes were prepared according to the procedure of Gilkes and Weeks (1977b), with several modifications. Harvested cells which had been washed with Bonner's salts were resuspended in buffer containing 10 mM Tris-Cl pH 7.5, 8.6% (w/v) sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM p-amino-benzamidine, centrifuged at 700 x g and resuspended in the same buffer at a density of $10^8$ cells/ml. The cells were broken by mechanical agitation with glass beads (0.45 to 0.5 mm diameter, 3.3 g/10^8 cells). Unbroken cells, nuclei and intact mitochondria were removed by centrifugation at 10,000 RPM for 15 minutes (10K pellet) in an SS34 rotor. The supernatant was collected and centrifuged at 100,000 x g for 1 hr. The resulting pellet (40K pellet) was resuspended in buffer containing 10 mM Tris-Cl pH 7.5, 20% (w/v) sucrose, 0.1 mM PMSF, 1 mM p-aminobenzamidine and 0.1 mM EDTA and was centrifuged at
100,000 x g. The pellet was then resuspended in 12 ml of the same buffer to give a protein concentration of approximately 15 mg/ml and 2 ml was layered onto each of 6 discontinuous sucrose gradients (15 ml 44%; 10 ml 41.6% and 10 ml 39.3% (w/v) sucrose in 10 mM Tris-Cl pH 7.5 buffer containing 0.1 mM PMSF, 0.1 mM EDTA and 1 mM p-aminobenzamidine). The gradients were centrifuged at 23,000 RPM in a Beckman SW27 rotor for 16 to 18 hours and the bands enriched for plasma membranes were collected.

The plasma membranes were diluted with 10 to 15 volumes of 10 mM Tris-Cl pH 7.5 and centrifuged at 100,000 x g for 1 hour. The resulting pellet was resuspended in 10 mM Tris-Cl pH 7.5, 100 mM KCl, 2 mM EDTA, centrifuged and washed once in 10 mM Tris-Cl pH 7.5. Following a final centrifugation at 100,000 x g the pellet was resuspended in buffer containing 10 mM Tris-Cl pH 7.5, 20% (v/v) glycerol, 40 μg/ml leupeptin and stored at -70°C.

Crude membranes were prepared in a similar manner. Cells were disrupted with glass beads and the homogenate centrifuged at 10,000 RPM in an SS34 rotor. The resulting supernatant was then centrifuged at 100,000 x g for 1 hour. The pellet was resuspended in 100 mM KCl/2mM EDTA in 10 mM Tris-Cl pH 7.5. Following an additional centrifugation at 100,000 x g the pellet was suspended in 10 mM Tris-Cl pH 7.5, 20% (v/v) glycerol and 40 μg/ml leupeptin and stored at -70°C.
For some experiments crude membranes were obtained according to the procedure of Das and Henderson (1983) with minor modifications. Cells were harvested and washed as described above, resuspended at a concentration of 2 x 10^8 cells/ml in buffer containing 5 mM Tris-Cl pH 8.5, 1 mM p-aminobenzamidine and 0.1 mM PMSF and lysed by forced passage through a Nucleopore polycarbonate filter (25 mm diameter, 5 μm pore size) placed in a Swin-Lok Holder (Nucleopore Corporation). The homogenate was then centrifuged at 5,000 x g for 30 minutes and the pellet was resuspended in 10 mM Tris-Cl pH 7.5 and stored at -70°C.

C) Solubilization of Plasma Membranes

The plasma membranes were resuspended at a protein concentration of 5 mg/ml in 10 mM Tris-Cl pH 7.5, 20% (v/v) glycerol. Detergent was added from a 10% (w/v) stock solution to give the desired final concentration and the solution was incubated, with stirring, at 0°C for 15 minutes. The supernatant from centrifugation at 100,000 x g was the solubilized protein fraction. The remaining pellet was resuspended in an equal volume of 10 mM Tris-Cl pH 7.5, 20% (v/v) glycerol.
D) Protein Determination

The Folin procedure, as modified by Sandermann and Strominger (1972), was used throughout.

E) Preparation of Phosphatidylcholine

Egg yolk (Type II) phosphatidylcholine was washed according to the procedure of Kogawa and Racker (1971), with several modifications. Approximately 700 mg of the phospholipid was stirred at 4°C for 48 hours in 100 ml acetone containing 1 mg/ml butylated hydroxytoluene. The acetone was then decanted and the phospholipid transferred to a 35 ml siliconized glass screw cap test tube and washed twice with 30 ml acetone. The phospholipid was dissolved in anhydrous ether and was transferred to a tared glass beaker. Most of the ether was then evaporated off under a stream of nitrogen and residual solvent removed by storing the lipid in vacuo for 24 hours.

The phospholipid was reconstituted to a concentration of 50 mg/ml in 10 mM MES/Tris pH 6.8 and sonicated on ice until clear. This preparation was then used for studies on ATPase activity.

F) Enzyme Assays

The ATPase activity was assayed at 30°C by the release of inorganic phosphate. Unless indicated otherwise in the figure legends, the reaction mixtures contained, in a final volume of 1.0 ml, 3 mM ATP, 10 mM MgCl₂ and enzyme protein in 10 mM MES/Tris pH 6.8. After a 10 minute pre-incubation
reactions were initiated by the addition of ATP and were terminated after 15 to 60 minutes by the addition of 0.1 ml 10% (w/v) TCA and 1% (w/v) SDS. Inorganic phosphate was measured as described by Ames (1966) by the addition of 0.1 ml of the reaction mixture to 0.2 ml 1% (w/v) SDS and 0.7 ml of the ammonium molybdate reagent (six parts 0.42% (w/v) ammonium molybdate, 0.5% (w/v) SDS; in 1N H$_2$SO$_4$ to one part 1% (w/v) SDS, 1% (w/v) ascorbic acid). Solutions were incubated at 45°C for 15 minutes and the absorbance was read at 660 nm.

In some cases [2,8-$^3$H]-ATP (specific activity 200,000 cpm/μmol) was used as the substrate. Following the termination of the reaction 0.025 ml aliquots were spotted onto PEI-cellulose TLC plates (Polygram CEL 300; Mackery-Nagel, West Germany). The plastic plates were developed initially with a mobile phase of 0.1 M LiCl to 4 cm above the origin, followed directly by 1 M LiCl to 13 cm above the origin. Nucleotide spots were visualized under UV light, excised with scissors and the radioactivity determined by liquid scintillation counting.

Alkaline phosphatase was determined by the hydrolysis of p-nitrophenyl phosphate. The incubation mixtures contained 1 mM p-nitrophenylphosphate (pNPP), 20 mM MgCl$_2$, 30 mM NaF, 20 mM Tris-Cl pH 8.5 and enzyme in a final volume of 1 ml. Reactions were carried out at 30°C and were terminated by the addition of 1.0 ml of 1 M Na$_2$CO$_3$. Enzyme activity was measured spectrophotometrically at 410 nm and
an extinction coefficient of $E_{1M} = 1.62 \times 10^4$ was used to calculate the quantity of p-nitrophenol formed.

Succinate dehydrogenase was assayed as described by Gilkes and Weeks (1977a) in a mixture containing 2 mM KCN, 10 mM EDTA, 0.1 mM dichlorophenol-indophenol (DCPIP), 0.2% (v/v) Triton X-100, 1 mg/ml phenazine methosulfate, 12.5 mM phosphate buffer pH 7.6, 20 mM sodium succinate and membrane protein (0.1 to 0.2 mg) in a final volume of 3.0 ml. Enzyme activity was measured by the decrease in absorbance at 600 nm and the amount of DCPIP reduced was calculated using the extinction coefficient $E_{1M} = 2.1 \times 10^4$.

G) Polyacrylamide gel electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (1970). The separation gel contained 7% acrylamide, 0.1% SDS in 0.15 M Tris-Cl pH 8.8 and the stacking gel contained 1% acrylamide, 0.1% SDS in 0.083 M Tris-Cl pH 6.8. The running buffer consisted of 0.2 M glycine, 0.083 M Tris-Cl and 0.1% SDS and the sample buffer contained glycerol, 0.083 M Tris-Cl pH 6.8 and 2% (w/v) SDS. The gels were subjected to 5 mA for 1 hour and then 30 mA until the bromophenol blue tracking dye had reached the bottom of the gel. The gels were fixed overnight in methanol:glacial acetic acid:de-ionized water (0.8:0.2:1.0, v/v/v) and silver stained the following day with the BioRad silver stain kit (BioRad Technical Bulletin # 1089), except that the oxidizing step
was replaced by treatment with 3 mg/ml dithiothreitol as described by Morrissey (1981).

H) Purification of ATPase Activity

All procedures were performed at 4°C.

i) Sephacryl S-300 gel filtration. Sephacryl S-300 was packed into a 2 x 80 cm column and equilibrated with 25 mM Tris-Cl pH 7.5 containing 1% BRIJ-35 or 0.5% CHAPS and 0.3 M NaCl. No more than 4 ml of the solubilized membrane preparation (approximately 20 mg protein) was applied to the column and fractions of 2.5 ml were collected and monitored for protein by absorbance at 280 nm and ATPase activity. Fractions expressing enzyme activity were pooled and dialyzed for 12 hours against at least 100 volumes of 10 mM Tris-Cl pH 7.5 containing 10% (v/v) glycerol and 0.1% CHAPS. The buffer was generally changed once during the dialysis procedure.

ii) Ion exchange chromatography on DEAE-Sephacel. Approximately 5 ml of DEAE-Sephacel was poured into a 1 x 10 cm column and equilibrated with 100 mls of 10 mM Tris-Cl pH 7.5 containing 0.5% BRIJ or CHAPS. Enzyme preparations were loaded onto the column and, after the unbound protein was removed by washing with 75 ml of equilibration buffer, bound protein was eluted by successively washing the column with 0.1, 0.3 and 0.5 M NaCl in equilibration buffer. Fractions of 1.5 ml were collected and monitored for protein and ATPase activity as described before. ATPase containing fractions were pooled and
dialyzed against 100 volumes of Tris-Cl pH 7.5 containing 10% (v/v) glycerol and 0.1% CHAPS.

iii) Glycerol density gradient centrifugation. Approximately 0.5 ml of the ATPase preparation was layered onto each of six 9 ml linear glycerol gradients (15 to 35% (v/v) glycerol, 0.25% (w/v) CHAPS in 10 mM Tris-Cl pH 7.5) and centrifuged at 120,000 x g for 24 to 36 hours in an SW41 swinging bucket rotor. Following this, the gradients were dripped from the bottom and 20 drop fractions were collected and assayed for enzyme activity. Protein was estimated by its absorbance at 280 nm, following the removal of glycerol by overnight dialysis against 10 mM Tris-Cl pH 7.5.

iv) Phenyl-Sepharose, Reactive Red-Agarose and ATP-Agarose chromatography. Phenyl-Sepharose (5 ml) was poured into a 1 x 10 cm column and equilibrated with 100 mls of 25 mM Tris-Cl pH 7.5 containing 0.5 M NaCl. Enzyme preparations were loaded onto the column and eluted first with equilibration buffer followed by 10 mM Tris-Cl pH 7.5 containing 10% (v/v) glycerol and finally by a linear gradient of 0 to 1% BRIJ-35 in 10 mM Tris-Cl pH 7.5.

Reactive Red-Agarose was swollen at room temperature (1 g dry beads/5 ml gel) in 10 mM Tris-Cl pH 7.5 for 4 hours. The gel was then poured into a 0.5 x 5 cm column and washed with 0.5 M NaCl as described by Coll and Murphy (1984) before being equilibrated with 10 mM Tris-Cl pH 7.5 containing 10% (v/v) glycerol and 0.5% (w/v) CHAPS. The ATPase preparations were applied to the column and eluted
first with equilibration buffer followed by equilibration buffer containing 0.4 mM adenyl 5'-yl imidodiphosphate (AMP-PNP).

Pre-swollen ATP-Agarose was packed into a 0.5 x 5 cm column and equilibrated with the same buffer as the reactive red column except that it contained 1 mM MgCl₂. Protein was eluted with the equilibration buffer followed by equilibration buffer containing 1 mM ATP.

I) 32P labeling experiments

Enzyme preparations were incubated in a reaction mixture containing 0.01 to 0.02 ml plasma membrane protein, 10 mM MgCl₂, 10 mM MES/Tris pH 6.8 and various concentrations of [γ-32P]-ATP (see appropriate figure legends for details) in a final volume of 0.05 ml. The reactions were initiated by the addition of ATP and were terminated by the addition of 0.05 ml of electrophoresis sample buffer. The samples were then subjected to electrophoresis according to the procedure of Laemmli (1970) at 4°C. The gels were then soaked overnight in 0.4 l of methanol:glacial acetic acid:de-ionized water (0.8:0.2:1), silver stained, dried and placed against radiographic X-ray film for 24 to 172 hours before being developed.
RESULTS

SECTION I. IDENTIFICATION OF ATPASE ACTIVITY IN PURIFIED D. DISCOIDEUM PLASMA MEMBRANES

a) Plasma Membrane Isolation

The initial step in this work was to select specific marker enzymes for the mitochondrial and plasma membranes. Alkaline phosphatase was selected as the cell surface marker since it had been used extensively in previous work on D. discoideum plasma membranes (Green and Newell, 1974; Rossomando and Cutler, 1975; Gilkes and Weeks, 1977a, 1977b). Recent cytochemical work has confirmed the plasma membrane localization of this enzyme (Glomp et al., 1985). Succinate dehydrogenase was selected as the mitochondrial membrane marker.

In order to establish the existence of a plasma membrane ATPase activity it was of crucial importance for plasma membrane preparations to be free of mitochondrial ATPase contamination. Initially a number of simple and rapid techniques, such as differential centrifugation and the two phase polymer partitioning system of Brunette and Till (1971), were used, but these techniques did not give satisfactory results and were abandoned (data not shown). A somewhat time consuming method, devised earlier in this laboratory (Gilkes and Weeks, 1977a, 1977b) was used and
yielded highly enriched plasma membranes as revealed by the distribution of the marker enzymes (Table I). Moreover, these membranes expressed a high level of ATPase activity (Table I), suggesting that there was a specific plasma membrane ATPase.

b) Characterization of the Plasma Membrane ATPase

The plasma membrane ATPase activity was found to be resistant to the mitochondrial ATPase inhibitors azide and oligomycin (Fig. 2), whereas the ATPase in the mitochondrial fraction that pelleted at 10,000 RPM was sensitive. These data provided further evidence that the plasma membrane activity was distinct from the mitochondrial enzyme.

Previous reports had suggested the presence of an ATP:pyrophosphohydrolase in D. discoideum plasma membranes (Rossomando and Sussman, 1973; Rossomando and Hodge-Jahngen, 1983), necessitating an analysis of the reaction products produced under the present ATPase assay conditions. When plasma membranes were incubated with $[^3H]$-ATP and the products separated by PEI-cellulose TLC it was found that ADP constituted the majority of the hydrolyzed radioactivity (Table II). Even after a 60 minute incubation, AMP accounted for only slightly more than 10% of the total product. This result indicated that the activity was attributable to a classical ATPase in that ATP was hydrolyzed to ADP and $P_i$. 

27
### TABLE I
**Distribution of Enzyme Markers during Plasma Membrane Purification**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Succinate dehydrogenase</th>
<th>Alkaline phosphatase</th>
<th>Mg$^{2+}$-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>22.5</td>
<td>6.4</td>
<td>75.2</td>
</tr>
<tr>
<td>10K pellet</td>
<td>34.0</td>
<td>5.0</td>
<td>92.0</td>
</tr>
<tr>
<td>40K pellet</td>
<td>4.7</td>
<td>17.2</td>
<td>41.6</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>1.1</td>
<td>65.0</td>
<td>160.0</td>
</tr>
</tbody>
</table>
Figure 2. Effect of Azide and Oligomycin on Plasma Membrane and Mitochondrial ATPase Activity. Plasma membranes (□) or 10K pellet fractions (○) were preincubated at 30°C for 10 min. in a mixture containing 30 mM imidazole-Cl pH 7.5, 3 mM MgCl₂ and the indicated amounts of oligomycin (A) and azide (B). Reactions were initiated by the addition of 3 mM ATP. The final volume of the reaction mixture was 1.0 ml. The data represents single experiments, although similar results were obtained upon repetition. The additional experiments are not included as the specific activities vary between preparations. One unit of ATPase activity represents 1 nmol Pi/min/mg protein.
TABLE II

DISTRIBUTION OF HYDROLYZED PRODUCTS RESULTING FROM INCUBATION OF PLASMA MEMBRANES WITH [³H]ATP

<table>
<thead>
<tr>
<th>Time of incubation (min)</th>
<th>Percentage total radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP</td>
</tr>
<tr>
<td>5</td>
<td>2.2</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>8.7</td>
</tr>
<tr>
<td>30</td>
<td>13.5</td>
</tr>
<tr>
<td>45</td>
<td>14.6</td>
</tr>
<tr>
<td>60</td>
<td>18.2</td>
</tr>
</tbody>
</table>

Reaction solutions contained, in addition to membrane protein, 3 mM MgCl₂, 30 mM imidazole-Cl pH 7.5 and 3 mM (2,8-³H)Na₂ATP (200,000 cpm/μmol). The final volume was 0.2 ml.
Hydrolysis of ATP was stimulated by millimolar concentrations of Mg\(^{+2}\) and to a lesser extent by Ca\(^{+2}\) (Fig. 3). However, when both of these cations were present at high concentrations, enzyme activity was substantially inhibited (Fig. 4). Neither ouabain (Table III) nor equimolar concentrations of Na\(^{+}\) and K\(^{+}\) (Fig. 4) had any effect on activity indicating that the enzyme was not a Na\(^{+}\)-K\(^{+}\)-ATPase. Five mM Na\(^{+}\) and K\(^{+}\) will result in full activation of the Na\(^{+}\),K\(^{+}\)-ATPase (Kyte, 1971,1974; Dixon and Hokin, 1974; Jorgensen, 1974). The plasma membrane ATPase activity was partially inhibited by DES, DCCD, thimerosal and vanadate (Fig. 5) and also by N-ethylmaleimide (NEM) (Fig. 6).

The plasma membrane ATPase was assayed for its ability to hydrolyze other nucleotides. Table IV shows that all the nucleoside triphosphates except UTP were utilized to a certain extent although ATP was hydrolyzed most rapidly. Neither AMP nor ADP were degraded to any significant extent (Table IV).

Because the plasma membrane bound enzyme was resistant to azide and oligomycin it was possible to assay selectively for its activity in crude membrane preparations. The results of an experiment where the oligomycin resistant activity of crude total membranes or a 100,000 x g pellet of a 10,000 RPM supernatant was assayed at various stages of development is illustrated in Fig. 7. The specific activity decreased as the cells proceeded through aggregation and
Figure 3. Stimulation of Plasma Membrane ATPase Activity by Mg$^{2+}$ and Ca$^{2+}$. Plasma membranes were incubated in a mixture containing 30 mM imidazole-Cl pH 7.5 and the indicated concentrations of MgCl$_2$ (♦) or CaCl$_2$ (◊). Reactions were initiated by the addition of 3 mM Na$_2$ATP and were allowed to proceed for 15 minutes. Incubation volumes were 1.0 ml. The experiments was repeated several times with similar results. Since the initial specific activity was variable from preparation to preparation the additional experiments are not shown.
Figure 4. The Effect of Various Cations on Plasma Membrane ATPase Activity. Plasma membranes were incubated at 30°C in 30 mM imidazole-Cl pH 7.5 with the indicated concentrations of choline-Cl (△), CaCl₂ (○) or an equimolar mixture of NaCl and KCl (□). Reactions were initiated by the addition of 3 mM Mg-ATP. The final reaction volume was 1.0 ml. The data represents single experiments, but similar results were obtained upon repetition. For the sake of clarity the additional experiments are not shown. One unit of ATPase activity equals 1 nmol Pi/min/mg protein.
**TABLE III**

EFFECT OF OUABAIN ON PLASMA MEMBRANE ATPASE ACTIVITY

<table>
<thead>
<tr>
<th>Ouabain (mM)</th>
<th>Specific Activity&lt;sup&gt;a&lt;/sup&gt; (nmol PO&lt;sub&gt;4&lt;/sub&gt;/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>56.4 ± 2.7</td>
</tr>
<tr>
<td>0.1</td>
<td>55.0 ± 1.8</td>
</tr>
<tr>
<td>0.01</td>
<td>55.0 ± 2.6</td>
</tr>
<tr>
<td>0.001</td>
<td>57.5 ± 1.1</td>
</tr>
<tr>
<td>no addition</td>
<td>59.2 ± 2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> The numbers are the means of 3 experiments ± the standard deviation.
Assay mixtures contained, in addition to enzyme protein, 3 mM MgCl<sub>2</sub>, 30 mM imidazole-Cl pH 7.5, 3 mM Na<sub>2</sub>ATP and the indicated amounts of ouabain. The final volume was 1 ml.
Figure 5. The Effects of Various Inhibitors on Plasma Membrane ATPase Activity. Plasma membrane ATPase activity was determined in a mixture containing 30 mM imidazole-Cl pH 7.5, 3 mM MgCl₂, and 3 mM Na₂ATP and the indicated amounts of the inhibitors DES (○), DCCD (▲), thimerosal (●) or sodium orthovanadate (△). The final reaction volume was 1.0 ml. Reactions were terminated after 15 minutes and inorganic phosphate was determined as described in Methods. Each curve represents the average of four separate experiments ± the standard deviation.
Figure 6. Inhibition of Plasma Membrane ATPase Activity by N-ethylmaleimide. Plasma membranes were incubated at 0°C in 0.1 ml 30 mM imidazole-Cl pH 7.5 and 5 mM N-ethylmaleimide. At the indicated times 0.9 ml of the ATPase assay mixture was added to give final concentrations of 3 mM ATP, 10 mM MgCl₂ and 30 mM imidazole-Cl pH 7.0 and incubated for a further 15 minutes at 30°C. This experiment was performed only once.
**TABLE IV**

**Utilization of Other Nucleotides by the Plasma Membrane of Mg\(^{2+}\)-ATPase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (nmol PO(_4) min(^{-1}) mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>1</td>
</tr>
<tr>
<td>ADP</td>
<td>1</td>
</tr>
<tr>
<td>UTP</td>
<td>1</td>
</tr>
<tr>
<td>CTP</td>
<td>6.1</td>
</tr>
<tr>
<td>GTP</td>
<td>7.0</td>
</tr>
<tr>
<td>ATP</td>
<td>21.2</td>
</tr>
</tbody>
</table>

Reaction solutions contained, in addition to membrane protein, 3 mM MgCl\(_2\), 30 mM imidazole-Cl pH 7.5 and 3 mM nucleotide. The final volume was 1 ml.
Figure 7. Developmental Changes in Plasma Membrane ATPase Activity. Cells were harvested and washed as described in Methods and 2 x 10^8 cells were plated onto each of 12 non-nutrient agar plates (2% agar in Bonners Salts). The cells were harvested at the indicated times and disrupted with glass beads as described in methods. Homogenates were then divided in half and a total membrane fraction (□) was obtained from one half by centrifugation at 100,000 x g. The other half was centrifuged at 10,000 RPM and the supernatant was decanted and recentrifuged at 100,000 x g to give a 40 K pellet fraction (○). ATPase activity was measured in a reaction mixture containing 30 mM imidazole-Cl (pH 7.5), 3 mM MgCl_2, 10 μg/ml oligomycin and 3 mM Na_2ATP. The final volume was 1.0 ml. Inorganic phosphate was determined as described in methods. Both the total membrane and 40 K pellets were resuspended in 5 mM Tris-Cl pH 7.5 following centrifugation. This experiment was performed only once.
then remained constant through pseudoplasmodium formation and migration (Fig. 7).

c) Solubilization of the Plasma Membrane ATPase Activity

All of the previously described results on the plasma membrane ATPase of *D. discoideum* were obtained using the wild type strain V₁₂M₂. However, initial results indicated that the ATPase in V₁₂M₂ and the axenic mutant Ax-2 had similar properties (data not shown, Pogge von Strandmann et al., 1983) and in view of the ease with which large amounts of Ax-2 could be obtained this strain was used for subsequent studies.

It was found that the specific activity of the plasma membrane bound ATPase activity increased upon washing the membranes with a mixture of 100 mM KCl and 2 mM EDTA (Table V). For this reason the membranes were routinely treated with KCl and EDTA, prior to solubilization. In order to establish the optimum conditions for the solubilization of the plasma membrane ATPase a variety of detergents were tested. The best detergent was lysolecithin (Fig. 8). This detergent stimulated total enzyme activity at all concentrations tested (Fig. 8a) and at the highest concentration, solubilized most of the activity (Fig. 8b). The highest specific activity of the solubilized ATPase was, however, obtained at low detergent concentration, concomitant with low total solubilization (Fig. 8c).
<table>
<thead>
<tr>
<th>Fraction</th>
<th>ATPase Activity (nmol Pi/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membranes</td>
<td>18.5</td>
</tr>
<tr>
<td>Washed Plasma membranes</td>
<td>33.5</td>
</tr>
</tbody>
</table>
Figure 8. Solubilization of Plasma Membrane ATPase Activity with Lysolecithin. Plasma membranes were treated with the indicated concentrations of lysolecithin and centrifuged at 100,000 x g as described in Methods. Figure A represents the total additive activity in pellets and supernatants, Fig. B the solubilized activity as a percent of the total activity and Fig. C the specific activity of the solubilized ATPase, at each detergent concentration.
The detergents CHAPS, octylglucoside, C_{12}E_9 and Triton X-100 were only stimulatory at low concentrations, higher concentrations being inhibitory (Fig. 9a), resulting in the maximum solubilization of only 50 to 65% of the initial activity (Fig. 9b). The highest specific activities of the solubilized material was again obtained at low detergent concentrations (Fig. 9c).

Deoxycholate and Z-14 gave poorer extraction of ATPase activity, ostensibly due to the even greater inhibitory effects of these detergents, rather than to insufficient extraction of membrane protein (Fig. 9a and b). Interestingly, the addition of exogenous phospholipid reversed the inhibitory effect of deoxycholate but no such phenomenon was observed for Z-14 (Fig. 10).

d) Evidence for a Labile DES Sensitive Plasma Membrane ATPase

A comparison of the membrane bound and octylglucoside solubilized activities revealed a loss of DES sensitivity upon extraction (Fig. 11). This finding, in conjunction with the fact that a certain amount of ATPase activity was routinely lost upon detergent treatment (Fig. 9a) suggested either the existence of a specific labile DES sensitive plasma membrane ATPase or the loss, during solubilization, of a cofactor which conferred DES sensitivity on the enzyme. Evidence supporting the former hypothesis is shown in Figure 12. Plasma membranes were solubilized with various
Figure 9. Detergent Solubilization of Plasma Membrane ATPase Activity. Plasma membranes were solubilized by incubation with the indicated concentrations of octylglucoside (▲), CHAPS (■), C12E9 (△), Z-14 (●), deoxycholate (○) and Triton X-100 (□). ATPase activity was determined in both pellets and supernatants following centrifugation at 100,000 x g as described in Methods. The figure designations are identical to those in Fig. 8.
Figure 10. Effect of Exogenous Phosphatidylcholine on Deoxycholate and Z-14 Extracted ATPase. Plasma membrane ATPase activity was solubilized with 1% deoxycholate (■) and Z-14 (▲) and the 100,000 x g supernatants were assayed for ATPase activity in the presence of the indicated concentrations of phosphatidylcholine as described in Methods. The results are representative of a single experiment which was performed twice with similar results.
Figure 11. Effect of DES on the Plasma Membrane Bound and Solubilized ATPase Activity. ATPase activity was determined in plasma membranes (■) and the 100,000 x g supernatant following solubilization with 1% octylglucoside (□) in the presence of the indicated concentrations of DES. The results represent the average of four separate experiments ± the standard deviation of the mean.
Figure 12. Inactivation of the DES Sensitive ATPase Upon Solubilization with Several Detergents. Plasma membranes were solubilized with various detergents as described in Methods and the ATPase activity was determined in the presence and absence of 0.2 mM DES. The DES insensitive activity (■) is given as the former value, while the DES sensitive activity (▲) is the difference between the activities in the presence and absence of DES. The detergents were Triton X-100 (A), octylglucoside (B), C_{12}E_{9} (C) and CHAPS (D).
detergents and the total DES sensitive and insensitive ATPase activities were determined. The DES sensitive ATPase was partially inactivated by all the detergents tested with \( C_{12}E_9 \) being the least and Z-14 the most inhibitory (Figs. 12 and 13). Indeed the enhanced inhibitory effect of Z-14 described earlier (Fig. 9) was due almost solely to its extremely deleterious effect on this enzyme (Fig. 13b). To test whether the DES sensitive ATPase could be reactivated by the addition of phospholipids solubilized plasma membrane ATPase activity was assayed in the presence of DES with or without 20 mg/ml exogenous phosphatidylcholine (PC). Addition of 0.2 mM DES resulted in a 57 and 22% inhibition of plasma membrane bound and detergent solubilized ATPase activity respectively (Table VI). Phosphatidylcholine failed to reconstitute the DES sensitive component to any significant extent (Table VI).

The DES insensitive enzyme was stimulated by lysolecithin (Fig. 13a) and inhibited by deoxycholate (Fig. 13c), but with the other detergents the activity remained relatively constant (Fig. 12).

e) Comparison of the Properties of the DES Sensitive and Insensitive ATPase Activities in Plasma Membranes

The DES sensitive ATPase was stimulated to a much larger extent by Mg\(^{+2}\) as compared to Ca\(^{+2}\) (Fig. 14) whereas the DES insensitive activity was stimulated equally well by both cations, further suggesting two distinct enzymes. This
Figure 13. Effect of Z-14, Deoxycholate and Lysolecithin Solubilization on DES Sensitive and Insensitive ATPase Activity. Plasma membranes were solubilized and DES sensitive and insensitive ATPase activities were determined exactly as described in Fig. 12, except that the detergents were lysolecithin (A), Z-14 (B) and deoxycholate (C). Triangles denote DES sensitive and squares DES insensitive ATPase activity.
<table>
<thead>
<tr>
<th>DES (mM)</th>
<th>Intact Plasma Membrane ATPase Activity (nmol Pi/min/mg protein)</th>
<th>Solubilized Plasma Membrane ATPase Activity&lt;sup&gt;a&lt;/sup&gt; (&lt;sup&gt;b&lt;/sup&gt; PC - PC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no DES</td>
<td>31.4</td>
<td>43.2</td>
</tr>
<tr>
<td>0.002</td>
<td>31.0</td>
<td>46.0</td>
</tr>
<tr>
<td>0.020</td>
<td>28.2</td>
<td>40.4</td>
</tr>
<tr>
<td>0.200</td>
<td>13.7</td>
<td>31.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Washed plasma membranes were solubilized with 1% C<sub>12</sub>E<sub>9</sub> as described in the methods.

<sup>b</sup> The phosphatidylcholine concentration was 20 mg/ml.
Figure 14. Effect of Ca$^{2+}$ and Mg$^{2+}$ on DES Sensitive and Insensitive ATPase Activity. Plasma membranes were incubated in the presence and absence of 0.2 mM DES in a mixture containing 3 mM Na$_2$ATP and the indicated concentrations of MgCl$_2$ (A) or CaCl$_2$ (B) in 10 mM MES/Tris pH 6.8. DES sensitive (■) and insensitive (□) ATPase activities were determined as described in Fig. 12. The data represents a single experiment but it was repeated several times with similar results.
conclusion was corroborated when enzyme activity was measured as a function of ATP concentration in the presence of either Mg$^{+2}$ or Ca$^{+2}$ (Fig. 15). In the presence of Mg$^{+2}$ DES sensitive ATPase activity increased with increasing levels of ATP to a saturation point at 1.0 mM (Fig. 15a). When Mg$^{+2}$ was replaced with Ca$^{+2}$ a similar saturation occurred, although the activity was considerably lower (Fig. 15b). In contrast, DES insensitive ATPase activity did not display ATP saturation in this concentration range with either cation (Fig. 15a and b). Double reciprocal plots of the data in Fig. 15 revealed that, while the DES sensitive ATPase was considerably less active in the presence of Ca$^{+2}$ rather than Mg$^{+2}$, the apparent Km of the enzyme was the same (Km = 0.42 mM) regardless as to which cation was present (Fig. 16). In contrast, the DES insensitive ATPase did not display Michaelis-Menten kinetics with either Mg$^{+2}$ or Ca$^{+2}$, as double reciprocal plots showed a distinct non-linear pattern (Fig. 17). Non-linear kinetics are characteristic of allosteric enzymes.

The two activities were assayed at different pH values the DES sensitive ATPase exhibited a bell-shaped curve with a pH optimum between pH 7.0 to 7.5 (Fig. 18), while the DES insensitive enzyme displayed a broad pH optimum between pH 5.5 and 7.5 (Fig. 16).
Figure 15. Effect of ATP Concentration on DES Sensitive and Insensitive ATPase Activity in the Presence of Mg$^{+2}$ or Ca$^{+2}$. Plasma membrane ATPase activity was measured as a function of the ATP concentration in the presence and absence of 0.2 mM DES. The reaction mixtures contained, in addition to the indicated concentrations of ATP, 10 mM MgCl$_2$ (A) or CaCl$_2$ (B) in 10 mM MES/Tris pH 6.8. DES sensitive (●) and insensitive (○) activity was determined as described in Fig. 12. The data represents a single experiment but it was repeated several times with similar results.
Figure 16. The Effect of Mg$^{+2}$ and Ca$^{+2}$ on ATP Hydrolysis Kinetics of the DES Sensitive ATPase. The ATP saturation curves illustrated in Fig. 15 for the DES sensitive enzyme were analyzed as double reciprocal plots of ATP concentration against velocity. Figures represent enzyme activity in the presence of (◊) 10 mM MgCl$_2$ or (◆) 10 mM CaCl$_2$. The line of best fit was determined by linear regression.
Figure 17. The Effect of Mg$^{2+}$ and Ca$^{2+}$ on ATP Hydrolysis Kinetics of the DES Insensitive ATPase. The ATP saturation curves illustrated in Fig. 15 for the DES insensitive enzyme were analyzed as double reciprocal plots of ATP concentration against velocity. Figures represent enzyme activity in the presence of (O) 10 mM MgCl$_2$ or (●) 10 mM CaCl$_2$. 
Figure 18. The Effect of pH on the DES Sensitive and Insensitive ATPase. Plasma membranes were incubated in the presence and absence of 0.2 mM DES in a mixture containing 2 mM Na₂ATP, 10 mM MgCl₂ and 10 mM MES/Tris at the indicated pH values. DES sensitive (■) and insensitive (□) activities were determined as described in Fig. 12. This experiment was performed once.
SECTION II. PURIFICATION OF THE PLASMA MEMBRANE ATPASE ACTIVITY

a) Fractionation of Solubilized ATPase Activity by Ion Exchange Chromatography

Plasma membranes were solubilized by 1% C_{12}E_{9}, conditions that resulted in optimum solubilization of ATPase activity with this detergent. When the solubilized membranes were applied to a DEAE-Sephacel column and eluted with a step gradient of NaCl, two peaks of ATPase activity were resolved, one eluting with 0.1 M NaCl (peak I) and the second (peak II) eluting with 0.3 M NaCl (Fig. 19).

Neither of the ATPase activities was inhibited by DES (Table VII), indicating a complete loss of the DES sensitive activity during chromatography. The specific activity of peak II was considerably higher than that for peak I (Table VIII), but both fractions contained a large number of protein bands as revealed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 20), indicating that neither activity was homogeneous.

b) Purification of ATPase Activity by Density Gradient Centrifugation

It had been shown that the plasma membrane ATPases from Neurospora crassa and Schizosaccharomyces pombe could be purified in one step by centrifugation of solubilized membrane proteins through glycerol or sucrose gradients.
Figure 19. DEAE-Sephacel Chromatography of Detergent Solubilized Plasma Membranes. Plasma membranes were treated with 1% C_{12}E_{9} and the 100,000 x g supernatant (6 to 7 ml, 50 mg protein) was loaded onto a DEAE-Sephacel column (1.0 x 10 cm) which had been equilibrated as described under Methods. Unbound protein was eluted with equilibration buffer and was found to contain no ATPase activity. Bound protein (●) and ATPase activity (○) were eluted stepwise with buffer containing 0.1, 0.3 and 0.5 M NaCl at the points indicated by the arrows. Protein was monitored by absorbance at 280 nm. The ATPase activity represents the absorbance at 660 nm that resulted when 0.2 ml aliquots of the fractions were assayed for enzyme activity as described in Methods, except that the incubation time was 1 hour.
<table>
<thead>
<tr>
<th>DES (mM)</th>
<th>Specific Activity (nmol Pi/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak I</td>
</tr>
<tr>
<td>No addition</td>
<td>11.9 ± 1.1</td>
</tr>
<tr>
<td>0.02</td>
<td>13.1 ± 0.9</td>
</tr>
<tr>
<td>0.20</td>
<td>12.8 ± 0.4</td>
</tr>
<tr>
<td>2.00</td>
<td>13.4 ± 0.7</td>
</tr>
</tbody>
</table>
### TABLE VIII

ATPASE ACTIVITY IN PEAKS I AND II FROM DEAE-SEPHACEL

<table>
<thead>
<tr>
<th>Protein (mg)</th>
<th>Total Activity (nmol Pi/min)</th>
<th>Specific Activity (nmol Pi/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak I</td>
<td>2.15</td>
<td>29.0</td>
</tr>
<tr>
<td>Peak II</td>
<td>4.30</td>
<td>400.0</td>
</tr>
</tbody>
</table>
Figure 20. SDS-polyacrylamide Gel Electrophoresis of Various Fractions obtained During the Purification of the DES Insensitive ATPase. Samples were electrophoresed through SDS-polyacrylamide gels and proteins were visualized using the silver staining procedure described in Methods. Lane A, 1% octylglucoside solubilized plasma membranes (20 µg protein). Lane B, glycerol gradient centrifugation of the solubilized ATPase (10 µg protein). Lane C, peak I from the DEAE-Sephacel chromatography (20 µg protein). Lane D, peak II from DEAE-Sephacel (10 µg protein). Lane E, ATPase fraction after glycerol gradient centrifugation of peak II (3 µg protein). The molecular weight markers were myosin (207 kDa), β-galactosidase (116 kDa), phosphorylase b (97.1 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa).
(Dufour and Goffeau, 1978; Bowman et al., 1981a; Addison and Scarborough, 1981). However, when \( C_{12}E_9 \) solubilized \( D. discoideum \) plasma membranes were centrifuged through a 15 to 35% (v/v) glycerol gradient, the ATPase fraction still contained considerable amounts of protein contamination (Fig. 20) indicating that density gradient centrifugation alone was insufficient for purification to homogeneity. In view of the higher specific and total ATPase activity in peak II from DEAE-Sephacel further purification efforts were directed toward this fraction.

Centrifugation of peak II activity through a 15 to 35% (v/v) glycerol gradient resulted in the separation of ATPase activity from the bulk of the protein (Fig. 21). Polyacrylamide gels revealed a single major protein band of apparent molecular weight 64 kDa and relatively few other bands (Fig. 20). That the 64 kDa band represented the ATPase was indicated by comparison of the sedimentation of ATPase activity in glycerol gradients with the sedimentation of other proteins of known molecular weight. A molecular weight of 65 to 77 kDa was calculated for the active ATPase (Fig. 22). Despite the relative success of this purification procedure the final yield of the ATPase was extremely low, indicating a substantial loss of activity (Table IX). Alternatives to the glycerol gradient were therefore explored.
Figure 21. Glycerol Gradient Centrifugation of Peak II ATPase. Aliquots (0.5 ml, 0.35 mg protein) of peak II ATPase were applied to each of six, 9 ml linear glycerol gradients (15 to 35% v/v) and centrifuged at 120,000 x g for 36 to 48 hours. Following this, fractions were collected from the bottom of the tubes and protein concentration (●) and ATPase activity (○) were determined. Protein is expressed as the OD at 280 nm. Enzyme activity was determined for 0.2 ml aliquots as described in Methods, except that the reaction time was 2 hours, and is expressed as the absorbance at 660 nm.
Figure 22. Calibration Curve of Protein Markers on Glycerol Density Gradients. Several proteins of known molecular weight were centrifuged through 15 to 35% (v/v) glycerol gradients as described in Methods and in Fig. 19. Standard proteins were glucose-6-phosphate dehydrogenase (102 kDa), bovine kidney alkaline phosphatase (96 kDa), E. coli alkaline phosphatase (86 kDa) and cytochrome c (12.4 kDa). Alkaline phosphatase was determined as described in Methods. Glucose-6-phosphate dehydrogenase activity was determined by measuring the rate of NAD$^+$ reduction at 340 nm. The reaction mixtures contained, in a final volume of 3 ml, 55 mM Tris-Cl pH 7.8, 3.3 mM MgCl$_2$ and 2 mM NAD$^+$ and 0.3 ml of glucose-6-phosphate dehydrogenase. The arrow indicates the $R_f$ and the broken line indicates the molecular weight range of the D. discoideum ATPase.
### TABLE IX

**PURIFICATION OF A DES-INSENSITIVE PLASMA MEMBRANE ATPASE**

<table>
<thead>
<tr>
<th></th>
<th>Protein (mg)</th>
<th>ATPase (nmol/min)</th>
<th>% Recovery of ATPase</th>
<th>Specific Activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed membranes</td>
<td>73.2</td>
<td>1200.0</td>
<td>100.0</td>
<td>16.4</td>
</tr>
<tr>
<td>1% octylglucoside</td>
<td>60.0</td>
<td>1100.0</td>
<td>91.7</td>
<td>18.3</td>
</tr>
<tr>
<td>extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>4.3</td>
<td>400.0</td>
<td>33.3</td>
<td>92.0</td>
</tr>
<tr>
<td>column</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol gradient</td>
<td>0.2</td>
<td>51.0</td>
<td>4.2</td>
<td>243.0</td>
</tr>
</tbody>
</table>
c) Affinity and Hydrophobic Chromatography

Affinity chromatography of peak II on either Reactive Red-Agarose (Coll and Murphy, 1984) or ATP-Agarose were unsuccessful in that the enzyme failed to bind and no additional purification was obtained (Table X). Hydrophobic chromatography of peak II on Phenyl-Sepharose was also unsuccessful (Table XI) as the activity did not bind to the column.

d) Purification by Gel Filtration

Initial attempts at fractionation of solubilized membranes by gel filtration on Sephacryl S-300 in the presence of 1% BRIJ-35 (w/v) were unsuccessful in that the enzyme activity and most of the protein eluted in the column void volume, suggesting that under these conditions the enzyme was highly aggregated (Fig 23). However, when applied to the column in the presence of 0.5% CHAPS (w/v), the elution of the enzyme was retarded and was well separated from the major protein peak (Fig. 24).

The gel filtration purified ATPase still contained a considerable number of protein bands as determined by SDS-PAGE (Fig. 25) and was therefore further fractionated by DEAE-Sephacel chromatography (Fig. 26). Almost all of the activity eluted with 0.3 M NaCl, corresponding to the peak II activity previously described (Fig. 19). This material exhibited a single major band of 64 kDa when subjected to SDS-PAGE (Fig. 25). Moreover, as shown in Table XII, the
### Table X

**Affinity Chromatography of Peak II ATPase Activity**

<table>
<thead>
<tr>
<th>Gel used</th>
<th>ATPase Activity (nmol Pi/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Applied</td>
</tr>
<tr>
<td>ATP-agarose</td>
<td>91.2</td>
</tr>
<tr>
<td>Reactive</td>
<td>86.5</td>
</tr>
<tr>
<td>Red-Agarose</td>
<td></td>
</tr>
<tr>
<td>Activity applied</td>
<td>ATPase Activity (nmol Pi/min/mg protein)</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>Activity applied</td>
<td>103.2</td>
</tr>
<tr>
<td>Elution with 10 mM Tris-Cl pH 7.5, 300 mM NaCl</td>
<td>90.1</td>
</tr>
<tr>
<td>Elution with 10 mM Tris-Cl pH 7.5, 10% (v/v) glycerol</td>
<td>0.0</td>
</tr>
<tr>
<td>Elution with 0-15% (w/v) BRIJ-35 gradient</td>
<td>0.0</td>
</tr>
<tr>
<td>Elution with 1% BRIJ-35</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Figure 23. Gel Filtration in the Presence of 1% (w/v) BRIJ-35 of Detergent Solubilized Plasma Membranes. Plasma membranes were solubilized with 1% C_{12}E_{9} and 4 ml (16 mg protein) of the 100,000 x g supernatant was applied to a Sephacryl S-300 column (3 x 80 cm) which had been equilibrated in 25 mM Tris-Cl pH 7.5 containing 1% (w/v) BRIJ-35 and 0.3 M NaCl. Fractions of 2.5 ml were collected and protein (●) was monitored by absorbance at 280 nm. ATPase activity (○) was determined for 0.1 ml of each fraction as described in Methods, except that a 1 hr incubation time was used, and is expressed as the absorbance at 660 nm. The arrow indicates the volume at which the maximum amount of Blue Dextran eluted and is a measure of the void volume of the column.
Figure 24. Gel Filtration in the Presence of 0.5% (w/v) CHAPS of Detergent Solubilized Plasma Membranes. Experimental procedures and conditions were identical to those of Fig. 21, except that 1% BRIJ-35 was replaced by 0.5% (w/v) CHAPS. Closed circles denote protein as monitored at 280 nm and the open circles indicate ATPase activity.
Figure 25. SDS-polyacrylamide Gel Electrophoresis of Fractions Obtained During the Purification of the DES Insensitive ATPase. Samples of solubilized plasma membranes (Lane A, 20 µg protein), ATPase fraction from Sephacryl S-300 (Lane B, 10 µg protein) and purified enzyme from DEAE-Sephacel (Lane C, 2.5 µg protein) were subjected to SDS-polyacrylamide gel electrophoresis as described in Methods. The molecular weight markers were the same as for Fig. 18. Proteins were visualized using the silver staining procedure described in Methods.
Figure 26. DEAE-Sephacel Chromatography of ATPase Fraction Eluted from the Gel Filtration Column. The ATPase containing fractions eluted from the Sephacryl S-300 column (0.8 mg protein) in the presence of CHAPS were pooled and dialyzed against 200 volumes of 10 mM Tris-Cl pH 7.5 containing 0.1% CHAPS and 10% v/v glycerol for 12 hours with one change of buffer. The dialyzed material was then applied to a DEAE-Sephacel column which had been equilibrated as described in Methods. Bound protein was eluted as described in Fig. 17. Protein was monitored by absorbance at 280 nm (●) and ATPase activity (○) was determined for 0.1 ml aliquots as described in Methods, except that incubations were for 2 hrs. Enzyme activity is expressed as the absorbance at 660 nm.
shown in Table XII, the recovery of activity after the combination of gel filtration and DEAE-Sephacel chromatography was approximately two-fold greater than that observed for the ion exchange/glycerol gradient preparation, although the relative enrichment was approximately the same. Interestingly, the activity eluted by 0.1 M NaCl (peak I, Fig. 19) was very much diminished (Fig. 24,) and in other experiments totally eliminated, when the solubilized enzyme was first subjected to gel filtration.
TABLE XII

PURIFICATION OF THE PLASMA MEMBRANE ATPASE

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Total Activity (nmol Pi/min)</th>
<th>% Recovery</th>
<th>Specific Activity (nmol Pi/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranes</td>
<td>22.0</td>
<td>680.0</td>
<td>100</td>
<td>31.0</td>
</tr>
<tr>
<td>1% octyl-glucoside extract</td>
<td>16.0</td>
<td>690.0</td>
<td>101</td>
<td>46.1</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>0.83</td>
<td>290.0</td>
<td>43</td>
<td>343.6</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>0.11</td>
<td>56.7</td>
<td>8.3</td>
<td>515.2</td>
</tr>
</tbody>
</table>
SECTION III PROPERTIES OF THE FRACTIONATED DES INSENSITIVE ATPases

a) Peak I from DEAE-Sephacel

The material eluted by 0.1 M NaCl from DEAE-Sephacel chromatography exhibited maximum ATPase activity at alkaline pH (Fig. 27). In addition to being insensitive to DES, this ATPase activity was also insensitive to vanadate (Table XIII). The relatively low ATPase activity of peak I suggested the possibility that ATP might not be the true substrate for the enzyme. When activity was assayed with a variety of phosphorylated substrates, the greatest activity was observed for inorganic pyrophosphate (Table XIII). UTP was hydrolyzed preferentially among the nucleotides tested and AMP and p-nitrophenylphosphate were not hydrolyzed at all (Table XIII). Interestingly, although no marked preference toward Ca\(^{+2}\) or Mg\(^{+2}\) was observed when the nucleoside triphosphates were hydrolyzed, the hydrolysis of pyrophosphate was considerably higher in the presence of Mg\(^{+2}\) (Table XIV).

b) Properties of Purified Peak II

The enzyme purified by a combination of gel filtration and DEAE-Sephacel chromatography that eluted with 0.3 M NaCl (peak II) exhibited a broad pH optimum between pH 6.0 and 8.0 (Fig. 28), a result similar to that observed earlier for the membrane bound DES insensitive enzyme (Fig. 18). The
Figure 27. Effect of pH on Peak I Activity. Peak I was eluted from DEAE-Sephacel and assayed as described in Methods, except that the pH was varied by titration of MES with Tris base. Similar results were obtained with other nucleoside triphosphates.
**TABLE XIII**

**SUBSTRATE SPECIFICITY OF PEAK I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific Activity (nmol Pi/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>12.8 ± 0.6</td>
</tr>
<tr>
<td>GTP</td>
<td>9.3 ± 1.1</td>
</tr>
<tr>
<td>CTP</td>
<td>10.9 ± 0.3</td>
</tr>
<tr>
<td>UTP</td>
<td>16.0 ± 1.9</td>
</tr>
<tr>
<td>AMP</td>
<td>0</td>
</tr>
<tr>
<td>ADP</td>
<td>7.4 ± 1.2</td>
</tr>
<tr>
<td>pNPP</td>
<td>0</td>
</tr>
<tr>
<td>PPI</td>
<td>82.1 ± 7.8</td>
</tr>
<tr>
<td>ATP + 1 mM VO₄⁺</td>
<td>12.8 ± 0.5</td>
</tr>
</tbody>
</table>

*Reactions were carried out in the presence of 10 mM MgCl₂. The final substrate concentrations were 3 mM for the nucleotides and 1 mM for pNPP and PPI. Since one mole of PPI yields two moles of Pi the activity with PPI was calculated as half the rate of Pi liberation. The data shown are the means of four independent experiments ± the standard deviation.*
**TABLE XIV**

**EFFECT OF DIVALENT CATIONS ON PEAK I ENZYME ACTIVITY**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (nmol Pi/min/mg protein)</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>13.6 ± 1.2</td>
<td>12.8 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>12.0 ± 2.3</td>
<td>9.3 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>CTP</td>
<td>10.6 ± 0.5</td>
<td>10.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>UTP</td>
<td>17.8 ± 0.7</td>
<td>16.0 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>PPI</td>
<td>10.0 ± 1.1</td>
<td>82.1 ± 7.8</td>
<td></td>
</tr>
</tbody>
</table>

*a* Reaction conditions were the same as for Table XV except in some cases Mg²⁺ was replaced with Ca²⁺. The data shown are the means for four independent experiments ± the standard deviation.
Figure 28. Effect of pH on the Purified ATPase Activity. Purified ATPase was assayed as described in Methods, except that the pH was varied by titration of MES with Tris base. This experiment was performed once.
ATPase was sensitive to vanadate and fluoride but was only marginally inhibited by thimerosal and was completely insensitive to DCCD (Fig. 29) and N-ethylmaleimide (Fig. 30). The ATPase was inhibited by millimolar concentrations of both Ca\(^+2\) and Mg\(^+2\) (Fig. 31), although it was stimulated equally well by either cation alone (Fig. 32, Table XVI). It should be noted that the 20 to 30% of maximum stimulated activity observed at zero or low Mg\(^+2\) or Ca\(^+2\) concentrations (Fig. 32) was not abolished by either EDTA or EGTA (data not shown). Monovalent cations failed to enhance the Mg\(^+2\) or Ca\(^+2\)-ATPase activity (Table XVI) or stimulate the enzyme in the absence of the divalent cations (Table XV). Enzyme activity was also greatly stimulated by Zn\(^+2\), Cu\(^+2\) and Mn\(^+2\) but less efficiently by Ba\(^+2\) (Table XV). As with Ca\(^+2\) and Mg\(^+2\) the stimulation necessitated millimolar divalent cation concentrations (Fig. 33) for maximum stimulation, although slight stimulation was obtained with 10 \(\mu\)M Zn\(^+2\).

ATP was the preferred substrate for the enzyme, although all the nucleoside triphosphates tested and ADP were hydrolyzed to a significant extent, but AMP and pNPP were not utilized at all (Table XVII).

When enzyme activity was measured as a function of the substrate concentration it was found that the ATP concentration curve was sigmoidal in the absence of divalent cations (Fig. 32). Addition of 1.0 and 10.0 mM Mg\(^+2\) or Ca\(^+2\) produced a positive allosteric effect resulting in an apparent increase in the affinity of the enzyme for ATP.
Figure 29. Effect of Various Inhibitors on Purified ATPase Activity. Purified ATPase was assayed as described in Methods, with the indicated concentrations of vanadate (♦), thimerosal (O), DCCD (●) and NaF (◇). The data represents the mean for three separate experiments ± the standard deviation.
Figure 30. Effect of N-ethylmaleimide on Purified ATPase Activity. Purified ATPase (100 ul) was preincubated at 0°C in 0.2 ml 25 mM Tris-Cl pH 8.0 and 10 mM N-ethylmaleimide. At the indicated times 0.8 ml of the ATPase assay mixture was added to give a final concentration of 4 mM ATP, 10 mM MgCl₂ and 10 mM MES/Tris pH 6.8 and further incubated for 2 hours. The reactions were terminated and inorganic phosphate was determined as described in Methods. The data represents the mean of three separate experiments ± the standard deviation.
Figure 31. Inhibition of Purified ATPase Activity by High Concentrations of Ca\(^{2+}\) in the presence of Mg\(^{2+}\). Purified ATPase activity was assayed in the presence of 5 mM Mg\(^{2+}\) and the indicated concentrations of CaCl\(_2\). Enzyme activity was determined as described in Methods. The experiment was performed only once.
Standard deviation are the average of three separate experiments. The results were determined as indicated. The M<sup>+</sup> concentration was varied as indicated. The results described in Methods except that the Mg<sup>2+</sup> and Ca<sup>2+</sup> activity were assayed as Figure 3Z. Effect of M<sup>+</sup> or Ca<sup>2+</sup> on purity ATPase ATPase (percent) ATPase (percent) ATPase (percent) ATPase (percent) ATPase (percent) ATPase (percent) ATPase (percent) ATPase (percent) ATPase (percent) ATPase (percent)
### TABLE XV

**EFFECT OF VARIOUS CATIONS ON PURIFIED ATPASE ACTIVITY**

<table>
<thead>
<tr>
<th>Addition&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Activity (nmol Pi/min)</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>1.1 ± 0.0</td>
<td>17.7</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>6.2 ± 0.1</td>
<td>100.0</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>6.7 ± 0.1</td>
<td>108.8</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.0 ± 0.0</td>
<td>16.1</td>
</tr>
<tr>
<td>Li&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.2 ± 0.1</td>
<td>18.7</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.1 ± 0.1</td>
<td>17.6</td>
</tr>
<tr>
<td>Rb&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.1 ± 0.0</td>
<td>17.6</td>
</tr>
<tr>
<td>Ba&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>3.1 ± 0.1</td>
<td>50.0</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>11.5 ± 0.3</td>
<td>186.3</td>
</tr>
<tr>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>9.2 ± 0.1</td>
<td>148.6</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>12.1 ± 0.6</td>
<td>195.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>In all cases the cation concentrations were 10 mM. The data represents the means of three separate experiments ± the standard deviation.
### TABLE XVI

EFFECT OF MONOVALENT CATIONS ON Mg\(^{2+}\) OR Ca\(^{2+}\) STIMULATED ATPASE ACTIVITY

<table>
<thead>
<tr>
<th>Cation(^{a})</th>
<th>Activity (%)(^{b})</th>
<th>Ca(^{2+})-ATPase</th>
<th>Mg(^{2+})-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Li(^{+})</td>
<td>90.8 ± 3.5</td>
<td>92.0 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>K(^{+})</td>
<td>94.6 ± 3.5</td>
<td>96.7 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Rb(^{+})</td>
<td>104.4 ± 1.9</td>
<td>100.7 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Na(^{+})</td>
<td>96.0 ± 5.4</td>
<td>101.4 ± 0.0</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Monovalent cation concentration was 50.0 mM.

\(^{b}\) Reaction mixtures contained 10 mM Mg\(^{2+}\) or Ca\(^{2+}\). Reactions were initiated by the addition of 4.0 mM Ca\(^{2+}\) or Mg\(^{2+}\)-ATP. The data shown are the means of four independent experiments ± the standard deviation.
Figure 33. Effect of Divalent Cations on Purified ATPase Activity. The purified ATPase was assayed as described in Fig. 30, in the presence of the indicated concentrations of Zn\(^{2+}\) (□), Cu\(^{2+}\) (▲), Mg\(^{2+}\) (△) and Mn\(^{2+}\) (■). The data represents a single experiment.
### TABLE XVII

**SUBSTRATE SPECIFICITY OF THE PURIFIED ATPASE**

<table>
<thead>
<tr>
<th>Substrate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
</tr>
<tr>
<td>GTP</td>
<td>33.7 ± 3.4</td>
</tr>
<tr>
<td>CTP</td>
<td>57.7 ± 2.6</td>
</tr>
<tr>
<td>UTP</td>
<td>26.7 ± 8.3</td>
</tr>
<tr>
<td>ADP</td>
<td>24.5 ± 5.5</td>
</tr>
<tr>
<td>AMP</td>
<td>0.0</td>
</tr>
<tr>
<td>pNPP</td>
<td>0.0</td>
</tr>
<tr>
<td>PPI</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>The final substrate concentrations were 4 mM for the nucleotides and 1 mM for pNPP and PPI. The data are the means of three separate experiments ± the standard deviations.
Figure 34. Effect of Mg$^{2+}$ and Ca$^{2+}$ on ATPase Activity Determined as a Function of the ATP Concentration. Purified ATPase activity was measured as a function of the ATP concentration in the presence and absence of various fixed concentrations of Mg$^{2+}$ (A) and Ca$^{2+}$ (B). Enzyme activity was measured as described in Methods. The various cation concentrations were 1.0 mM (▲), 10 mM (□), 100 mM (■) and no cation (△). The data is an average of three separate experiments. Standard deviations have been omitted for the sake of clarity.
Increasing the cation concentration to 100 mM resulted in a slight increase in the apparent ATP affinity relative to zero cation but the reaction velocity was decreased (Fig. 34).

The fact that the enzyme was stimulated by Ca$^{+2}$ raised the possibility that it may be a plasma membrane Ca$^{+2}$ pump. However, the ATPase activity was not stimulated by micromolar concentrations of Ca$^{+2}$ and Mg$^{+2}$, nor was it stimulated by Ca$^{+2}$/calmodulin (Table XVIII). There was no evidence for a Ca$^{+2}$/calmodulin stimulated ATPase activity in the original plasma membrane preparations (Table XVIII), although these membranes had been extensively washed with either EGTA or KCl/EDTA to ensure complete removal of endogenous calmodulin (Jarrett and Penniston, 1978).

c) Phosphorylation of the Plasma Membrane ATPase

Initial attempts to phosphorylate the ATPase in plasma membranes which had been stored at -70°C were unsuccessful in that most of the radioactively labelled proteins failed to enter the gel (Fig. 35). When freshly prepared membranes were used a few phosphorylated bands were faintly visible but there was no apparent phosphorylation of a 64 kDa protein. In addition, most of the radioactivity was again found at the top of the gel (Fig. 36), a phenomenon which could not have been due to protein aggregation as Coomassie staining revealed an abundance of protein bands. No 64 kDa
**TABLE XVIII**

THE EFFECT OF EXOGENOUS CALMODULIN ON PURIFIED AND PLASMA MEMBRANE BOUND ATPASE ACTIVITY

<table>
<thead>
<tr>
<th>Ca(^{2+}) Concentration ((\mu\text{M}))</th>
<th>Purified ATPase</th>
<th>EGTA Washed Membranes(^a)</th>
<th>KCl/EDTA Washed Membranes(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Cal. (^c)</td>
<td>- Cal. (^c)</td>
<td>+ Cal. (^c)</td>
</tr>
<tr>
<td>0.01</td>
<td>54.0</td>
<td>51.0</td>
<td>5.9</td>
</tr>
<tr>
<td>0.05</td>
<td>46.0</td>
<td>48.0</td>
<td>5.2</td>
</tr>
<tr>
<td>0.10</td>
<td>36.0</td>
<td>48.0</td>
<td>5.2</td>
</tr>
<tr>
<td>0.50</td>
<td>44.0</td>
<td>50.0</td>
<td>5.9</td>
</tr>
<tr>
<td>1.00</td>
<td>40.0</td>
<td>45.0</td>
<td>6.2</td>
</tr>
<tr>
<td>10.00</td>
<td>44.0</td>
<td>48.0</td>
<td>5.2</td>
</tr>
</tbody>
</table>

\(^a\) Plasma membranes were washed once with 10mM Tris-Cl, pH 7.5 containing 2mM EGTA and twice with 10mM Tris-Cl, pH 7.5.

\(^b\) Plasma membranes were washed as described in the methods.

\(^c\) 5 \(\mu\)g bovine brain calmodulin was added.
Figure 35. Autoradiography of $^{32}$P-labeled Detergent Solubilized Plasma Membranes. Plasma membranes which had been stored at -70°C for several days were solubilized with 1% octylglucoside as described in Methods. Solubilized proteins were labeled for 5 (Lanes A), 10 (Lanes B) or 15 (Lanes C) seconds in reaction mixtures containing, in a final volume of 0.05 ml, 10 mM MES/Tris pH 6.8, 0.02 mg protein, 10 mM MgCl$_2$ and (2) 0.03, (3) 0.3 or (4) 3.0 mM [γ-$^{32}$P]-ATP (specific activities 3.3 mCi/umol, 0.33 mCi/umol and 0.033 mCi/umol, respectively). Lane 1 represents a zero time control. The reactions were terminated by the addition of 0.05 ml electrophoresis sample buffer containing 0.083 M Tris-Cl pH 6.8, glycerol, de-ionized water (1:1:6, v/v/v), 0.05 M 2-mercaptoethanol and 5% (w/v) SDS. The samples were subjected to electrophoresis at 4°C as described in Methods. The gel was then soaked for 12 hrs, with two changes, in methanol/acetic acid/de-ionized water (0.8:0.2:1, v/v/v), dried and placed against radiographic X-ray film for 1 week before being developed.
Figure 36. Autoradiography of $^{32}$P-labeled crude membranes. Crude membranes were prepared according to the modified procedure of Das and Henderson (1983) as described in Methods and were phosphorylated immediately after preparation. Reaction mixtures were as described in Fig. 34 and the incubations were performed in duplicate. The final concentrations of $[\gamma^{32}\text{P}]$-ATP were (2) 0.03, (3) 0.3 and (4) 3.0 mM. Lane 1 represents a zero time point. Reactions were terminated and gels run and treated as described in Fig. 33.
phosphoprotein was detected when similar experiments were performed with purified enzyme (data not shown).

d) Effect of DIF on ATPase Activity

It has been proposed that DIF effects stalk cell development by interacting directly with a cell surface proton pumping ATPase (Gross et al., 1983). Since it was possible that at least one of the ATPases described in this thesis was involved in proton pumping the effects of DIF on enzyme activity were studied. As shown in Table XIX partially purified DIF (Kay et al., 1983) had no effect on either total plasma membrane bound or partially purified peaks I and II ATPase activity.
<table>
<thead>
<tr>
<th>Treatment(^a)</th>
<th>Relative Activity (%)</th>
<th>Plasma Membranes</th>
<th>Peak I</th>
<th>Peak II</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>5 (\mu)l DIF</td>
<td>93</td>
<td>-</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>10 (\mu)l DIF</td>
<td>98</td>
<td>102</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>20 (\mu)l DIF</td>
<td>-</td>
<td>-</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>10 (\mu)l DIF</td>
<td>-</td>
<td>-</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>sonicated</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 (\mu)l Ethanol</td>
<td>-</td>
<td>-</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>sonicated</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) 5 \(\mu\)l DIF induced 64% stalk cell differentiation in a standard DIF assay (Sobolewski et al., 1983). DIF was added as an ethanolic solution. Where indicated the DIF solution was sonicated along with the ATPase for a total of 30 seconds in 10 second bursts. Enzyme activity was determined as described in Methods. For peaks I and II 40 \(\mu\)g protein was used and for the plasma membranes 95 \(\mu\)g protein was used.
DISCUSSION

Plasma Membrane Purification

At the outset of this work only two reports existed describing plasma membrane ATPases in *D. discoideum*. Parish and Wiebel (1980) observed that intact cells hydrolyzed extracellular $[^{14}C]$-ATP, indicating the presence of an ecto-ATPase. This enzyme was inhibited by the Na+,K+-ATPase inhibitor suramin but not by oligomycin or ouabain. Moreover, Parish and Wiebel (1980) also suggested that the enzyme may be coupled to Ca$^{2+}$ uptake in that the addition of ATP to a cell suspension led to an enhanced uptake of $^{45}$Ca$^{2+}$. The $^{45}$Ca$^{2+}$ uptake was reduced when cells were incubated with uncouplers, suramin or non-hydrolyzable ATP analogues, although in most cases the reduction was less than 50%. The ATPase had a comparatively high affinity for ATP with a $K_m$ of between 1 to 25 $\mu$M and had a requirement for Mg$^{2+}$. In addition, Mg$^{2+}$ could not be replaced by any other ion.

In the other report, Blanco (1982) described the existence of a Na+,K+ stimulated, ouabain resistant ATPase activity in *D. discoideum* plasma membranes prepared using the two phase system of Brunette and Till (1971). This ATPase was also strongly inhibited by azide and thimerosal. No data was included with regard to the purity of the plasma membranes.
A wide variety of plasma membrane purification methods for *D. discoideum* have been published and considerable effort was initially directed toward finding the method most appropriate for ATPase characterization. Parish and Muller (1976) and Condeelis (1978) stabilized plasma membranes with ConA before disrupting the cells with Triton X-100. Plasma membranes were then isolated following low speed centrifugation and ConA was removed by washing with α-methylmannoside. A disadvantage of this technique was that a considerable loss of plasma membrane alkaline phosphatase, and possibly other proteins, occurred, presumably through detergent solubilization (Parish and Muller, 1976). In view of this drawback this technique was not used. The two phase polymer partitioning system of Brunette and Till (1971), that had been applied previously to *D. discoideum* (Siu *et al*., 1977; Ono *et al*., 1978; Blanco, 1982), was also tried but a considerable amount of mitochondrial contamination persisted in the plasma membrane fraction (data not shown).

Jacobson (1980) and Chaney and Jacobson (1983) isolated membranes by coating the cells with either polycationic beads or colloidal silica, then breaking the cells by mechanical agitation (Jacobson, 1980) or explosive decompression (Chaney and Jacobson, 1983). When this technique was used it was found that only very small amounts of material could be processed and consequently very little ATPase activity was obtained (data not shown).
Differential centrifugation was also attempted with *Dictyostelium* membranes, but resulted in only marginal enrichment of plasma membranes (data not shown). Such a technique had been utilized to obtain a 35-fold enrichment of plasma membrane ATPase activity from *Neurospora crassa* (Bowman et al., 1981b).

A somewhat laborious centrifugation of crude membranes through sucrose gradients (Gilkes and Weeks, 1977b) resulted in relatively pure plasma membrane preparations which were free of mitochondrial contamination, as assessed by succinate dehydrogenase activity, and contained a high level of ATPase activity (Table I). Moreover, the ATPase activity was refractory to the usual mitochondrial ATPase inhibitors azide and oligomycin, confirming the absence of mitochondria (Figure 2). The more recently described sucrose density gradient procedure of Das and Henderson (1983) was also used during the course of this work and yielded plasma membranes free of mitochondrial contamination. However, this procedure was no better than the Gilkes and Weeks (1977b) method and it was found that the ATPase activity was lost upon fractionation of the solubilized membranes a phenomenon that was apparently due to the 50 mM glycine pH 8.5 buffer used throughout (data not shown).

**Identification of plasma membrane ATPase activities**

The plasma membrane ATPase activity was not stimulated by equimolar Na\(^+\) and K\(^+\) (Fig. 5) and it was insensitive to ouabain (Table III), indicating that the enzyme was not a
Na\textsuperscript{+},K\textsuperscript{+}-ATPase. This result suggested that the azide sensitive Na\textsuperscript{+},K\textsuperscript{+}-ATPase in \textit{D. discoideum} described earlier (Blanco, 1982) may have been due to mitochondrial contamination, although the mitochondrial ATPase from \textit{D. discoideum} has never been characterized.

Solubilization of plasma membranes revealed the existence of two distinct ATPase activities, one being sensitive and the other insensitive to DES (Figs. 12 and 13). The DES sensitive ATPase was unstable when removed from its native membrane bound state and fractionation of the solubilized membranes resulted in a complete loss of activity. In contrast, the DES insensitive activity was stable to detergent solubilization and could be fractionated. Solubilization of some plasma membrane DES sensitive H\textsuperscript{+}-ATPases has resulted in a loss of activity which could be restored by the reconstitution of the enzyme with exogenous phospholipids (Bowman \textit{et al.}, 1981a; Vara and Serrano, 1982; Cocucci and Marré, 1984). In the case of the \textit{D. discoideum} DES sensitive ATPase, however, addition of phosphatidylcholine to C\textsubscript{12}E\textsubscript{9} solubilized plasma membranes failed to reconstitute a DES sensitive ATPase (Table VI). Moreover, loss of DES sensitive enzyme activity occurred even when plasma membranes were solubilized with lysolecithin (Fig. 13a). Thus, the inactivation of DES sensitive ATPase was probably not due to delipidation.

In addition to their dissimilar stabilities the DES sensitive and insensitive activities had other distinctive

102
properties. The DES sensitive ATPase displayed a pH optimum between pH 7.0 to 7.5 while the DES insensitive ATPase had a broad optimum between pH 6.0 and 7.5 (Fig. 18).

Furthermore, while the DES insensitive ATPase expressed similar activities in the presence of either Mg$^{+2}$ or Ca$^{+2}$, the DES sensitive enzyme displayed a distinct preference for Mg$^{+2}$ (Figs. 14 and 15). Interestingly, replacement of Mg$^{+2}$ with Ca$^{+2}$ did not result in a change in the apparent Km ($K_m = 0.42$ mM) of the DES sensitive ATPase for substrate but rather affected only the velocity of the reaction (Fig. 16). Double reciprocal plots of ATP concentration against velocity for the DES insensitive ATPase did not yield linear relationships (Fig. 17), indicating that this enzyme did not display Michaelis-Menten kinetics.

Comparison of ATPase to Previously Identified Plasma Membrane ATPases from D. discoideum

While this work was in progress Pogge-von Strandmann et al. (1984) also described a DES sensitive plasma membrane ATPase, in D. discoideum, that was solubilized by lysolecithin but not by other detergents. The enzyme was fractionated over sucrose gradients and DES sensitive proton translocation was demonstrated in reconstituted phosphatidylcholine vesicles. In addition, this enzyme was reported to have a broad pH optimum around pH 6.8 with less than 50% of the activity below pH 5.7 and above pH 8.0. The activity was also sensitive to the sulfhydryl group reagents Cu$^{+2}$ and Hg$^{+2}$. Pogge-von Strandmann et al. (1984) also
reported a Km of 0.9 mM for Mg$^{2+}$-ATP hydrolysis measured by phosphate release and 0.5 to 1.5 mM for Mg$^{2+}$-ATP hydrolysis measured by proton translocation. It is possible that the DES sensitive ATPase described in this thesis is the same enzyme that was described by Pogge-von Strandmann et al. (1984) but all attempts to demonstrate proton pumping in either membrane vesicles or reconstituted vesicles were unsuccessful (data not shown).

It was not clear if Pogge-von Strandmann et al. (1984) were measuring a combination of the DES sensitive and DES insensitive activities in their study, as no reference was made to a DES insensitive ATPase. In addition, only data pertaining to DES sensitive proton pumping as opposed to ATPase activity was presented. It is surprising that they obtained such poor solubilization with detergents such as CHAPS and Triton X-100 and successful solubilization with lysolecithin, considering the fact that in the present study CHAPS and Triton X-100 were only slightly less efficient than lysolecithin.

Serrano et al. (1985) have also recently reported an ATPase activity which was inhibited 75% by 150 μM DES in D. discoideum plasma membranes. This activity was also inhibited by DCCD, vanadate and sulfhydryl reagents such as Cu$^{2+}$ and p-chloromercuriphenylsulfonate. The pH optimum for this enzyme was reported at pH 6.5 with a 50% reduction of activity at pH 7.5. The enzyme had a requirement for Mg$^{2+}$ but was inactive with Ca$^{2+}$. The ATPase expressed hyperbolic
kinetics with respect to Mg$^{2+}$-ATP with an apparent Km of 0.1 mM. No attempt was made to solubilize the activity or demonstrate proton translocation and no reference was made to a DES insensitive component.

Although the DES sensitive ATPase described in this thesis is possibly analogous to the well characterized electroneutral proton pumps in the plasma membrane of Neurospora and yeast, at least two differences exist. One of these differences, noted earlier, is the irreversible instability of the Dictyostelium DES sensitive ATPase upon solubilization.

The second difference is that when DES sensitive ATPase activity was measured as a function of the Mg$^{2+}$ concentration, the amount of ATP hydrolysis increased with increasing Mg$^{2+}$ to a point at which there was a 16-fold excess in the level of Mg$^{2+}$ relative to the ATP concentration (Fig. 14). As the Mg$^{2+}$ concentration was increased further the enzyme activity decreased. In contrast to the D. discoideum results, Brooker and Slayman (1983b, 1983c) characterized the kinetics of ATP hydrolysis with regard to divalent cation requirements for the Neurospora DES sensitive H$^+$-ATPase, and found that the enzyme functioned optimally in the presence of equimolar concentrations of Mg$^{2+}$ and ATP. Excess Mg$^{2+}$ was also found to inhibit the Neurospora enzyme (Brooker and Slayman, 1983c), but at much lower concentrations than those observed for the Dictyostelium DES sensitive ATPase. The
Dictyostelium and Neurospora DES sensitive ATPases were similar in that Mg$^{2+}$ was preferred over Ca$^{2+}$. The former enzyme, however, did express some enzyme activity in the presence of Ca$^{2+}$, whereas the Neurospora ATPase was completely inactive when Ca$^{2+}$ served as the cation (Bowman et al., 1981; Brooker and Slayman, 1983b).

Developmental Role of the DES Sensitive ATPase

The potential developmental role of the DES sensitive ATPase remains to be established. Gross et al. (1983) suggested that intracellular pH changes, effected by an inhibitory interaction between the stalk cell differentiation inducing factor (DIF) and a cell surface proton pump, play an important determinative role in stalk cell development. Compelling evidence supporting this hypothesis was obtained when it was observed that DES mimicked the effect of DIF in inducing stalk cell development in low density monolayers (Gross et al., 1983). However, partially purified DIF had no effect whatsoever on ATPase activity (Table XIX). In addition, recent work by Kay et al. (1986) failed to show a decrease in intracellular pH, as determined by $^{31}$P-NMR spectroscopy, when differentiating cells were exposed to DIF or DES.

Although it appears that DIF and DES probably effect differentiation by some mechanism other than influencing intracellular pH it should be noted that the findings of Kay et al. (1986) and others (Jentoft and Town, 1985; Ratner, 1986) which fail to show quantitative changes in
intracellular pH do not necessarily mean that pH changes do not occur. It is entirely possible that any pH change may be localized at a specific point e.g. the inner surface of the plasma membrane, since if this were the case it is unlikely that such a pH change would be detected. In addition, Kay et al. (1986) did not examine the effects of DIF on ATPase activity and it is possible that the observation made in this thesis, showing that DIF had no effect on ATPase activity (Table XIX), may have been due to the removal of a factor necessary to confer DIF sensitivity to the enzyme. Clearly, proton flux plays some role in D. discoideum development since Gross et al. (1983) observed that weak acids or mildly acidic conditions influenced stalk cell differentiation whereas neutral or mildly alkaline conditions favoured spores when cells were plated out in submerged monolayers, but it remains to be established whether the DES sensitive ATPase plays any role in these proton movements.

Jamieson et al. (1984) have presented evidence suggesting a possible role of increased cellular pH in the initiation of D. discoideum development, mediated through a Na+/H+ antiporter, rather than a DES sensitive H+-ATPase. They found that a transient (10 min) intracellular alkalination in cells which had been starved for two hours was abolished when the cells were exposed to the Na+/H+ antiport antagonist amiloride. Further evidence supporting the role of a putative antiporter was obtained when it was
observed that aggregation was delayed when cells were plated out in the presence of amiloride (Jamieson et al., 1984). Previously it had been noted that a low external Na\(^+\) concentration also delayed aggregation (Marin and Rothman, 1980).

Malchow et al. (1978a,b) had previously observed extracellular pH oscillations in suspensions of aggregation competent cells. Moreover, these pH pulses were shown to be dependent on cAMP signals (Malchow et al., 1978b, Gottman and Weijer, 1986). The exact nature of these oscillations is not known. They are possibly due in part to the hydrolysis of extracellular cAMP by phosphodiesterase but the magnitude of the proton pulse was considerably higher than that of the cAMP pulse (Malchow et al., 1978b) and weak acid and CO\(_2\) extrusion were also cited as possible alternative sources for the pH change (Malchow et al., 1978a, 1978b). It is possible that the pH oscillations are mediated by proton extrusion through a putative Na\(^+\)/H\(^+\) antiporter. If this were the case it should be possible to abolish the pH pulses by the addition of amiloride. Similarly, if the putative proton pump plays a role in the regulation of these pH oscillations it should be possible to abolish the pulses by the addition of DES.

Comparison to Pyrophosphatase

When solubilized plasma membranes were fractionated over an ion exchange column two distinct DES insensitive activities (Peaks I and II) were detected (Fig. 19).
Pyrophosphate was hydrolyzed more rapidly than nucleoside triphosphates by peak I enzyme (Table XIII), suggesting that this enzyme might be an inorganic pyrophosphatase. The enzyme also had a distinct preference for Mg\(^{2+}\) when pyrophosphate was the substrate and activity was greater at alkaline pH (Fig. 27). This enzyme was also insensitive to vanadate (Table XIII).

Enzymes with characteristics similar to the pyrophosphate hydrolyzing activity described above have been isolated from the tonoplast membrane of oat roots (Wang et al., 1986) and beets (Karlsson, 1975; Walker and Leigh, 1981b; Rea and Poole, 1985, 1986; Table XX). These enzymes were found to be vanadate insensitive and were stimulated considerably by K\(^{+}\) (Karlsson, 1975; Walker and Leigh, 1981b; Wang et al., 1986) and to a lesser extent by Rb\(^{+}\) (Karlsson, 1975) and Cs\(^{+}\) (Wang et al., 1986). The pyrophosphatases also expressed a divalent cation requirement for Mg\(^{2+}\) with a small amount of activity with Mn\(^{2+}\) but none with Ca\(^{2+}\) (Walker and Leigh, 1981; Rea and Poole, 1986; Wang et al., 1986) and enzyme activity was greatest at alkaline pH (Rea and Poole, 1985).

The tonoplast inorganic pyrophosphatase was also shown to translocate protons (Rea and Poole, 1986; Wang et al., 1986), a phenomenon which was sensitive to fluoride, NEM and DCCD (Table XX), but was insensitive to 4,4-diisothiocyanostilbene-2,2-disulfonate (DIDS) and KNO\(_3\) (Wang et al., 1986). Rea and Poole (1985, 1986) also
TABLE XX

COMPARISON OF THE PURIFIED D. DISCOIDEUM PLASMA MEMBRANE ATPASE WITH SEVERAL OTHER MEMBRANE BOUND PHOSPHATASES

<table>
<thead>
<tr>
<th>ATPase</th>
<th>D. discoideum</th>
<th>DES insensitive</th>
<th>H⁺-ATPase</th>
<th>(Ca²⁺:Mg²⁺)-ATPase</th>
<th>Ca²⁺ or Mg²⁺-ATPase</th>
<th>Tonoplast ATPase</th>
<th>Pyrophosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vanadate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DES</td>
<td>-</td>
<td>+</td>
<td>N.D.</td>
<td>N.D.</td>
<td>+</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>DCCD</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>N.D.</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>fluoride</td>
<td>+</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ouabain</td>
<td>N.D.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NEM</td>
<td>-</td>
<td>+</td>
<td>N.D.</td>
<td>N.D.</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Membrane origin</td>
<td>P.M.</td>
<td>P.M.</td>
<td>P.M.</td>
<td>P.M.</td>
<td>Vacuolar membrane</td>
<td>Vacuolar membrane</td>
<td></td>
</tr>
<tr>
<td>Molecular weight</td>
<td>64 kDa</td>
<td>104 kDa</td>
<td>130-140 kDa</td>
<td>N.D.</td>
<td>Several subunits</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20 - 90 kDa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.D. = not determined; P.M. = plasma membrane. For references see the appropriate sections of the text.
reported a complete lack of enzyme activity when the pyrophosphatase of beet root was assayed with nucleotides. It is possible that the *D. discoideum* pyrophosphate hydrolyzing activity also functions as a proton pump.

Enrichment of pyrophosphatase activity was not followed during the course of this thesis and it is conceivable that the apparent cell surface localization of the pyrophosphate hydrolyzing activity was due to contamination of the plasma membrane preparation with contractile vacuolar or lysosomal membranes. Vacuolar membranes from *Dictyostelium* have not been purified, possibly due to the lack of knowledge regarding specific membrane markers. Quiviger et al. (1978, 1980) and Glomp et al. (1985) have provided histochemical evidence for alkaline phosphatase on *D. discoideum* contractile vacuolar membranes of and, since the vacuolar and plasma membranes fuse at the terminal stage of the contractile process (Murray, 1982), it is possible that the two membrane types are similar in terms of their molecular composition.

Peak II activity was vanadate sensitive and was purified via two different procedures. The first method involved separation on an ion exchange column, followed by centrifugation through a glycerol gradient. This procedure resulted in a relatively homogeneous preparation with a single major protein band of 64 kDa, as shown by SDS-PAGE (Fig. 20), but the recovery of enzyme was low (Table IX). A second method which used an initial separation by gel
filtration on Sephacryl S-300 in the presence of 0.5% CHAPS, followed by ion exchange chromatography, gave the same 64 kDa protein (Fig. 23) with a two-fold higher yield (Table XIII). A comparison of the sedimentation velocities of several proteins of known molecular weights revealed the apparent molecular weight of the active ATPase to be between 65 and 77 kDa.

In order to confirm that the 64 kDa band corresponded to the ATPase, attempts were made to phosphorylate the protein, since phosphoprotein intermediates have been observed for many other plasma membrane ATPases (Lane et al., 1973; Wallick et al., 1978; Dame and Scarborough, 1980; Amory et al., 1980; Brisken and Poole, 1983). Initial results using membranes which had been stored at -70°C revealed that the phosphorylated proteins failed to enter the gel (Fig. 35), a problem which had previously been encountered with phosphorylated alkaline phosphatase from D. discoideum (P. Bhanot, 1986). Specific phosphorylated bands were faintly detected when freshly prepared membranes were used, indicating that some phosphoproteins were entering the gel, but there was no phosphorylation of the 64 kDa protein (Fig. 36). No phosphorylation was observed with the purified protein. It must be stressed that if the ATPase formed a $\beta$-aspartylphosphate intermediate it might be susceptible to hydrolysis under the electrophoretic conditions used here. The same experiments were attempted using acid gels (Amory et al., 1980), but the proteins failed to migrate as
discrete bands and no phosphoproteins were detected (data not shown).

It is likely that this ATPase does form a phosphoprotein intermediate since the enzyme is vanadate sensitive (Fig. 29). Vanadate sensitivity appears to be a common property among phosphohydrolases which form covalent attachments with phosphate during the course of their reaction cycles (Sze, 1985). It is believed that the inhibitory effect of vanadate is due to its competition with phosphate for binding, thus preventing the rapid turnover of the enzyme (Sze, 1985). Neither the mitochondrial nor the tonoplast ATPases form phosphoprotein intermediates and neither of these enzymes are sensitive to vanadate (Amzel et al., 1983; Sze, 1985).

Characterization of the purified DES insensitive ATPase from D. discoideum

The purified DES insensitive ATPase is unique in a number of ways when compared to other well characterized cell surface ATPases (Table XX). The apparent molecular weight of the enzyme was considerably less than that observed for the H^+-ATPases (104 kDa) of Neurospora (Bowman et al., 1981a; Addison and Scarborough, 1981), yeast (Dufour and Goffeau, 1978) and plants (Sze, 1985), for the (Ca^{2+}:Mg^{2+})-ATPases (130 to 140 kDa; De Smedt et al., 1983; Debetto and Cantley, 1984; Ansah et al., 1984) and for the catalytic subunit of the Na^+,K^+-ATPases (approximately 121 kDa; Craig and Kyte, 1980). The purified Dictyostelium ATPase was also stimulated by divalent cations, including
Zn$^{2+}$ and Cu$^{2+}$, but not by monovalent cations (Fig. 33, Table XVI). In contrast, the H$^+$-ATPases of Neurospora and plants were not stimulated by Ca$^{2+}$ or Zn$^{2+}$ (Bowman et al., 1981a; O'Neill and Spanswick, 1984b), although a certain degree of activity was observed with Mn$^{2+}$ (Table XX), but were stimulated by monovalent cations. The H$^+$-ATPase of radish (Cocucci and Marré, 1984) and the Ca$^{2+}$-ATPase (Ansah et al., 1984) of pancreatic acinar cell plasma membranes were also comparatively active with Zn$^{2+}$ as the cation, although in both cases the activity was lower than that seen with Mg$^{2+}$.

The purified _D. discoideum_ ATPase was also insensitive to NEM (Fig. 30) suggesting that the NEM inhibition of plasma membrane bound ATPase activity (Fig. 6) was due to inactivation of the DES sensitive ATPase. A wide variety of ATPases (Table XX), including the Na$^+$,K$^+$-ATPase (Wallick et al., 1978), are sensitive to NEM. Walderhaug et al. (1985) have determined the amino acid sequence of the active sites of the Na$^+$,K$^+$-ATPase, the K$^+$,H$^+$-ATPase and the H$^+$-ATPase from corn root plasma membranes to be -cys-(ser/thr)-asp-lys- and it is believed that NEM inhibition is due to the covalent modification of the cysteine residue (Brooker and Slayman, 1982, 1983a,b). These data suggest a fundamental difference in the active site between these ATPases and the purified _D. discoideum_ ATPase.

Although the purified ATPase was stimulated by Mg$^{2+}$ or Ca$^{2+}$, enzyme activity was increasingly inhibited as the concentration of either of these cations was increased.

114
above 10 mM (Fig. 32). The enzyme was also inhibited by millimolar concentrations of Mg\(^{2+}\) and Ca\(^{2+}\) when both ions were present (Fig. 31) although at lower total cation concentrations than that observed when the ions were present individually. The plasma membrane H\(^{+}\)-ATPases of *Neurospora* (Brooker and Slayman, 1983b) and radishes (Coccuci and Marré, 1984) were also shown to be inhibited by Ca\(^{2+}\) in the presence of Mg\(^{2+}\). In the case of the *Neurospora* enzyme it was suggested that Ca\(^{2+}\) inhibited ATPase activity by competing with Mg\(^{2+}\) at a high affinity binding site at which Mg\(^{2+}\) binding was necessary for the activation of the enzyme (Brooker and Slayman, 1983b). Such a mechanism is, however, insufficient to explain the combined Ca\(^{2+}\)/Mg\(^{2+}\) induced inhibition of the purified *D. discoideum* ATPase as this enzyme was stimulated equally be either Mg\(^{2+}\) or Ca\(^{2+}\) alone (Fig. 32, Table XV). Neither the *Neurospora* nor the radish H\(^{+}\)-ATPase displayed any activity when Mg\(^{2+}\) was replaced with Ca\(^{2+}\).

A substantial increase in substrate affinity was observed with a Mg\(^{2+}\) or Ca\(^{2+}\) concentration (1 mM) at which most of the ATP would be uncomplexed with the divalent cation (Fig. 34). A ten-fold increase in the Mg\(^{2+}\) or Ca\(^{2+}\) concentration did not result in a substantial increase in substrate affinity over and above that observed at 1 mM cation (Fig. 34). Thus, the increase in substrate affinity in the presence of Mg\(^{2+}\) or Ca\(^{2+}\) was not due to the enzyme expressing a greater affinity for the ATP-divalent cation.
complex. From these data it is possible to postulate the presence of at least one distinct divalent cation binding site at which binding of Mg$^{2+}$ or Ca$^{2+}$ serves to activate the enzyme by increasing its affinity for substrate. The reduction in velocity observed with 100 mM Mg$^{2+}$ or Ca$^{2+}$ may indicate an additional divalent cation binding site, of lower affinity than the first, at which Mg$^{2+}$ or Ca$^{2+}$ binding inhibits, rather than activates, the enzyme. The inhibition induced under conditions of excess divalent cation (Figs. 32 and 34) was not simply an ionic effect, as enzyme activity was unaffected by high concentrations of NaCl or KCl.

**Physiological Role of the D. discoideum DES Insensitive ATPase**

The physiological role of the purified *D. discoideum* ATPase is not known. It is unlikely that it corresponds to the *D. discoideum* plasma membrane ATP-pyrophosphohydrolase described by Rossomando and Hodge-Jahngen (1983), as the latter enzyme was reported to be vanadate insensitive. It is also unlikely that the purified ATPase is a vacuolar ATPase of the type described in plants (Randall and Sze, 1986), yeast (Uchida *et al.*, 1985) and Neurospora (Bowman, 1983; Table XX). The vacuolar ATPases are insensitive to vanadate (Churchill and Sze, 1984; Sze, 1985; Bowmann, 1983; Uchida *et al.*, 1985) and fluoride (Wang *et al.*, 1986) but are sensitive to DCCD (Walker and Leigh, 1981a; Bowman, 1983; Uchida *et al.*, 1985; Randall and Sze, 1986) and DES (Walker and Leigh, 1981; Uchida *et al.*, 1985). Bowman
(1983) and Bowman et al, (1985) have suggested that the vacuolar ATPase is similar to the mitochondrial $F_0F_1$-ATPase.

A more likely possibility is that the purified ATPase is a non-specific Ca$^{+2}$ or Mg$^{+2}$-ATPase similar to the enzymes that have been identified in the plasma membranes of placenta (Shami and Radde, 1971), kidney (Parkinson and Radde, 1971), liver (Garnett and Kemp, 1975; Lotersztajn et al., 1981), corpus luteum (Verma and Penniston, 1981) and neutrophil cells (Ochs and Reed, 1984). Initial work on the enzymes from placenta and rat kidney cortex revealed that ATPase activity was stimulated by Mn$^{+2}$ in addition to Ca$^{+2}$ and Mg$^{+2}$, but not by Sr$^{+2}$ (Shami and Radde, 1971; Parkinson and Radde, 1971). Stimulation by Mn$^{+2}$ did not exceed that observed with either Mg$^{+2}$ or Ca$^{+2}$. Both of these enzymes were resistant to azide and expressed optimal ATPase activity between pH 7.5 to 9.0, values that are somewhat higher than those observed for the purified Dictyo3telium enzyme (Fig. 26). Very little subsequent work has been done on the nonspecific Ca$^{+2}$ or Mg$^{+2}$-ATPases and their physiological role remains unclear, although Avissar et al. (1975) suggested the erythrocyte plasma membrane Ca$^{+2}$ or Mg$^{+2}$-ATPase to be a myosin-like ATPase and possibly play some role in the contractile process of the cell, since the activity was stimulated by actin. Thus it is conceivable that the D. discoideum ATPase might have a function in motility.
Penniston (1983) has suggested that one of the criteria to be addressed in order to ascribe a Ca\(^{+2}\) pumping function to a Ca\(^{+2}\) stimulated ATPase is that it should be stimulated by free Ca\(^{+2}\) concentrations \(\leq 10 \, \mu\text{M}\) in the presence of calmodulin. The purified *D. discoideum* ATPase was not stimulated by Ca\(^{+2}\) concentrations under 1 mM (Fig. 32) nor was enzyme activity stimulated by Ca\(^{+2}\)/calmodulin (Table XVIII). However, the possibility that this ATPase functions as a high affinity Ca\(^{+2}\) pump cannot be ruled out as it is possible that the calmodulin binding site was either lost or denatured during the purification procedure. If this were the case then the low Ca\(^{+2}\) affinity could be explained as the action of calmodulin would be to increase the apparent affinity of the ATPase for Ca\(^{+2}\) (Roufagalis, 1979; Niggli et al., 1981; Kotagal et al., 1983; Penniston, 1983, Ansah et al., 1984; Carafoli et al., 1984)

Plasma membrane bound ATPase from *D. discoideum* was similarly unaffected by exogenous calmodulin even though the membranes had been extracted with EGTA or KCl/EDTA to ensure removal of any endogenous calmodulin (Jarrett and Penniston, 1978). Again, this negative result could be due to the removal of an integral membrane component necessary in conferring calmodulin sensitivity to a putative Ca\(^{+2}\) pump. It should be noted that these assays were not performed in a Ca\(^{+2}\)/EGTA buffer and that the failure to observe a calmodulin stimulated, high affinity Ca\(^{+2}\)-ATPase activity could be due to the absence of EGTA. It has been
suggested (Penniston, 1983; Carafoli et al., 1984) that the high Ca$^{+2}$ affinity of the (Ca$^{+2}$:Mg$^{+2}$)-ATPases requires the presence of EGTA. Indeed, Kotagal et al. (1983) have observed EGTA to mimic the effect of calmodulin in activating the (Ca$^{+2}$:Mg$^{+2}$)-ATPase from islet cell plasma membranes.

Presumably *D. discoideum* plasma membranes do contain a Ca$^{+2}$ transporter since Ca$^{+2}$ flux has been measured during cAMP signalling and $^{45}$Ca$^{+2}$ uptake has been observed (Wick et al., 1978; Bumann et al., 1984; Parish and Weibel, 1980; Europe-Finner and Newell, 1985). This putative Ca$^{+2}$ transporter may be a high affinity (Ca$^{+2}$-Mg$^{+2}$)-ATPase which has eluded detection in the present study and it is possible that the ecto-ATPase described by Parish and Weibel (1980) is a Ca$^{+2}$ pump. Evidence for a facilitated diffusion mechanism for Ca$^{+2}$ uptake in *D. discoideum* has been presented (Europe-Finner and Newell, 1985a), but the putative transporter has not yet been characterized. It is also possible that *D. discoideum* plasma membranes contain a Na$^{+}$/Ca$^{+2}$ exchanger of the type described in the giant axon of squid, heart tissue and other excitable cells. (Carafoli et al., 1984).

Finally, it is possible that the purified *D. discoideum* ATPase is not a plasma membrane enzyme but represents contamination with a non mitochondrial intracellular membrane, and is involved in the regulation of internal Ca$^{+2}$ stores. Internal Ca$^{+2}$ mobilization has recently been
implicated in the synthesis of cGMP during the early stages of *D. discoideum* differentiation (Europe-Finner and Newell, 1985; Europe-Finner *et al*., 1985; Small *et al*., 1986; Europe-Finner and Newell, 1986). Rabbit skeletal muscle transverse tubule membranes have been shown to contain a Ca\(^{2+}\) or Mg\(^{2+}\)-ATPase which was capable of transporting Ca\(^{2+}\) (Fernandez *et al*., 1980; Hidalgo *et al*., 1983), although the molecular weight of this enzyme (112 kDa) was considerably higher than that of the purified *D. discoideum* ATPase.
REFERENCES


pumping ATPase from oat roots: direct effects of Cl−, NO3− and a disulfonic stilbene. Plant Physiol. 76:490-
497.

activated, vanadate sensitive Mg2+-ATPase from radish

ATPase of sarcoplasm reticulum by affinity

Condeelis, J. (1979). Isolation of ConA caps during various
stages of formation and their association with actin

weight of the minimum asymmetric unit of canine renal

the hydrolytic moiety of the Neurospora plasma membrane
H+-ATPase and demonstration of a phosphoryl-enzyme
intermediate in its catalytic mechanism. Biochemistry
19:2931-2937.

the phosphorylated intermediate of the Neurospora
plasma membrane H+-ATPase a S-aspartylphosphate. J.

Darmon, M., Brachet, P. and Da Silva, L.H. (1975).
Chemotactic signals induce cell differentiation in
72:3162-3166.

gentle lysis of eukaryotic cells. Isolation of plasma
membranes from Dictyostelium discoideum. Biochim.
Biophys. Acta. 736:45-56.

Ca2+ stimulated Mg2+ dependent ATPase in Friend murine
erythroleukemia cell plasma membranes. J. Biol. Chem.
259:13824-13831.

De Smedt, H., Parys, J.B., Borghgruef, R. and Wrytak, F.
(1983). Phosphorylated intermediates of (Ca2+ + Mg2+)-
ATPase and alkaline phosphatase in renal plasma


Lotersztajn, S., Hanoune, J. and Pecker, F. (1981). A high affinity Ca$^{2+}$ stimulated Mg$^{2+}$ dependent ATPase in rat liver plasma membranes. Dependence on an endogenous


the cellular slime mold Dictyostelium discoideum. FEBS Letts. 175:422-428.


Winter, C.G. and Moss Jr., A.J. (1979). Ultracentrifugal analysis of the enzymatically active fragments produced by digitonin action on Na\(^+\),K\(^+\)-ATPase. in Na\(^+\),K\(^+\)-ATPase: