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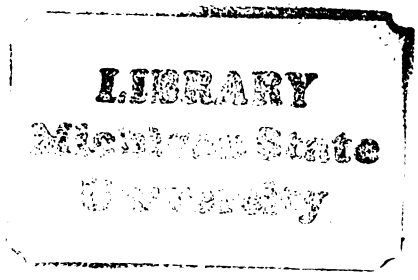

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PHOSPHORYLATION OF LYSOSOMAL MEMBRANE COMPONENTS

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PHOSPHORYLATION OF LYSOSOMAL MEMBRANE COMPONENTS

By

Christine Ann Collins

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ABSTRACT

PHOSPHORYLATION OF LYSOSOMAL MEMBRANE COMPONENTS

By

Christine Ann Collins

In order to examine whether regulation of lysosomal function could be mediated by modification of membrane components through phosphorylation, lysosomal membranes were treated with [γ-^{32}P]ATP and the labeled products were characterized. Two phosphorylated components were detected. One of these was found to contain ^{32}P in an acyl linkage. This phosphorylated product exhibited rapid turnover and sensitivity to pH and hydroxylamine characteristic of other acylphosphorylated enzymes, most of which are cation pump ATPases. Acylphosphate formation occurred in the absence of a divalent metal cation, but the rate and extent of phosphorylation were increased in the presence of Mg^{2+}. Calcium did not stimulate ^{32}P incorporation. However, dephosphorylation was stimulated by either Ca^{2+} or Mg^{2+}. Acylphosphate formation was decreased by treatment of the membrane with inhibitors of the lysosomal membrane ATPase, such as N,N'-dicyclohexylcarbodiimide and sulfhydryl reagents. Polyacrylamide gel electrophoresis and autoradiography demonstrated a labeled band, approximately 180,000 daltons, exhibiting properties of the acylphosphate moiety. Phosphoryl transfer activities similar to those found for other acylphosphorylated ATPases were detected in lysosome

preparations. These results are consistent with the identification of the acylphosphate as a covalent reaction intermediate of a lysosomal membrane ATPase.

The second phosphorylated material was identified as phosphatidylinositol 4-phosphate and a trace of phosphatidylinositol 4,5-bisphosphate based on its chromatographic properties on silicic acid. Chromatographic and electrophoretic analysis of the deacylated lipids further substantiated this conclusion. The enzyme which carried out the phosphorylation reaction, phosphatidylinositol kinase, was characterized with respect to assay conditions required for optimal activity and the use of exogenous phosphatidylinositol as a substrate. The specific activity of the kinase in lysosomes was comparable to the activity found in liver microsomes and plasma membrane, the previously recognized sources of this enzyme. Stimulation of phosphoinositide metabolism has been observed in many tissues in response to hormones and other agents which modify calcium flux in the cell. It is possible that some of the recognized effects of hormones on liver lysosome function may be mediated through regulation of polyphosphoinositide turnover in the lysosomal membrane.

To My Parents

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TABLE OF CONTENTS

page

LIST OF TABLES..... vii

LIST OF FIGURES.....viii

ABBREVIATIONS..... ix

INTRODUCTION..... 1

LITERATURE REVIEW

The vacuolar apparatus of the cell..... 3
Receptor-mediated endocytosis..... 3
Lysosome isolation methods..... 4
Characterization of the lysosomal membrane..... 5
Acidification of the lysosomal compartment..... 8
Hormonal regulation of lysosomal activity..... 11
Membrane fusion..... 11
Calcium and polyphosphoinositide metabolism..... 12

REFERENCES..... 15

CHAPTER

I. CHARACTERIZATION OF ENDOGENOUS PROTEIN PHOSPHORYLATION IN ISOLATED RAT LIVER LYSOSOMES..... 23

Abstract..... 24

Introduction..... 24

Experimental Procedures

Materials..... 24
Membrane preparation..... 24
Standard phosphorylation assay..... 25
Polyacrylamide gel electrophoresis..... 25
Other assays..... 25

Results

Time course of membrane phosphorylation..... 25
Polyacrylamide gel electrophoresis of labeled products. 25
Effect of cyclic nucleotides on phosphorylation..... 26
Substrate specificity of the phosphorylating activity.. 26
Chemical characterization of the phosphorylated products..... 26

Discussion..... 27

References..... 28

II. IDENTIFICATION OF PHOSPHATIDYLINOSITOL KINASE IN RAT LIVER LYSOSOMAL MEMBRANES..... 29

Abstract..... 30

Introduction..... 30

Experimental Procedures

Materials..... 30
Lysosome preparation..... 30
Lysosome phosphorylation..... 30
Extraction of lipids..... 31
Chromatography of lipids..... 31
Chromatography of deacylated products..... 31
Phosphatidylinositol kinase determination..... 31
Protein determination..... 31

Results

Identification of ^{32}P-labeled phosphatidylinositol 4-phosphate..... 31
Properties of lysosomal phosphatidylinositol kinase
Assay conditions..... 32
Effect of Triton X-100..... 32
Effect of phosphatidylinositol..... 32
Time course of phosphatidylinositol 4-phosphate production..... 32
PI kinase in normal rat liver lysosomes..... 33
Subcellular localization of PI kinase..... 33
Inhibition of PI kinase..... 33

Discussion..... 33

References..... 34

III. CHARACTERIZATION OF AN ACYLPHOSPHATE INTERMEDIATE OF A LYSOSOMAL MEMBRANE ATPase..... 35

Abstract..... 36

Introduction..... 37

Experimental Procedures

Materials..... 39
Lysosome preparation..... 39
Phosphorylation assay..... 40
Polyacrylamide gel electrophoresis..... 40
ATPase assays..... 41
NTP-NDP phosphate exchange..... 42

ATP-P_i exchange..... 42
Protein determination..... 43

Results

Substrate specificity of the acylphosphorylation reaction..... 44
Polyacrylamide gel analysis of the acylphosphate.... 44
Cation requirement for acylphosphorylation..... 50
Cation requirement for dephosphorylation..... 50
Substrate specificity of the lysosomal membrane ATPase..... 55
Effect of inhibitors on acylphosphate formation and ATPase activity..... 55
Phosphoryltransfer reactions..... 58

Discussion..... 64

References..... 74

SUMMARY..... 77

LIST OF TABLES

Table — Page

Literature Review

I. Lipid Composition of the Lysosomal and Plasma Membranes... 6

II. Carbohydrate Composition of the Lysosomal and Plasma Membranes..... 7

Chapter I

I. Effect of Cyclic Nucleotides on Membrane Phosphorylation.. 26

II. Substrate Specificity of Lysosomal Phosphorylation Activity..... 26

III. Characterization of Reaction Products..... 27

Chapter II

I. Activity of PI Kinase from Lysosomes, Plasma Membrane, and Microsomes..... 33

II. Activity of Lysosomal PI Kinase in the Presence of Inhibitors..... 33

Chapter III

I. Acylphosphate Formation in Lysosomal Membranes..... 49

II. Substrate Specificity of Lysosomal Membrane ATPase..... 56

III. Effect of ATPase Inhibitors on ATPase Activity and Acylphosphate Formation..... 57

IV. Effect of Sulfhydryl Reagents on ATPase Activity and Acylphosphate Formation..... 61

V. Phosphoryl Transfer Reactions of the Lysosomal Membrane... 63

VI. Summary of ATPase Partial Reactions under Selected Experimental Conditions..... 70

LIST OF FIGURES

Figure — Page

Chapter I

1. Time Course of Endogenous Phosphorylation in Rat Liver Lysosomal Membranes..... 25
2. Time Course of Endogenous Phosphorylation..... 25
3. TDAB-Acid Gel Electrophoresis of Phosphorylated Lysosomal Membranes..... 26
4. Factors Affecting Lysosomal Membrane Phosphorylation..... 26
5. Effect of pH on the Rate of Hydrolysis of Phosphorylated Products..... 27

Chapter II

1. Analysis of the ^{32}p-labeled Material from Lysosomes..... 31
2. Assay Conditions for Lysosomal PI Kinase..... 32
3. Effect of Triton-X 100 on Lysosomal Phosphatidylinositol Kinase..... 32
4. Effect of Phosphatidylinositol Concentration on PI Kinase Activity..... 32
5. Time Course of the Phosphatidylinositol Kinase Reaction... 32
6. Time Course of DPI Formation in Normal Liver Lysosomes.... 32

Chapter III

1. Nucleotide Specificity of Phosphorylation Reaction..... 45
2. Analysis of Acylphosphate on LDS Polyacrylamide Gels..... 47
3. Metal Ion Specificity for Phosphorylation..... 51
4. Metal Ion Requirement for Dephosphorylation..... 53
5. Effect of pH on Vanadate Inhibition of ATPase..... 59

ABBREVIATIONS

Abbreviations not listed below are standard usage according to the Instructions to Authors, 1983, in the Journal of Biological Chemistry.

ATPγS	adenosine 5'-[γ-thio]triphosphate
CaM	calmodulin
CDTA	trans-cyclohexane-1,2-diamine-N,N,N',N'-tetraacetic acid
DCCD	N,N'-dicyclohexylcarbodiimide
DPI	phosphatidylinositol 4-phosphate
EGTA	ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetra-acetic acid
FITC	fluorescein isothiocyanate
LDS	lithium dodecylsulfate
MOPS	4-morpholino-propane sulfonic acid
NEM	N-ethylmaleimide
NDP	nucleoside diphosphate
NTP	nucleoside triphosphate
pCMB	p-chloromercuribenzoic acid
PI	phosphatidylinositol
SDS	sodium dodecylsulfate
TDAB	tetradecyltrimethyl ammonium bromide
TPI	phosphatidylinositol 4,5-bisphosphate
2-ME	2-mercaptoethanol

INTRODUCTION

Hormones, particularly insulin and glucagon, are known to have effects on proteolysis in liver cells (1). Glucagon greatly stimulates cellular autophagy, proteolysis, and lysosomal membrane swelling and fragility (2). In the fasting state, increased degradation of intracellular proteins by the lysosomes provides a pool of amino acids. Insulin and exogenous amino acids counteract this effect. Thus, glucagon and insulin control intracellular protein catabolism, which may be a major regulatory point in hepatic gluconeogenesis.

The studies to be described here were undertaken with the objective of determining whether phosphorylation of lysosomal membrane components occurs. The effects of glucagon on cellular metabolism are in most part carried out through a phosphorylation-dephosphorylation mechanism modulated by cyclic AMP (3). Insulin also affects the phosphorylation of soluble and membrane proteins, including its own receptor (4-6). Zahlten et al. reported that glucagon stimulated the net uptake of $^{32}P_i$, in vivo, into rat liver proteins of microsomes, mitochondria, and lysosomes (7). An initial study of lysosomal phosphorylation, in vitro, also found stimulation of ^{32}P labeling by cyclic AMP (8). This effect was later found to be due to contamination of the crude lysosomes with soluble or other organelle-associated protein kinases. The phosphorylation of more purified lysosomal membranes has been examined and the results of these studies are presented here.

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LITERATURE REVIEW

The vacuolar apparatus of the cell. Lysosomes ("lytic particles") were first identified as a membrane-bound, or latent, form of acid phosphatase (9). Lysosomes are now known to contain over 60 acid hydrolases, most of which are glycoproteins (10). This organelle is unique in that its size is quite variable and the lysosomal contents are heterogeneous. In fact, the word lysosome refers to a variety of organelles, termed the vacuolar apparatus (11), which are involved in the digestion of every class of biological material from endogenous and exogenous sources. Among these organelles, primary lysosomes are those whose enzymes have not engaged in a digestive event. Secondary lysosomes are sites of present or past digestion.

Receptor-mediated endocytosis. Secondary lysosomes may arise from fusion of primary lysosomes with vesicles derived from the plasma membrane. These vesicles have been referred to as endosomes, endocytic vesicles, and receptosomes (12, 13). The process of endocytosis and subsequent transfer of material to the lysosomal compartment has been examined in some detail for such exogenous ligands as low-density lipoprotein, transferrin, asialoglycoproteins, peptide hormones, bacterial toxins, viruses, and lysosomal enzymes themselves (12, 14). The initial formation of an endocytic vesicle relies on the binding of these ligands to a plasma membrane receptor, invagination of this area of the membrane (coated pit), and then release of the coated vesicle into the cytoplasm. The clathrin coat is rapidly shed and the smooth

vesicle may then fuse with another endosome or with a lysosome.

Lysosomes may in some cases also phagocytize endogenous material (15). A portion of cytosol or internal membrane structure becomes segregated by a limiting membrane of as yet unknown origin. This phagosome fuses with the lysosomal membrane to form an autophagolysosome. These secondary lysosomes may be involved with many digestive events until undigested material builds up within the lysosomal membrane. The structure is then referred to as a residual body, containing electron dense material, membrane remnants, and a highly pigmented material, lipofuscin (9). This also occurs in pathological states of lysososmal storage disease, where the lack of a specific hydrolase leads to a build up of unmetabolized material in the lysosome (16).

Lysosome isolation methods. It is very difficult to purify liver lysosomes away from other cellular organelles. Differential centrifugation schemes do not result in adequate separation of lysosomes from mitochondria and peroxisomes. The method currently in wide use involves injection of rats with the detergent Triton WR-1339 (17). This material is taken up by the liver by adsorptive pinocytosis and is accumulated in the lysosomes where it cannot be degraded. The secondary lysosomes containing this material have a lowered density and can therefore be separated from contaminating cellular components with a relatively high yield. The disadvantages of this method are that only secondary lysosomes are obtained, i.e., those lysosomes which have fused with endosomes carrying the detergent, and the possible alteration in membrane structure and enzymatic activity caused by this material (17-20). Other purification methods include: 1, uptake of dextran-500 by lysosomes with a corresponding increase in density (21); 2, density gradient centrifugation in metrizamide (22); and 3, free

flow electrophoresis (19). These methods also result in a relatively high degree of purification. However, these methods also probably select for only secondary lysosomes. Percoll density gradient centrifugation (23) has been utilized to separate two populations of lysosomes, probably corresponding to primary and secondary lysosomes (24). The yield and degree of purification are not as high as for the other methods, however.

Characterization of the lysosomal membrane. In order to carry out cellular hydrolytic functions, the limiting membrane of the lysosome must fuse with that of the vesicle carrying the substrate. Therefore, the regulation of lysosomal activity in the cell may well occur at the level of membrane interactions. In order to examine lysosomal membrane function, several studies of the lipid (19, 25-30), protein (25, 30-35), and carbohydrate (25, 31, 36) composition of the membrane have been carried out. The lipid composition (Table 1) is quite similar to that of the plasma membrane. These membranes and the Golgi apparatus are characterized by large amounts of cholesterol and sphingomyelin. In addition, the fatty acids found in lysosome and plasma membrane lipids show a high degree of saturation (25, 37). The lysosomal membrane has a unique lipid, bis(monoacylglyceryl)phosphate, which is synthesized from lysophosphatidylglycerol and an acyl donor, probably phosphatidyl-inositol, on the lysosomal membrane (38).

The similarity between the lysosomal and plasma membranes has led some researchers to suggest that large amounts of plasma membrane are incorporated into secondary lysosomes (19, 29). However, the carbohydrate contents of the lysosomal membrane (Table II) is higher than that of the plasma membrane, and the protein and glycoprotein composition is entirely different from that of other subcellular

Table I. Lipid Composition of the Lysosomal and Plasma Membranes.

Lipid	Lysosomal Membrane				Plasma Membrane	
	% of lipid phosphorous					
phosphatidylcholine	41.4[a]	36.2[b]	33.6[c]	39.7[d]	26[e]	43.1[f]
lysophosphatidylcholine	0.9	1.2	7.8	7.4	4	1.8
phosphatidylethanolamine	26.3	19.7	10.2	14.1	16	19.8
lysophosphatidylethanolamine	2.9	1.7	1.1	n.d.[g]	n.d.	n.d.
phosphatidylserine	3.0	3.1	3.9	1.7	13	3.7
phosphatidylinositol	9.7	4.5	5.9	4.5	8	6.5
sphingomyelin	7.6	28.4	21.0	20.3	21	23.1
cardiolipin	4.0	1.9	-	1.0	2	-
bis(monoacylglyceryl)phosphate	4.2	3.3	12.8	7.0	7	-
unidentified			3.7	0.6		

[a]Lysosomes prepared by free-flow electrophoresis (19).

[b,c,d]Triton-filled lysosomes (19, 27, 28).

[e,f]Plasma membrane preparations from Triton-treated rats (29, 30).

[g]Not determined.

Table II. Carbohydrate Composition of the Lysosomal and Plasma Membranes.

Carbohydrate	Lysosomal Membrane			Plasma Membrane
			µg/mg protein	
neutral sugars	45.6[a]	135.1[b]	197[c]	28.0[a]
glucosamines	25.4	37.0	14	trace
galactosamines	5.5		25	8.6
sialic acid	16.1	52.1	28	10.4

[a]Lysosomes and plasma membranes from Triton-treated rats (25).

[b]Triton-filled lysosomes (31).

[c]Normal lysosomes (36).

organelles (25, 35). There do not appear to be any enzymatic activities common to both the lysosomal membrane and the plasmalemma (33, 34). In addition, the rate of turnover of lysosomal membrane proteins and carbohydrate residues is different from that of plasma membrane (35). This suggests that either the region of the plasma membrane which invaginates to form endocytic vesicles is of a much different composition than other areas of the membrane, or that the components of the endosome do not remain with the lysosome but are recycled back to the cell surface. Evidence has been accumulating in support of the second hypothesis (12, 14, 34, 39-41). Plasma membrane markers are constantly recycled between surface and internal compartments. The half-life of cell surface receptors is much longer than would be calculated based on the rate of endocytosis and degradation of the receptor-associated ligand. Hence, there must be extensive reutilization of endosome membrane components and little incorporation of these into the lysosome.

Acidification of the lysosomal compartment. The majority of lysosomal hydrolases have a pH optimum in the acid range (37). The pH of the lysosome in living cells has been measured by the uptake of a pH-sensitive fluorescent dye (42), and determined to be in the range of 3-5. Maintenance of the acid pH was dependent on energy, since the addition of metabolic inhibitors led to an increase in lysosomal pH and a resulting inability of the lysosomes to carry out hydrolytic functions. The presence of an energy-dependent proton pump has been postulated by Mego (43), based on the increased proteolytic activity of isolated lysosomes incubated at pH 8 when ATP was added to the medium. No stimulation by ATP was observed at pH 5, suggesting that the lysosomal proteases were already optimally active at this pH. Studies measuring the ATP-dependent uptake of basic dyes (44), methylamine (45),

and amino acid methyl esters (46) into lysosomes have also led to the conclusion that a proton pump is active in the lysosomal membrane. The uptake of these substances is based on their diffusion across the membrane in an uncharged state and subsequent protonation in an acidic compartment. The compounds in the ionized form are then unable to cross the membrane. This mechanism may also hold true for the anti-inflammatory drug, chloroquine, which is concentrated over 1000-fold in the lysosomes compared with the cytosol (47). This compound, other "lysosomotropic" amines, and NH_4Cl inhibit a number of lysosomal functions, such as protein degradation (48-50), receptor recycling (40, 51, 52), and fusion of the lysosome with endosomes and phagosomes (53-56). The inhibitory effect of the amines is believed to be due to an elevation of the lysosomal pH. Continuous uptake of these compounds as observed in cultured cells would therefore require constant adjustment of the pH by means of an energy requiring proton pump (58). A second mechanism for maintaining low pH is a Donnan equilibrium established by negatively charged glycoproteins within the lysosome. This mechanism probably accounts for the difference of 1 pH unit found between isolated lysosomes and their external medium (59), although it is not responsible for formation of the initial acid conditions (60, 61).

Direct evidence for the existence of a proton pump in lysosomal membranes has been obtained by Ohkuma et al. (57, 62). They examined the fluorescence of a dextran-dye conjugate which had been sequestered in secondary lysosomes. The fluorescence of the isolated organelles was proportional to the pH of the internal compartment. ATP was found to decrease the fluorescence due to the lowered pH within the lysosomes. Various metabolic inhibitors and ionophores were found to inhibit this activity, further substantiating the existence of an ATP-driven proton

pump in the lysosomal membrane.

An ATPase activity in the lysosomal membrane has been analyzed by Schneider and others (46, 63-65). It was suggested that this enzyme carries out the translocation of protons across the membrane (45). In the studies described in Chapter III, various inhibitors of known proton pump ATPases were found to also inhibit the lysosomal enzyme. Since this activity is non-latent and exhibits a neutral pH optimum, it has been suggested that the active site of the enzyme is located on the cytoplasmic face of the lysosomal membrane (64).

An ATP-driven proton pump in the membrane of adrenal medulla chromaffin granules has been well characterized (67-69). The physiological function of the acid pH in these granules is to promote the accumulation of the basic catecholamines against a concentration gradient. This proton pump exhibits sensitivity to ionophores and metabolic inhibitors and has been characterized as having an electrogenic mechanism. An ATPase activity has been measured in chromaffin granule membranes which is thought to be responsible for acidification (70, 71). The chromaffin granule and lysosomes may be related in both structure and function since they are both derived from the Golgi (39), and lysosomal hydrolases are secreted from the adrenal cell along with the catecholamines. A similar acidification activity has also been observed in the sperm acrosome, which is a modified lysosome at the head of the sperm which fuses with the plasma membrane in the capacitation reaction (74, 75). An energy-dependent proton pump has also been postulated for the membranes of yeast vacuoles and secretory vesicles from many sources (76-83). The possible role lysosomes may play in fusion of these vesicles with the plasma membrane and subsequent secretion has been reviewed (72, 73, 84-86). Proton pump

activity has also been found in endosome membranes (87, 88). The acid pH within this vesicle promotes dissociation of ligands from their receptor. The receptor may then be recycled back to the cell surface and the ligand may diffuse into the cytoplasm or be delivered to the lysosome for degradation (12).

Hormonal regulation of lysosome activity. Most studies of lysosomal function involve the measurement of proteolysis under various hormonal (89) and nutritional (90, 91) conditions. Glucagon has been shown to enhance proteolysis in the perfused liver and in isolated hepatocytes (92-94), while insulin and excess amino acids inhibit breakdown. It was proposed that the glucagon effect is mediated by a decrease in intracellular amino acids, particularly glutamine (95). Ogawa et al. have examined the perfused liver microscopically and have found that glucagon or cyclic AMP in the perfusate stimulates the formation of autophagolysosomes (96, 97). Lysosomes in these electron micrographs were seen to elongate and wrap around intracellular structures such as mitochondria. This suggests that the lysosomal membrane may be altered in some way by glucagon treatment. Glucocorticoids have also been found to stimulate proteolysis in isolated hepatocytes (98). It has been postulated that this occurs independently of glucagon and insulin effects, perhaps by stimulation of membrane protein synthesis required for autophagy. These experiments suggest that the regulation of lysosome fusion with other membranes may be the important factor in modulating lysosomal hydrolytic activity.

Membrane fusion. Studies of lysosome fusion with plasma membrane and phagosomes have been carried out in vivo and in vitro (66, 99-103). A recent report has demonstrated the fusion of isolated endocytic vesicles with lysosomes (104). Vesicles which retained their clathrin

coats were unable to fuse. Uncoated endososmes, and those stripped of their surface proteins by protease treatment were capable of fusion, suggesting that lipid components are of primary importance in this process. Fusion events cannot be indescriminant, however, since lysosomes only fuse with newly formed endocytic vesicles, only rarely with older vacuoles, and never directly with mitochondria or the nucleus (105). Secondary lysosomes may fuse several times with endosomes, phagosomes, or other secondary lysosomes, however. It has been suggested that the similarity of the plasma membrane and the lysosomal membrane allows recognition and fusion of these elements (30, 105). However, as discussed before, the protein and carbohydrate composition of these membranes is quite different. Again, it may be that the lipid components, which are quite similar for these membranes but different from other cellular structures, are the important recognition point. The high concentration of sphingomyelin, cholesterol, and saturated fatty acids in these two membrane systems confers impermeability and a large degree of structural rigidity (25). The only unique lipid in the lysosomal membrane, bis(monoacylglyceryl)phosphate, arises from interaction of the lysosome with other membranes which contain phosphatidylglycerol or cardiolipin. Secondary lysosomes, e.g. Triton-filled lysosomes, have been found to contain much higher levels of this lipid than primary lysosomes (27). The effect of this lipid on membrane structure and function, however, is unknown.

Calcium and polyphosphoinositide metabolism. Calcium is known to play a role in membrane fusion, both from studies with artificial lipid bilayers (106, 107) and in studies of secretion, where secretory vesicles fuse with plasma membrane during exocytosis (108-110). Extracellular calcium is required for the release of both catecholamines and

lysosomal enzymes from the adrenal gland (111). Calcium is also required for the release of acid hydrolases from the polymorphonuclear leukocyte (66). Some investigators have suggested a role for a Ca^{2+}-dependent ATPase on the membrane of fusion competent vesicles (66, 108, 112). ATP stimulates fusion of secretory vesicles with plasma membrane (112, 113), but the mechanism of this action is not completely understood.

The class of myo-inositol containing phospholipids has been proposed to play a role in membrane fusion in secretory cells (66, 114-116), myoblasts (117), erythrocytes (118), and lysosome fusion in leukocytes (116). The polyphosphoinositides, phophatidylinositol 4-phosphate (DPI), and phosphatidylinositol 4,5-bisphosphate (TPI), exhibit rapid turnover, particularly in response to hormones and other agents which stimulate calcium flux in their target cells (119, 120). Phosphatidylinositol kinase has been found in liver plasma membrane (121), microsomes (122, 123), nuclear envelope (124), and in adrenal chromaffin granule membranes. The data to be presented in Chapter II indicate a lysosomal membrane localization for this enzyme as well. The further phosphorylation of DPI to TPI is catalyzed by a particulate enzyme in erythrocytes (126) and kidney (127), and by a soluble enzyme in brain (128). The degradation of the polyphosphoinositides has been extensively studied with regard to soluble and membrane bound phosphomonoesterases (122, 129), as well as soluble and membrane bound Ca^{2+}-dependent phosphodiesterases (130-132). The latter enzyme may be stimulated by hormones binding to their cell surface receptor and the subsequent increase in cytosolic calcium levels (133-135). The reaction product, 1,2-diacylglycerol, may reenter the biosynthetic scheme through phosphatidic acid, cytidine diphosphoglyceride, and back

to phosphatidylinositol. The inositolphosphates also produced may be further degraded by phosphomonoesterases (129). The enzymology of this phosphoinositide turnover phenomenon has been recently reviewed (136). The phosphatidylinositol cycle is thought to occur largely on the plasma membrane. However, the presence of polyphosphoinositides on the lysososmal membrane leaves open the possibility that this pool of lipid is also hormonally controlled (137).

Besides their possible function in calcium translocation and membrane fusion, polyphosphoinositides have been implicated in the propagation of nerve action potentials, plasma membrane ion and solute transport, and the orientation and modulation of enzymes (119). Phosphoinositides have been found to serve as anchorage sites for several plasma membrane-associated enzymes (138). A requirement for phosphatidylinositol for the maintenance of membranous ATPase activities has also been found (139, 140). Although the polyphosphoinositides probably constitute only a few percent of the total membrane phospholipid, membrane structure may be substantially altered by the loss of the highly charged inositolphosphate head group by phosphodiesterase activity. TPI and DPI may bind divalent metal ions, which render the lipids more hydrophobic and alter their orientation in the membrane (141). Turnover of the phosphoinositides in the lysosomal membrane may have a physiological role in the alteration of membrane structure (137) and, therefore, the function of this organelle in the liver cell.

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CHAPTER I

CHARACTERIZATION OF ENDOGENOUS PROTEIN PHOSPHORYLATION IN ISOLATED RAT LIVER LYSOSOMES

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Characterization of Endogenous Protein Phosphorylation in Isolated Rat Liver Lysosomes*

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Membranes prepared from highly purified rat liver lysosomes contain endogenous protein-phosphorylation activities. The transfer of phosphate to membrane fractions from [γ-^{32}P]ATP was analyzed by gel electrophoresis under acidic denaturing conditions. Two phosphopeptides were detected, with molecular weights of 3,000 and 14,000. Phosphorylation of these proteins was unaffected by the addition of cAMP, cGMP, or the heat-stable inhibitor of cAMP-dependent protein kinase. No additional phosphorylation was observed when cAMP-dependent protein kinase was included in the reaction or when exogenous protein kinase substrates were added. The 14,000-dalton ^{32}P-labeled product was formed rapidly in the presence of low concentrations (250 μM) of either Ca^{2+} or Mg^{2+}. This product was labile under both acidic and alkaline conditions, suggesting that this protein contains an acyl phosphate, present presumably as a catalytic intermediate in a phosphotransferase reaction.

The lower molecular weight species required a high concentration (5 mM) of Mg^{2+} for phosphorylation, and micromolar concentrations of Ca^{2+} stimulated the Mg^{2+}-dependent activity. The addition of Ca^{2+} and calmodulin stimulated the phosphorylation reaction to a greater extent than with Ca^{2+} alone. This activity was strongly inhibited by 0.2 mM $LaCl_3$ and to a lesser extent by 50 μM chlorpromazine or trifluoperazine. These results suggest that the 3000-dalton peptide may be phosphorylated by a Ca^{2+}, calmodulin-dependent kinase associated with the lysosomal membrane.

Many membrane systems have been studied which show reversible phosphorylation of membrane-associated proteins (1). In most cases, the presence of membrane-bound protein kinases as well as the endogenous protein substrates has been demonstrated. While membrane phosphorylation may be sensitive to hormonal regulation, via cAMP or other effectors, a change in function or activity upon phosphorylation has yet to be demonstrated for the majority of these proteins. However, the phosphorylation-dephosphorylation of membrane proteins could provide a mechanism for the alteration of membrane structure and regulation of transport and enzymatic activities (2).

In 1972, Zahlten *et al.* (3) showed that glucagon stimulated the incorporation of ^{32}P, into membrane proteins of rat liver microsomes, mitochondria, and lysosomes *in vivo*. This result suggests that phosphorylation of lysosomal membrane proteins may occur by a cAMP-dependent process. Lysosome phosphorylation may, therefore, mediate some of the known effects of glucagon on hepatic lysosome function, such as increased proteolysis in the nutritionally deprived rat (4, 5) and formation of flattened vesicles and autophagolysosomes in livers perfused with glucagon or cAMP (6, 7).

We previously reported the *in vitro* phosphorylation of rat liver lysosomes isolated by Percoll density gradient centrifugation (8). Phosphorylation of lysosomes isolated from 3-day starved rats was stimulated by cAMP, whereas the activity in lysosomes from fed rats was unaffected or inhibited by the same concentration of cyclic nucleotide. In the present report, we examine the phosphorylation of more highly purified liver lysosomal membranes prepared from rats injected with the detergent Triton WR-1339.

EXPERIMENTAL PROCEDURES

Materials—[^{32}P]Orthophosphate, carrier free, was purchased from New England Nuclear. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (9), as modified by Reimann *et al.* (10). Calmodulin was purified to homogeneity from frozen bull testis according to the procedure of Dedman *et al.* (11). Materials for gel electrophoresis were purchased from Bio-Rad. Trifluoperazine was provided by Smith, Kline, and French Laboratories. Chlorpromazine, tetradecyltrimethyl ammonium bromide (TDAB),[1] bovine heart protein kinase inhibitor, type I cAMP-dependent protein kinase, cyclic nucleotides, and protein kinase substrates were obtained from Sigma. Triton WR-1339 was from the Ruger Chemical Co. All other chemicals for enzyme assays were of reagent grade.

Membrane Preparation—Triton WR-1339-filled lysosomes were prepared from rat liver by the method of Leighton *et al.* (12). Holtzman strain rats weighing between 150 and 250 g were injected intraperitoneally with Triton WR-1339 (85 mg/100 g body weight) 3 days before sacrifice. Lysosomes were isolated by differential centrifugation and flotation through 34.5% (w/w) sucrose in the presence of phenylmethylsulfonyl fluoride (75 mg/liter) and soybean trypsin inhibitor (100 mg/liter). Lysosomes obtained from the sucrose gradient were washed by diluting with an equal volume of cold 20 mM Tris-HCl, pH 7.5. After centrifugation at 40,000 × *g* for 20 min, the pellets were suspended in 10 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol. This lysosome preparation was purified 60-fold over the whole homogenate based on hexosaminidase specific activity (13). Contamination of the preparation by mitochondria, Golgi, and peroxisomes was negligible based on fumarase (14), galactosyltransferase (15), and urate oxidase (16) activities, respectively. Analysis of NADH cytochrome *c* reductase (17) indicated less than 5% contamination by endoplasmic reticulum. Analysis of 5′-nucleotidase (18) or alkaline phosphodiesterase (19) indicated less than 4% contamination of the lysosomes with plasma membrane. Membranes were prepared by 1 cycle of freeze-thawing and collected by centrifugation at 80,000 × *g* for 45 min. The resulting pellet was resuspended in 10 mM Tris-HCl.

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[1] The abbreviations used are: TDAB, tetradecyltrimethyl ammonium bromide; CaM, calmodulin.

pH 7.5, and 1 mM dithiothreitol and stored at −70 °C. Membranes prepared in this manner typically contained 35% of the protein present in the intact lysosome fraction.

Microsomes were isolated from rat liver as described in Ref. 20 and were purified 3-fold over the whole homogenate based on glucose 6-phosphatase and NADH cytochrome *c* reductase activity. Plasma membrane, isolated according to the method of Emmelot *et al.* (21), was purified 25-fold based on alkaline phosphodiesterase activity.

Standard Phosphorylation Assay—The reaction mixture contained lysosomal membranes (50–100 μg of protein), 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 0.1 mM [γ-^{32}P]ATP (100–200 cpm/pmol) in a final volume of 60 μl. The reaction was initiated by the addition of membrane sample and incubation at 30 °C was carried out for various times as indicated. The reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid containing 10 mM Na pyrophosphate and 20 μl of bovine serum albumin (5 mg/ml). After 10 min on ice, the samples were filtered through Whatman 3MM filter paper discs and washed with 10 ml of 10% trichloroacetic acid. The filters were dried and counted in a Beckman model 7000 liquid scintillation spectrometer. For gel electrophoresis, the reactions were stopped with 0.5 ml of 10% trichloroacetic acid containing 2 mM ATP. After 5 min on ice, the samples were centrifuged at 12,000 × *g* for 5 min. The precipitated proteins were washed by centrifugation with 0.5 ml of 10% trichloroacetic acid, 2 mM ATP, and with 0.5 ml of 50 mM KH_2PO_4/H_3PO_4, pH 2.0. The pellet was solubilized in 50 μl of 0.25 M sucrose, 2% 2-mercaptoethanol, 35 mM TDAB, 100 mM KH_2PO_4 (pH 4.0), and 10 μg/ml of methyl green.

Polyacrylamide Gel Electrophoresis—Cationic detergent acid slab gels containing 10% (w/v) acrylamide were prepared according to Amory *et al.* (22). The gel solution contained 0.08% (w/v) TDAB and 75 mM KH_2PO_4/H_3PO_4, pH 2.0. The 4% stacking gel solution was prepared with 125 mM KH_2PO_4, pH 4.0. The method of Jordan and Raymond (23) was used for polymerization. The electrode buffer containing 75 mM glycine and 0.125% (w/v) TDAB adjusted to pH 3.0 with H_3PO_4. The gel was run at constant current of 40 mA for 4 h at 4 °C.

After electrophoresis, the gels were soaked for 5 min in 2% glycerol, dried, and exposed to x-ray film for autoradiography. Molecular weights of phosphoproteins were estimated by comparison with prestained molecular weight standards obtained from Bethesda Research Laboratories. To quantitate the radioactivity in some cases, protein bands were cut from the dried gel, rehydrated in 0.5 ml of H_2O for 30 min, and counted in 5 ml of scintillation mixture.

Other Assays—Protein content was determined by the method of Lowry (24) using bovine serum albumin as a standard. Calmodulin content was determined by the procedure of Sharma and Wang (25).

RESULTS

Time Course of Membrane Phosphorylation—The incubation of lysosomal membranes with [γ-^{32}P]ATP resulted in a time- and concentration-dependent incorporation of radioactivity into trichloroacetic acid-insoluble material. The extent of phosphorylation after 1 min of incubation increased linearly with the amount of membrane sample up to 2 mg of protein/ml (data not shown). A rapid incorporation of label was seen (Fig. 1*A*) which peaked at 30 s and then diminished. A second component was phosphorylated at a slower rate. This product was stable to treatment with hot trichloroacetic acid as indicated in Fig. 1*B*, whereas the early peak disappeared. The difference plot derived from these curves is shown in Fig. 2*A*, indicating the time course of the acid-labile ^{32}P-labeled product. The data in Fig. 2*B* show that under conditions of low Ca^{2+} or Mg^{2+} concentration, an early burst of phosphorylation also occurs. A slow increase in ^{32}P incorporation is then seen in the presence of Ca^{2+} but not Mg^{2+}.

Polyacrylamide Gel Electrophoresis of Labeled Products—The time course and acid-alkali stability of the phosphorylated products were examined by TDAB-acid polyacrylamide gel electrophoresis and autoradiography (Fig. 3). After 30 s of incubation, both a 3,000 and 14,000-dalton band were labeled. However, by 2 min of incubation, the 14,000-dalton band disappeared. Treatment of the reaction mixture after 1 min of incubation with 10% trichloroacetic acid at

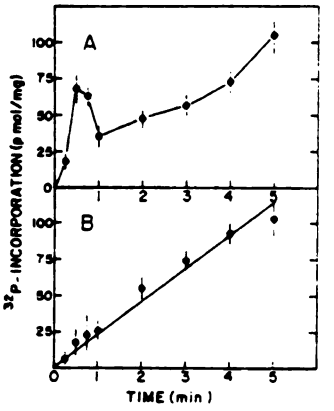

FIG. 1. **Time course of endogenous phosphorylation in rat liver lysosomal membranes.** *A*, incorporation of ^{32}P into total membrane proteins. Assays were conducted as described under "Experimental Procedures" for the times indicated. *B*, incorporation of ^{32}P into an acid-stable product. Samples phosphorylated as above were treated with 10% trichloroacetic acid at 90 °C for 20 min. After cooling the tubes on ice for 10 min, the protein precipitate was collected by filtration as described. Each point represents the mean ± S. E. of 4 experiments.

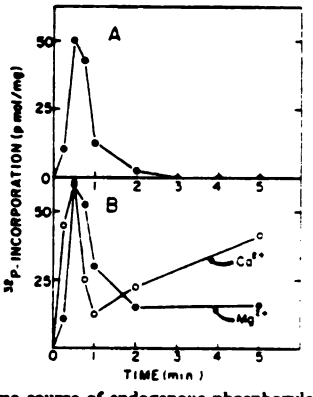

FIG. 2. **Time course of endogenous phosphorylation.** *A*, incorporation of ^{32}P into acid-labile product. These values were obtained by difference from the curves presented in Fig. 1. *B*, phosphorylation under conditions of low metal ion concentration. Assays were carried out as usual except for the substitution of 250 μM Ca^{2+} (○——○) or 250 μM Mg^{2+} (●——●) for the 5 mM Mg^{2+} in the assay mixture. Each point represents the mean of 2 experiments.

90 °C resulted in the loss of the 14,000-dalton product but only a slight decrease in the intensity of the 3,000-dalton band. Treatment with 1 N NaOH at 90 °C degraded both phosphorylated products.

Other factors were tested for their ability to affect phosphorylation of the lysosomal membrane (Fig. 4). In the presence of low metal ion concentration, only the 14,000-dalton band was present (*lanes a* and *c*). The 3,000-dalton band appeared when 5 mM Mg^{2+} was included in the reaction; 5 mM Ca^{2+} did not effectively replace Mg^{2+} (*lane b*). The phosphorylation of the 14,000-dalton product was inhibited by these higher salt concentrations. The addition of Ca^{2+} and CaM resulted in increased phosphorylation of the lower molecular weight

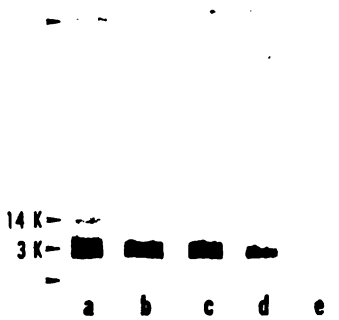

FIG. 3. **TDAB-acid gel electrophoresis of phosphorylated lysosomal membranes.** The time course and acid base stability of the phosphorylated products were examined. Samples were treated as described below and prepared for electrophoresis and autoradiography as described under "Experimental Procedures." The *arrowheads* indicate the position of the top of the stacking gel and the dye front. *a–c*, assays were conducted at 30 °C for the times indicated. *a*, 30-s incubation; *b*, 1 min; *c*, 2 min. *d* and *e*, The samples were treated as described after a 1-min reaction; *d*, 20-min incubation with 10% trichloroacetic acid at 90 °C. *e*, 10-min incubation with 1 N NaOH at 90 °C.

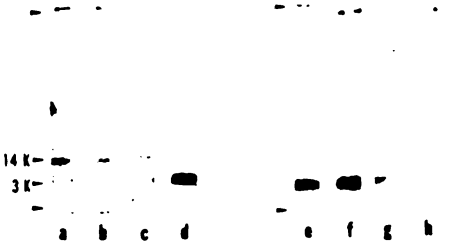

FIG. 4. **Factors affecting lysosomal membrane phosphorylation.** All assays were carried out for 1 min at 30 °C with additions to the reaction as indicated below. TDAB-gel electrophoresis and autoradiography were carried out as before. *a*, 250 μM Ca^{2+} (no Mg^{2+}); *b*, 5 mM Ca^{2+} (no Mg^{2+}); *c*, 250 μM Mg^{2+}; *d* and *e*, 5 mM Mg^{2+}; *f–h*, 5 mM Mg^{2+}, 0.8 μg of CaM, and 50 μM Ca^{2+}; *f*, no further addition; *g*, 50 μM chlorpromazine; *h*, 0.2 mM $LaCl_3$.

product. This stimulation was blocked by 50 μM chlorpromazine (*lane g*). The phosphorylation of this band was completely inhibited by the addition of 0.2 mM $LaCl_3$ (*lane h*).

To test whether the phosphorylation observed was due to the presence of small amounts of other membranes in the lysosomal preparation, we examined the phosphorylation of purified microsomes and plasma membrane. Lysosomes, microsome, and plasma membrane preparations were phosphorylated and subjected to TDAB-gel electrophoresis and autoradiography. Phosphorylated products similar in molecular weight to those found in the lysosome sample were observed in both the microsome and plasma membrane samples. To quantitate ^{32}P incorporation, these portions of the gel were cut out and counted. The radioactivity in the 3,000-dalton region of the gel was 2.2-fold higher in the lysosome sample than in the microsome and 9-fold higher than in plasma membrane with equal amounts of protein applied in each case. Multiple bands were apparent at molecular weights greater than 10,000 in the microsome and plasma membrane lanes, but the lysosome sample contained at least 2-fold more ^{32}P in the 14,000-dalton region of the gel. These data indicate that the phosphorylated products detected are of lysosomal origin and can not be explained by contamination with other membranes.

Effect of Cyclic Nucleotides on Phosphorylation—The phosphorylation of lysosomal membranes was not significantly affected by the addition of cAMP or cGMP (Table I). cAMP-dependent kinase inhibitor also had no effect. The addition of cAMP and type I cAMP-dependent protein kinase did not result in additional incorporation of radioactivity. These experiments were also carried out by analyzing the products of the reaction on TDAB-acid gels. No additional radioactivity was detected in either phosphopeptide, and no other phosphoprotein was labeled by exogenous cAMP-dependent protein kinase.

Substrate Specificity of the Phosphorylating Activity—The ability of lysosomal membranes to phosphorylate exogenous substrates was examined (Table II). In experiment 1, the commonly used substrates of cAMP-dependent kinase were tested. In experiment 2, known substrates of Ca^{2+}-dependent protein kinase were examined. There was no increase in ^{32}P incorporation in the presence of these substrates beyond the activity seen with the membrane sample alone. Analysis of the reaction products by autoradiography following gel electrophoresis revealed no incorporation of ^{32}P into the exogenous proteins.

Chemical Characterization of the Phosphorylated Products—The products of the phosphorylation reaction after 30 s and 5 min of reaction were analyzed as described in Table

TABLE I

Effect of cyclic nucleotides on membrane phosphorylation

Lysosomal membranes were phosphorylated for 5 min as described under "Experimental Procedures" with the indicated additions made to the assay buffer.

Additions	^{32}P-incorporated[a]
	pmol/mg protein
None	90.7 ± 17.3 (100)
5 μM cGMP	97.1 ± 15.0 (107)
5 μM cAMP	87.6 ± 12.7 (97)
5 μM cAMP + 15 μg PkIn[b]	74.8 ± 20.8 (82)
5 μM cAMP + 10 μg cAMP-dependent protein kinase	65.4 ± 11.0 (72)

[a] Mean value ± S.E. of 4 experiments. Numbers in parentheses are per cent of control assay.

[b] cAMP-dependent protein kinase inhibitor.

TABLE II

Substrate specificity of lysosomal phosphorylation activity

Phosphorylation assays were conducted for 5 min as described under "Experimental Procedures" with the addition of the indicated substrates.

Added substrate	^{32}P-incorporated[a]
	pmol/mg protein
Experiment 1[b]	
None	152 (100)
Casein	155 (102)
Histone II-A	132 (87)
Protamine	105 (69)
Bovine serum albumin	103 (68)
Experiment 2[c]	
None	114 (100)
Phosphorylase *b*	108 (94)
Myosin	74 (65)

[a] Mean of 2 determinations. Numbers in parentheses are per cent of control assay containing no additional substrate.

[b] 250 μg of the indicated protein were added in a final reaction volume of 100 μl.

[c] Assays were conducted in the presence of 100 μM Ca^{2+} and 100 μg of the indicated substrate in a volume of 60 μl.

III. The data indicate that the product formed at 30 s is an acyl phosphate. The material was unstable in hot acid and alkali. The 12% of radioactivity remaining after hot trichloroacetic acid treatment is due to the small amount of the phosphate ester formed by 30 s. The phosphate ester comprised 95% of the total phosphorylated product at 5 min of incubation. Neither product was solubilized by extraction with organic solvents, indicating that phospholipids do not make up a significant portion of the ^{32}P-labeled material. Trypsin treatment released approximately one-half of the radioactivity compared with that found in a control reaction incubated under the same conditions in the absence of added protease. However, the incubated control value for the 30-s product was decreased by 80% compared with unincubated control, indicating hydrolysis of the acyl phosphate under these conditions.

TABLE III

Characterization of reaction products

Lysosomal membranes were phosphorylated for the times indicated below as described under "Experimental Procedures." The ^{32}P-labeled products were mixed with 100 μg of bovine serum albumin and precipitated with 10% trichloroacetic acid and 10 mM Na pyrophosphate and collected by centrifugation at 12,000 × *g* for 5 min. The samples were treated as described below and the remaining radioactivity determined by collecting the trichloroacetic acid-insoluble material on paper filters as before. 1) Control samples were resuspended in 1 ml of cold 10% trichloroacetic acid. 2) Samples suspended as for control were heated at 90 °C for 20 min and then chilled on ice. 3) Samples solubilized in 0.2 ml of 1 N NaOH were heated at 90 °C for 10 min and precipitated with 1 ml of cold 10% trichloroacetic acid. 4) Samples were washed in 1 ml of cold distilled water and extracted 3 times with 1 ml of chloroform/methanol (2:1, v/v). 5) Samples were treated as in 4 and then extracted 3 times with 0.5 ml of chloroform/methanol containing 0.25% HCl. 6) Samples precipitated without serum albumin were solubilized in 0.2 ml of 0.25 M Tris-HCl, pH 7.5, and treated with 10 μg of protease/100 μg of lysosomal protein at 30 °C for 1 h. 1 ml of cold trichloroacetic acid was added and the precipitated protein collected as above. The control for this treatment consisted of incubation of the sample in the absence of protease.

Treatment	Radioactivity remaining[a]	
	30 s	5 min
	%	
1. Control	100	100
2. Hot trichloroacetic acid	11.9	94.6
3. Hot NaOH	0	0
4. Chloroform/methanol	91.5	90.6
5. Chloroform/methanol/HCl	89.1	84.7
6. Trypsin	44.3	51.8

[a] Results are the mean of 3 separate experiments.

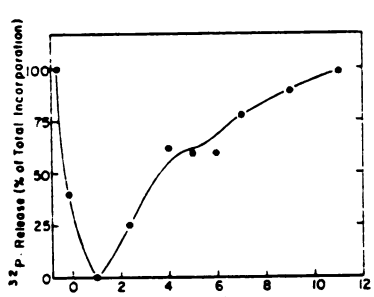

FIG. 5. **Effect of pH on the rate of hydrolysis of phosphorylated product.** Lysosomal membrane samples were phosphorylated in the presence of 250 μM Ca^{2+} for 30 s at 30 °C. The reactions were stopped by the addition of 1 ml of 10% trichloroacetic acid containing 10 mM PP, and 20 μl of bovine serum albumin (5 mg/ml) as usual. The protein was collected by centrifugation at 12,000 × *g* for 5 min. The pellets were washed with cold distilled water. Each membrane sample was suspended in 0.2 ml of solution at the indicated pH (HCl, 0.2 M acetate, citrate, Tris-Cl, or bicarbonate) and incubated for 30 min at 30 °C. After addition of 1 ml of 10% trichloroacetic acid, the membrane samples were collected by centrifugation as before. The sediment was dissolved in 0.1 ml of 1 M Tris-HCl, pH 7.5, for scintillation counting.

The hydrolysis characteristics of the 30-s phosphorylated product formed in the presence of 250 μM Ca^{2+} were determined as shown in Fig 5. The ^{32}P-labeled material was most stable at pH 1, with rapid hydrolysis occurring at both pH extremes. The hydrolysis rate of the acyl phosphate in 0.1 M acetate buffer, pH 3.5, at 40 °C was determined (26). The rate constant was calculated to be 0.012 ± 0.002 min^{-1} and the half-life of the acyl phosphate under these conditions was 56 min. This value is in close agreement with the rate constant observed for the acyl phosphate intermediate of the (Na^+, K^+)-ATPase (26). Similar results were observed for hydrolysis rate and pH effects using the usual assay conditions (5 mM Mg^{2+} and 1-min incubation) and correcting for the amount of stable phosphorylated product remaining.

DISCUSSION

We have shown in this report that a membrane fraction derived from Triton WR-1339-filled lysosomes contained 2 distinct phosphorylation systems. The time course of phosphorylation was biphasic, with a rapidly labeled component which decreased with time and a second more slowly phosphorylated product.

The rapidly labeled component was sensitive to treatment with hot trichloroacetic acid and was the major product formed under low Ca^{2+} or Mg^{2+} concentrations. It was not extracted by organic solvents ruling out the presence of labeled phospholipids. The phosphoprotein contained an acyl phosphate by the following criteria. 1) It was not detected in standard sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie blue in acidic methanol[2]; 2) the ^{32}P label was removed by treatment with hot trichloroacetic acid or NaOH; and 3) it showed a pH profile and rate constant of hydrolysis which is distinctive and comparable with that observed for other proteins containing an acyl phosphate linkage. Analysis of the phosphorylated products by cationic detergent-polyacrylamide gel electrophoresis at pH 2 revealed phosphopeptides of M_r = 3,000 and 14,000. Based on the time course of its appearance and its susceptibility to hydrolysis, we conclude that the 14,000-dalton band contains the acyl phosphate moiety. Acyl phosphates have been isolated as catalytic intermediates (in (Na^+, K^+)-ATPase (26, 27), (Ca^{2+}, Mg^{2+})-ATPase (28), and other phosphotransferases (29). Work in this laboratory and others has shown the presence of ATPase activity(s) in the lysosomal membrane (30–32). It is, therefore, likely that the acyl phosphate observed here is the covalent intermediate of the catalytic subunit of an ATPase or other phosphohydrolase associated with the lysosomal membrane.

The second component was also protein in nature and appears to contain a more stable phosphate ester. This peptide was presumably the substrate for a protein kinase associated with the lysosomal membrane. We attempted to assay for kinase activity by adding exogenous substrates to lysosomal membranes (Table II). So far we have been unable to detect stable phosphorylation of any protein other than the endogenous 3,000-dalton peptide. Some of the added protein substrates led to an inhibition of the endogenous activity, perhaps by nonspecific binding to the membrane site where phosphorylation normally occurs. The protein kinase may, therefore, be highly specific for this substrate or may require the correct

[2] C. A. Collins and W. W. Wells, unpublished results.

orientation and proximity of the substrate in the membrane.

Various modulators of protein kinase activity were tested for their effect on lysosomal membrane phosphorylation. In our highly purified preparations, neither cyclic nucleotides nor the cAMP-dependent kinase inhibitor had any effect on the endogenous phosphorylation, contrary to what we suggested previously (8). We feel that earlier preparations were contaminated with cAMP-dependent kinase from the cytosol or other membranes. The increase in lysosomal membrane phosphorylation in response to glucagon observed by Zahlten *et al.* (3) may be due to contaminating phosphoproteins from mitochondria or other sources in their preparation. Alternatively, phosphorylation of lysosomes by loosely associated cAMP-dependent kinase may be an important means of regulating lysosomal function *in vivo*. Interestingly, we found no additional phosphorylation of lysosomal membrane components by adding type I cAMP-dependent protein kinase and cAMP to the reaction mixture. Tsung and Weissman (33) have observed cAMP-independent phosphorylation in a lysosome-rich fraction from human polymorphonuclear leukocytes. They reported the presence of heat-labile inhibitor of soluble cAMP-dependent kinase in the leukocyte lysosome preparation and attributed the lack of cAMP binding and stimulation of kinase activity in various fractions to the action of this inhibitor. We have also observed inhibition of soluble cAMP-dependent kinase activity by purified rat liver lysosomes using histone as substrate[2] but have not determined what effect this activity may have on our measurement of endogenous lysosomal membrane phosphorylation.

Ca^{2+} stimulates a number of cAMP-independent protein kinases, most of which require the presence of the Ca^{2+} modulator protein, calmodulin, for activity (34). We reported a small stimulation of phosphorylation by 1 mM Ca^{2+} in our previous work (8). Here we examined a much lower Ca^{2+} concentration and the effect of CaM and inhibitors on the Ca^{2+}-stimulated activity. We found variable degrees of stimulation by Ca^{2+} and CaM between preparations, but 50 μM Ca^{2+} generally stimulated phosphorylation of the 3,000-dalton peptide 2-fold. Addition of CaM caused a further stimulation of 60%. Since the lysosomal membranes are prepared in the absence of metal chelators, it is likely that there is Ca^{2+} already present in the sample. In addition, we have found that lysosomal membranes stimulate activator-deficient cAMP phosphodiesterase in the standard assay for calmodulin.[2] This suggests that Ca^{2+} and calmodulin already present in the lysosomal membrane stimulate the endogenous kinase. The addition of 0.2 mM $LaCl_3$, which is known to block Ca^{2+} transport and competes for Ca^{2+} binding sites (35), completely inhibited the phosphorylation of the 3,000-dalton peptide in the presence or absence of added Ca^{2+} and CaM. In addition, 50 μM chlorpromazine or trifluoperazine, drugs which inhibit CaM binding (36), decreased the Ca^{2+}, CaM-dependent stimulation of phosphorylation. These data suggest that Ca^{2+} may regulate the phosphorylation of the 3,000-dalton peptide through the action of a Ca^{2+}, CaM-dependent protein kinase associated with the lysosomal membrane. The existence of this Ca^{2+}-regulated phosphopeptide in lysosomes may provide a mechanism for the mediation of hormonal effects on lysosomal function.

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CHAPTER II

IDENTIFICATION OF PHOSPHATIDYLINOSITOL KINASE IN RAT LIVER LYSOSOMAL MEMBRANES

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Identification of Phosphatidylinositol Kinase in Rat Liver Lysosomal Membranes*

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Liver lysosomes from Triton-injected or normal rats were found to rapidly incorporate ^{32}P from [γ-^{32}P]ATP into a lipid component of the membrane, *in vitro*. The lipid was identified as phosphatidylinositol 4-phosphate based on its chromatographic behavior on Silica Gel H thin layer plates as compared with standard phosphoinositides. The deacylation product, glyceryl-phosphorylinositol phosphate, was compared with standards in chromatographic and electrophoretic systems to further substantiate the identification of the radioactive material. A trace of phosphatidylinositol 4,5-bisphosphate was also found. The properties of the lysosomal membrane phosphatidylinositol kinase were examined using both endogenous lipid and exogenous phosphatidylinositol as substrate. The enzyme was active at neutral pH in the presence of 20 mM $MgCl_2$. The addition of 0.4% Triton X-100 stimulated the enzyme activity toward endogenous substrate, and the highest activity was observed in the presence of detergent and 1 mM phosphatidylinositol. Degradation of the product was seen only in the presence of Triton X-100. The specific activity of the lysosomal phosphatidylinositol kinase is comparable to the detergent-stimulated activity of liver microsomes and plasma membrane, the previously recognized sources of this enzyme in the liver cell.

Primary lysosomes from livers of starved rats (1) and those from livers perfused with glucagon or cAMP (2–5) rapidly undergo striking structural transformations in the process of autophagy. In attempts to understand the biochemical basis for this process, we have investigated the possibility that endogenous phosphorylation of highly purified rat liver lysosome components may affect membrane structure and function (6). In this previous study only two low molecular weight phosphorylated species were detected when membranes from Triton WR-1339-filled lysosomes were incubated with [γ-^{32}P] ATP. One was an acyl phosphate-containing protein of 14,000 daltons. We have suggested that this polypeptide represents the catalytic intermediate of a phosphotransferase reaction. The second phosphorylated component migrated in the 3,000-dalton region of sodium dodecyl sulfate or cationic detergent gels. Identification of this material was complicated by the copurification of small polypeptides with the ^{32}P-labeled product. These behaved as proteolipids in their solubility in chloroform and elution from Sephadex G-100 and LH-20 columns. We have now shown that the low molecular weight material contains predominantly DPI[1] and a trace of TPI. Previous analyses of lysosomal membrane phospholipids have not demonstrated the presence of these polyphosphoinositides (7–9). Nevertheless, Michell (10) noted that DPI has been found in adrenal chromaffin granule membranes (11, 12), and both polyphosphoinositides were present in the plasma membrane fraction from rat liver (13, 14). He, therefore, speculated that membranes of the Golgi complex and lysosomes, which are functionally related to these other membranes, may normally contain the acidic polyphosphoinositides. We now provide evidence for the presence of phosphatidylinositol 4-phosphate and phosphatidylinositol kinase (EC 2.7.1.67) in rat liver lysosomes.

EXPERIMENTAL PROCEDURES

Materials—[^{32}P]Orthophosphate, carrier free, was purchased from New England Nuclear. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (15), as modified by Reimann *et al.* (16). Phosphatidylinositol and other phospholipid standards were obtained from Serdary Research Laboratories. Polyphosphoinositide standards, metrizamide, and cyclohexanediaminetetraacetic acid were obtained from Sigma. Silica Gel H and cellulose (Avicel) thin layer plates were purchased from Analtech. Glass distilled organic solvents were obtained from MCB. Triton WR-1339 was from the Ruger Chemical Co. Triton X-100 was from Research Products International. An Aminex A-27 anion exchange high pressure liquid chromatography column (250 × 4 mm) was obtained from Bio-Rad.

Lysosome Preparation—Triton WR-1339 filled lysosomes were prepared from rat liver by the method of Leighton *et al.* (17) as previously reported (6), omitting the wash step after sucrose gradient centrifugation. These lysosomes were purified 60-fold over whole homogenate based on hexosaminidase-specific activity (18). Analysis of NADH cytochrome c reductase (19) and alkaline phosphodiesterase (20) indicated less than 5% contamination by either endoplasmic reticulum or plasma membrane. Contamination by other organelles was less than 1%.

Rat liver lysosomes were also purified by metrizamide gradient centrifugation according to the procedure of Wattiaux *et al.* (21), starting with the light mitochondrial fraction of DeDuve *et al.* (22). They were purified 50-fold over whole homogenate based on hexosaminidase-specific activity. Contamination by mitochondria, microsomes, and plasma membrane, was 1, 15, and 15%, respectively.

Plasma membrane and microsomes were prepared from rat liver as described previously (6).

Lysosome Phosphorylation—To obtain material for identification of the reaction products, 0.5 mg of lysosomal protein was incubated with 2 mM [γ-^{32}P]ATP, 30 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.5, in a final volume of 0.5 ml for 5 min at 30°C. The ^{32}P-labeled material was extracted and analyzed by thin layer chromatography as described below.

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[1] The abbreviations used are: DPI (diphosphoinositide), phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; TPI (triphosphoinositide), phosphatidylinositol 4,5-bisphosphate.

Extraction of Lipids—The phosphorylated lysosomes were extracted with 1.5 ml of chloroform:methanol (1:2, v/v) followed by 0.5 ml of 2.4 N HCl and 0.5 ml of chloroform as described by Schacht (23). The extraction mixture was mixed thoroughly and centrifuged (1000 × *g* for 10 min) in 12-ml heavy walled Pyrex tubes. The lower phase was removed to another tube and the upper layer and interface were extracted again with 1 ml of chloroform. The combined lower phases were washed with 2 ml of methanol:1 N HCl (1:1, v/v), and the upper phase was discarded after centrifugation. The lower phase was rapidly neutralized by suspending small plastic cups (Kontes Glass Co.) containing 15.1 N NH_4OH above the extract in a rubber sealed atmosphere for 5 min. The chloroform layer was evaporated to dryness under a stream of nitrogen at 35–40 °C. The lipid residue was dissolved in a small volume (200–300 μl) of chloroform:methanol:H_2O (75:25:2, v/v/v) in preparation for thin layer chromatography. For some preparations, 0.5 to 1.0 mg of authentic phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate were added as carriers.

Chromatography of Lipids—Aliquots of the lipid extract were applied to Silica Gel H thin layer plates (20 × 20 cm) previously heated at 110 °C for 1 h and chromatographed with appropriate phospholipid standards in 1-propanol:4.3 M NH_4OH containing 10 mM cyclohexanediaminetetraacetic acid (65:35, v/v) (24). After development, chromatograms were exposed to x-ray film for autoradiography. For phospholipid detection, the chromatograms were either sprayed with the phosphate reagent of Bochner *et al.* (25) or with 50% sulfuric acid containing 0.6% sodium dichromate and heated at 140 °C for 30 min.

Chromatography of Deacylated Products—Aliquots of the phosphorylated lipid fraction were deacylated following the procedure outlined by Kates (26). The water-soluble products were chromatographed on cellulose thin layer plates in 2-propanol:concentrated NH_4OH:H_2O (6:3:1, v/v/v). After 2 passes of the solvent mixture, the chromatogram was exposed to x-ray film and then sprayed with the phosphate reagent of Bochner *et al.* (25). Other aliquots of the water-soluble products were electrophoresed on cellulose plates (20 × 20 cm) in 0.1 M sodium oxalate, pH 1.5, with a potential of 800 V for 45 min. The dried plates were exposed to x-ray film followed by treatment with the phosphate reagent. The deacylated products were subjected to ion exchange high performance liquid chromatography on a column (250 × 4 mm) of Aminex A-27 in the formate form and equilibrated with a 0.1 M ammonium formate, 20 mM ammonium borate buffer, pH 9.5, at a flow rate of 0.6 ml/min. For the separation of the glycerylphosphoryl derivatives of the corresponding phospholipid precursors, a gradient was generated with 30 ml of 0.1 M and 0.75 M ammonium formate each combined with 20 mM ammonium borate.

Phosphatidylinositol Kinase Determination—The assay mixture contained 50 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 2 mM [γ-^{32}P]ATP (20–100 cpm/pmol), and lysosome sample in a final volume of 0.1 ml. Except where indicated otherwise, the final protein concentration was 1 mg/ml. The reaction was started by the addition of ATP-Mg^{2+} after a 5-min preincubation of the other components at 30 °C. After extraction of lipids by the method of Schacht (23) as described above, the chloroform phase was dried under N_2 and the residue suspended in a small volume of chloroform:methanol (2:1, v/v). The lipids were separated on Silica Gel H thin layer plates in the unidirectional 2-solvent system of Hauser *et al.* (24). The ^{32}P-labeled lipids were visualized by autoradiography, and PI, DPI, and TPI standards were detected by staining with I_2. Areas of the thin layer plate containing radioactivity were scraped off into vials containing fluor for scintillation counting. In some experiments the combined chloroform extracts were washed twice with methanol:1 N HCl (1:1, v/v) and counted directly to measure ^{32}P incorporation.

Protein Determination—Protein was determined by the method of Lowry *et al.* (27) with bovine serum albumin as the standard.

RESULTS

Identification of ^{32}P-labeled Phosphatidylinositol 4-Phosphate

The chloroform extract of ^{32}P-labeled lysosomes was mixed with carrier DPI and TPI and subjected to chromatography on Silica Gel H thin layer plates. The solvent system contained a calcium chelator, cyclohexanediaminetetraacetic acid, which facilitated the migration of the polyphosphoinositides from the origin (Fig. 1*A*). It is apparent that the major ^{32}P-labeled component corresponds in mobility to standard phosphatidylinositol 4-phosphate. A trace of radioactivity was detected in the region of phosphatidylinositol 4,5-bisphosphate. This varied from 3 to 5% of the label incorporated into DPI.

The deacylated water-soluble products of the phosphorylated lysosomal preparation were compared with the deacylation products of standard phosphatidic acid, phosphatidylinositol, DPI, and TPI in three different separation systems. Fig. 1*B* illustrates that the radioactive material comigrates with

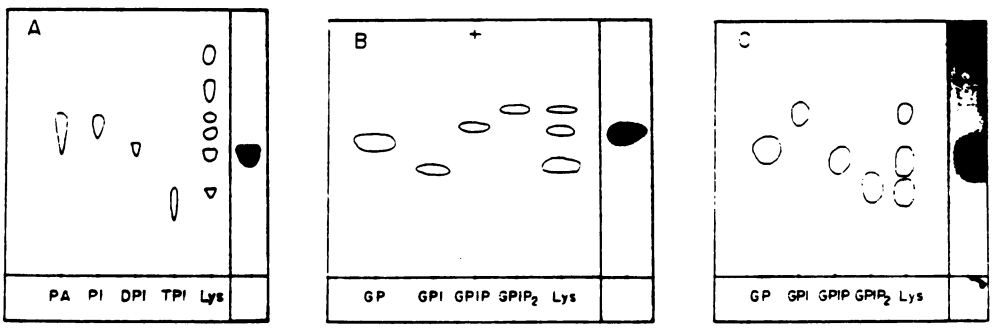

FIG. 1. **Analysis of the ^{32}P-labeled material from lysosomes.** *A*, thin layer chromatography of lysosomal lipid extract. Lysosomes were phosphorylated and extracted, and the extract was subjected to chromatography on Silica Gel H as described under "Experimental Procedures." The positions of lipid standards are indicated as detected by phosphate spray reagent. Lanes 1–4 contained standard phosphatidic acid (*PA*); PI; DPI; and TPI. The lysosome extract in lane 5 also contained carrier DPI and TPI. The *last lane* shows the position of the lysosomal ^{32}P-labeled product in an autoradiograph of lane 5. *B*, electrophoresis of lipid deacylation products. Aliquots of lysosomal ^{32}P-labeled lipid along with carrier DPI and TPI were subjected to deacylation in mild alkali (26). The water-soluble products were electrophoresed as described under "Experimental Procedures." Lanes 1–4 contained the deacylation products of lipid standards: glycerol phosphate (*GP*); glycerylphosphorylinositol (*GPI*); glycerylphosphorylinositol 4-phosphate (*GPIP*); glycerylphosphorylinositol 4,5-bisphosphate (*GPIP$_2$*). Lane 5 contained the ^{32}P-labeled lysosome extract and lane 6 is an autoradiograph of the separated deacylation products. *C*, thin layer chromatography of deacylation products. The deacylation products of standards and the ^{32}P-labeled lysosome extract as described for *B* were separated on a cellulose thin layer plate in 2-propanol:1.5 N NH_4OH:H_2O (6:3:1, v/v/v). The lipids were detected by the phosphate spray reagent and autoradiography as before.

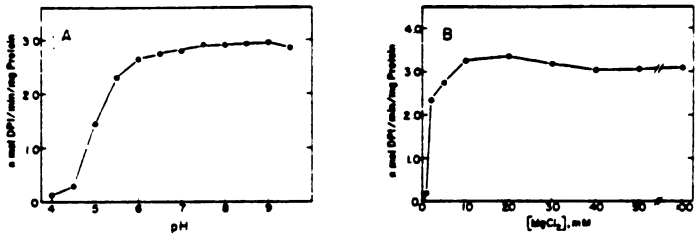

FIG. 2. **Assay conditions for lysosomal PI kinase.** *A*, pH profile of PI kinase activity. Assays were conducted as described under "Experimental Procedures" in the presence of 1 mM PI and 0.4% Triton X-100 at the indicated pH. The following buffers were used at a final concentration of 50 mM: sodium citrate, Tris-maleate, Tris-Cl, glycine-NaOH. The results are the mean of 4 determinations. *B*, effect of Mg^{2+} concentration on PI kinase activity. Assays were conducted for 2 min at 30 °C in the presence of 1 mM PI, 0.4% Triton X-100, and the indicated concentration of $MgCl_2$. 1 mM EGTA was added to the reaction mixture to measure activity in the absence of the cation. The results are the mean of 3 determinations.

glycerylphosphorylinositol 4-phosphate when subjected to electrophoresis on cellulose thin layer plates. Similarly, chromatography in 2-propanol:15 N NH_4OH:H_2O (6:3:1, v/v/v) on cellulose plates revealed a single spot comigrating with the deacylation product of authentic DPI (Fig. 1*C*). In agreement with these analyses, anion exchange high pressure liquid chromatography of the deacylated material resulted in a major peak of radioactivity that eluted with glycerylphosphorylinositol 4-phosphate as detected by phosphate analysis. Again, a minor peak of radioactivity was found to elute with the deacylation product of TPI (data not shown).

Properties of Lysosomal Phosphatidylinositol Kinase

Assay Conditions—Lysosomal PI kinase activity was found to be optimal at neutral pH and in the presence of at least 20 mM $MgCl_2$ (Fig. 2). The K_m for ATP determined in the presence of 20 mM $MgCl_2$ was 0.13 mM. A concentration of 2 mM ATP was utilized for subsequent experiments.

Effect of Triton X-100—The nonionic detergent Triton X-100 stimulated PI kinase activity at a concentration of 0.4% (w/v) (Fig. 3). Inhibition of DPI formation occurred at higher concentrations of detergent using either endogenous lipid or added PI. The addition of 0.5 mM PI in the absence of detergent did not enhance DPI synthesis. The effect of Triton X-100 was found to be dependent on the sample concentration in the assay mixture. At concentrations of lysosomal protein less than 1 mg/ml, Triton X-100 was inhibitory at all concentrations tested in the absence of exogenous PI (data not shown).

Effect of Phosphatidylinositol—Exogenous PI was utilized as a substrate for lysosomal PI kinase as shown in Fig. 4. Maximum incorporation of ^{32}P into DPI was observed with 1 mM PI in the presence of 0.4% Triton X-100. These conditions were also found to give optimal incorporation of label into DPI of plasma membrane and microsome preparations. In the presence of both detergent and 1 mM PI, the reaction rate was linear with respect to sample concentration in the assay mixture up to 2 mg of protein/ml (data not shown).

Time Course of Phosphatidylinositol 4-Phosphate Production—Analysis of the ^{32}P-labeled reaction products on Silica Gel H plates showed that the major species was DPI. The rate of ^{32}P incorporation into DPI is shown in Fig. 5. Using endogenous lipid as the substrate, the reaction rate proceeded linearly for only a short time, after which a plateau was reached. To assess whether this was due to depletion of ATP in the reaction mixture, ATPase activity was measured under these assay conditions. By 10 min of incubation at 30 °C, 75% of the ATP had been hydrolyzed to give $^{32}P_i$. If more ATP was added after 10 min of reaction, an additional burst of ^{32}P incorporation was observed (data not shown). The fact that a plateau was reached would suggest that little degradation of the product, DPI, occurred.

In the presence of 0.4% Triton X-100, the reaction rate was stimulated 2-fold. However, after reaching a peak of ^{32}P incorporation at 10 min, there was a steady decrease in the amount of labeled DPI. In the presence of an optimal concentration of exogenous substrate, the activity was further stimulated and followed the reaction course observed in the presence of detergent alone.

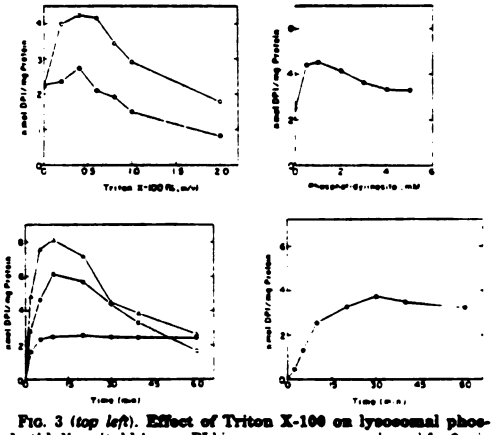

FIG. 3 (*top left*). **Effect of Triton X-100 on lysosomal phosphatidylinositol kinase.** PI kinase assays were conducted for 2 min at 30 °C as described under "Experimental Procedures" using 100 μg of lysosomal protein and the indicated concentration of Triton X-100. The reaction utilized endogenous lipid as substrate (●——●) or 0.5 mM PI added to the standard assay mixture (○——○).

FIG. 4 (*top right*). **Effect of phosphatidylinositol concentration on PI kinase activity.** Assays were conducted for 2 min at 30 °C in the presence of 0.4% Triton X-100 and the indicated concentration of phosphatidylinositol.

FIG. 5 (*bottom left*). **Time course of the phosphatidylinositol kinase reaction.** Assays were conducted as described under "Experimental Procedures" with 100 μg of lysosomal protein. ●——●, PI kinase activity using endogenous substrate; ○——○, PI kinase activity in the presence of 0.4% Triton X-100; △——△, activity using 1 mM PI as substrate in the presence of 0.4% Triton X-100.

FIG. 6 (*bottom right*). **Time course of DPI formation in normal liver lysosomes.** The PI kinase assay was carried out as usual but with lysosomes prepared by metrizamide gradient centrifugation as the source of enzyme and substrate, at a final concentration of 0.5 mg of protein/ml.

PI Kinase in Normal Rat Liver Lysosomes—Lysosomes prepared by metrizamide gradient centrifugation were analyzed for the presence of phosphatidylinositol kinase activity (Fig. 6). The activity was similar to that observed in Triton-filled lysosomes; however, DPI synthesis occurred up to 30 min. ATPase activity was lower in this preparation, degrading only 50% of the ATP by 60 min.

Subcellular Localization of PI Kinase—Plasma membrane and microsome fractions were isolated and assayed for PI kinase activity under the same conditions as for Triton-filled lysosomes (Table I). Using added PI as substrate, the specific activities of the kinase from all three preparations were similar. However, the plasma membrane preparation was strongly inhibited by Triton X-100 in the absence of exogenous substrate.

Inhibition of PI Kinase—$CaCl_2$ and $LaCl_3$ were tested for their effect on PI kinase activity (Table II, A). The stimulation observed in the presence of EGTA may be due to the chelation of inhibitory metal ions other than Ca^{2+}, since Ca^{2+} at low concentrations led to a small increase in DPI formation. At 1 mM Ca^{2+}, inhibition of DPI synthesis was observed. La^{3+} had been found to inhibit lysosomal membrane phosphorylation (6). Here it was examined for its effect of PI kinase activity. At a concentration of 0.2 mM, DPI synthesis was inhibited 78%.

The ability of other phospholipids to inhibit PI kinase activity was examined (Table II, B). In the presence of 1 mM PI, 0.5 mM phosphatidic acid was slightly inhibitory. A slight but reproducible increase in activity in the presence of phosphatidylcholine was observed. Other phospholipids had little effect.

TABLE I

Activity of PI kinase from lysosomes, plasma membrane, and microsomes

PI kinase assays were carried out for 1 and 2 min at 30 °C as described under "Experimental Procedures." The initial rate of reaction was determined in the presence of added detergent and substrate as indicated, with protein at 1 mg/ml for each sample.

Additions	DPI Formation*		
	Lysosomes	Plasma membranes	Microsomes
	nmol/min/mg protein		
None	0.79	1.46	0.63
0.4% Triton X-100	1.40	0.48	1.31
0.4% Triton X-100 +1 mM PI	2.38	2.07	1.98

* The results are the mean of 2–4 determinations.

TABLE II

Activity of lysosomal PI kinase in the presence of inhibitors

PI kinase assays were carried out for 2 min at 30 °C after a 5-min preincubation with all assay components except ATP and $MgCl_2$. The DPI formed was extracted as described under "Experimental Procedures." The results are expressed as a per cent of the control incubation which contained 0.4% Triton X-100 and 1 mM PI.

Additions	DPI formation*
	%
A.	
None	100
EGTA, 1 mM	111
$CaCl_2$	
10 μM	107
100 μM	105
1 mM	55
$LaCl_3$	
20 μM	79
200 μM	22
B.	
Phosphatidic acid	
0.2 mM	96
0.5 mM	71
Phosphatidylserine	
0.2 mM	98
0.5 mM	85
Phosphatidylcholine	
0.2 mM	114
0.5 mM	124
Phosphatidylinositol 4-phosphate	
0.2 mM	105
0.5 mM	93

* The results are the mean of 2 experiments. The control PI kinase specific activity is 2.33 nmol of DPI/min/mg of protein.

DISCUSSION

The presence of phosphatidylinositol kinase in Triton-filled lysosomes and the formation of phosphatidylinositol 4-phosphate, *in vitro*, establishes the occurrence of polyphosphoinositides in lysosomal membranes. Previous reports of lysosomal membrane phospholipid composition did not identify the polyphosphoinositides, presumably due to the very small levels present and their lability under most extraction conditions. Using ^{32}P-labeled ATP, it was possible to detect the synthesis of DPI *in vitro* and study the properties of the membrane-associated phosphatidylinositol kinase.

Recently, Behar-Bannelier and Murray (28) have reported that mouse liver microsomal membrane fractions incorporated significant amounts of ^{32}P from [γ-^{32}P]ATP into phosphatidylinositol 4-phosphate. Their experience was similar to ours (6), that is, the observance of a fast moving band on polyacrylamide gels of trichloroacetic acid-precipitated membranes following phosphorylation. In our studies, measurement of lysosomal membrane protein kinase activity gave erroneously high results by the trichloroacetic acid precipitation-filtration assay, since much of this activity is now known to result from lysosomal phosphatidylinositol kinase as reported herein. Consequently, we agree with the warning of Behar-Bannelier and Murray (28) that the formation of the polyphosphoinositides *in vitro* may lead to an appreciable overestimation of the amount of ^{32}P incorporated into endogenous membrane proteins.

A difficulty in the detection of PI kinase activity in previous studies of subcellular localization was the degree of lysosome purification. In their early study of phosphatidylinositol kinase from rat liver, Michell *et al.* (13) reported PI kinase activity in a lysosome fraction that was enriched 4.28-fold over the homogenate based on β-glucuronidase relative specific activity. The relative specific activity of the PI kinase was 0.59 in this fraction. These authors concluded that the enzyme distribution does not parallel that of nuclei, mitochondria, lysosomes, or endoplasmic reticulum. In an extension of this work, Harwood and Hawthorne (14) examined the PI kinase in rat liver, brain, kidney, heart, skeletal muscle, testis, and in human erythrocytes. In all cases they found the activity predominantly in fractions shown to be enriched in plasma membranes. However, they also detected a detergent-stimulated PI kinase activity associated with crude lysosome and microsome fractions from rat liver and kidney.

We report in this study the formation of phosphatidylinositol 4-phosphate by the action of a lysosomal membrane-associated phosphatidylinositol kinase from highly purified rat liver lysosomes. Like the PI kinase activity reported for microsomes of liver and kidney (14, 29), and microsomes and chromaffin granules from bovine adrenals (30), the lysosomal enzyme exhibited the highest specific activity in the presence of a nonionic detergent and exogenous PI (Table I). However, rapid degradation of the product, DPI, also occurred in the presence of Triton X-100. Preliminary experiments suggest

that degradation occurs through the action of both phospholipase C and phosphomonoesterase activities. There is no evidence to suggest that these enzymes are from the lysosome matrix, since degradation occurs at neutral pH. In addition, the acid phospholipase C in lysosomes has been reported to be unable to degrade lysosomal membrane phosphatidylinositol (31). Polyphosphoinositide phosphatases have been detected in both soluble and membrane fractions of rat kidney (32). Polyphosphoinositide-specific phosphodiesterase has also been reported to be both soluble and membrane bound (33, 34).

Lysosomal PI kinase was inhibited by Ca^{2+} as has been reported for other PI kinases (14, 30) and was also strongly inhibited by La^{3+}, which is known to compete for Ca^{2+}-binding sites (35) (Table II). Unlike the enzyme activity reported in liver homogenates by Michell *et al.* (13), lysosomal PI kinase was not inhibited by the addition of other phospholipids.

Phosphatidylinositol kinase was also found in this study to be associated with microsomes and plasma membrane (Table I). However, the high specific activity of the Triton-filled lysosome preparation and the occurrence of PI kinase in normal liver lysosomes (Fig. 6) would argue that the phosphatidylinositol kinase in our purified fractions is of lysosomal origin and not contamination from these other organelles. Studies with rat hepatocytes cultured in the presence of $^{32}P_i$ have shown that both TPI and DPI of lysosome fractions become labeled.[2] Since only a small amount of labeled TPI was synthesized in isolated lysosomes, we speculate that a cytoplasmic diphosphoinositide kinase may be required to complete the sequential phosphorylation reactions observed in intact liver cells. A cytoplasmic location for this enzyme has been found in rat parotid gland (36) and brain (37). In rat kidney, DPI kinase was enriched in the Golgi complex but was also found in the plasma membrane and supernatant fractions (29, 38).

Stimulation of phosphoinositide metabolism has been observed in many different target tissues in response to agents which modify Ca^{2+} flux in the cell (10). Calcium appears to be involved in the regulation of phospholipase C activities that specifically degrade the polyphosphoinositides giving rise to diacylglycerol and inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate from DPI and TPI, respectively (34, 40–42). Hormones that mobilize Ca^{2+} in hepatocytes such as vasopressin, angiotensin, and epinephrine acting at α_1 receptors provide the rapid degradation of liver cell polyphosphoinositides (43). It will, therefore, be of interest with respect to hormonal control of autophagy and membrane fusion to determine whether the polyphosphoinositides in the lysosomal membrane are subject to the same regulation.

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[2] M. A. Seyfred and W. W. Wells, unpublished results.

CHAPTER III

CHARACTERIZATION OF AN ACYLPHOSPHATE INTERMEDIATE OF A LYSOSOMAL MEMBRANE ATPase

ABSTRACT

Lysosomes prepared from liver of Triton-injected rats incorporate ^{32}P from either [γ-^{32}P]ATP or [γ-^{32}P]GTP into a membrane protein of 180,000 daltons. The phosphate is present in an acyl linkage as determined by sensitivity to alkaline pH and hydroxylamine treatment. Acylphosphate formation occurred in the absence of a divalent metal cation, but the rate and extent of phosphorylation were increased in the presence of $MgCl_2$. Ca^{2+} did not stimulate ^{32}P-incorporation and did not substitute for Mg^{2+} in the presence of the divalent metal chelator, trans-cyclohexane-1,2,-diamine-N,N,N',N'-tetraacetic acid (CDTA). However, dephosphorylation was stimulated by either Ca^{2+} or Mg^{2+}. The rate of dephosphorylation in the presence of CDTA and a 50-fold excess of unlabeled ATP was found to be 0.21 s^{-1}. This dephosphorylation rate was equal to ATPase turnover as measured by [γ-^{32}P]ATP hydrolysis in the presence of the chelator. Acylphosphate formation and the lysosomal membrane ATPase were similarly inhibited by dicyclohexylcarbodiimide, NaN_3, fluorescein isothiocyanate, and sulfhydryl reagents. Na_3VO_4 was found to inhibit ATPase activity in a pH dependent manner. The lysosomal membrane preparation catalyzed phosphate exchange reactions between a nucleoside triphosphate and nucleoside diphosphate or P_i. These results suggest that the lysosomal membrane ATPase catalyzes cleavage of nucleotides by means of a phosphorylated intermediate.

INTRODUCTION

The existence of an ATP-dependent H^+ pump on the lysosomal membrane has been postulated to account for the stimulation of proteolysis (1) and uptake of basic dyes (2), methylamine (3), and amino acid methyl esters (4) by lysosomes in the presence of ATP. Direct evidence for the existence of a lysosomal H^+ pump has been obtained by Ohkuma et al. (5), who measured acidification of lysosomes filled with fluorescein isothiocyanate-dextran by the change in fluoresence in response to ATP. An ATPase on the lysosomal membrane has been characterized (6) and it is suggested that this activity is functionally related to proton translocation (3). The lysosomal ATPase and acidification properties are similar to the electrogenic proton pump ATPase of adrenal medulla chromaffin granules (7). A proton pump activity has also been identified in membranes of yeast vacuoles (8), secretory vesicles (9), and endocytic vesicles (10). The ATPases of the lysosome and chromaffin granule are clearly distinct from the H^+ pump ATPases of mitochondria, chloroplasts, and bacterial membranes (11) in their lack of sensitivity to oligomycin and in their response to other inhibitors.

Another class of H^+ pump ATPases has been identified in the plasma membrane of fungal cells (12). These enzymes are sensitive to the H^+ pump inhibitor, DCCD, as are the other ATPases mentioned above (3, 7, 11). The fungal ATPase reaction procedes by means of an acylphosphate intermediate on a protein of 100,000 daltons (13-15). In this respect, its mechanism of action is similar to that of the cation pump ATPases of plasma membrane (16-19), sarcoplasmic reticulum (20, 21), and gastric mucosa (22). The Na^+/K^+ and Ca^{2+} pump ATPases form a phosphorylated reaction intermediate on a 100,000 dalton subunit during the course of

ATP hydrolysis. These cation pumps are dependent on the transported ion for activity and are not inhibited by DCCD.

In this Chapter, the characteristics of an acylphosphorylated protein in the lysosomal membrane are examined. Its properties suggest that it may be the catalytic intermediate of a lysosomal membrane ATPase.

EXPERIMENTAL PROCEDURES

Materials - (^{32}P)Orthophosphate, carrier free, and [^{14}C]ADP (58 mCi/mmol) were purchased from ICN. [γ-^{32}P]ATP and [β-^{32}P]GTP were prepared by the method of Glynn and Chappell (23), as modified by Reimann et al. (24). [^{35}S]Adenosine 5'-[γ-thio]triphosphate (65 Ci/mmol) was obtained from New England Nuclear. Unlabeled adenosine 5'-[γ-thio]-triphosphate (ATPγS) was obtained from Boehringer Mannheim. Other nucleotides, ATPase inhibitors, and lithium dodecylsulfate were purchased from Sigma. Cellulose (Avicel) thin layer plates were from Analtech. PEI-Cellulose coated plastic sheets and glass distilled organic solvents were from MCB. Reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad.

Lysosome Preparation - Triton WR-1339 filled lysosomes were prepared from rat liver as described previously (25). Protease inhibitors, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, and soybean trypsin inhibitor, were included in the homogenization medium at a concentration of 1, 5, 75, and 100 mg/liter, respectively. Protease inhibitors were also added to the final lysosome preparation buffered at pH 7.5 with 10 mM Tris-MOPS. Lysosomal membranes were prepared by 1 cycle of freeze-thawing in hypoosmotic sucrose and collected by centrifugation at 80,000 x g for 45 min. The resulting pellet was resuspended in 10 mM Tris-MOPS, pH 7.5, 0.25 M sucrose and stored at -70°C. Membranes were also prepared by dilution of frozen lysosomes with an equal volume of 0.2 M NaCl, 50 mM Tris-MOPS, pH 8.0, 1 mM EDTA followed by centrifugation and resuspension of the pellet as before. Lysosomal membranes prepared in either manner contained 25% of the protein present in the intact lysosome fraction and 85% of the ATPase activity.

Phosphorylation Assay - The standard assay mixture contained 50 mM Tris-Cl, pH 7.5, 0.1 mM [γ-^{32}P]ATP (500 - 2000 cpm/pmol) and lysosomal membrane (10 - 25 μg of protein) in a final volume of 50 μl. Additions of divalent metal cations and chelators were made as indicated in the figures. The reaction was initiated by the addition of ATP and incubation at 30°C was carried out for the times indicated. The reaction was quenched by adding 1 ml of cold 10% trichloroacetic acid containing 10 mM $NaPP_i$ and 2 mM ATP. For zero-time controls the ^{32}P-labeled nucleotide was added after the trichloroacetic acid. Bovine serum albumin (20 μl of 5 mg/ml) was added as a carrier. The precipitates were collected on Whatman GF/C filters and washed with 10 ml of 10% trichloroacetic acid containing $NaPP_i$ and ATP. The filters were dried and counted in 5 ml of scintillation fluid.

Polyacrylamide Gel Electrophoresis - Low pH gels as described by Lichtner and Wolf (26) were prepared containing 7.5% acrylamide and 0.1% (w/v) LDS. The gel solution was buffered with 80 mM citric acid, 10 mM phosphoric acid adjusted to pH 3.5 with Tris base. The gels were polymerized with $FeSO_4$ and H_2O_2 as detailed by Jones et al. (27). The electrode buffer contained 100 mM citric acid, 12.5 mM phosphoric acid, and Tris, pH 3.5, with 0.1% LDS. For analysis on polyacrylamide gels, membrane samples were phosphorylated as described above and precipitated by adding 0.5 ml of cold 10% trichloroacetic acid, 2 mM ATP. The protein was collected by centrifugation and washed once by resuspension in cold distilled water and centrifugation. The pellet was solubilized in 25 μl of 10 mM Tris base containing 2% LDS. An equal volume of sample buffer containing electrode buffer, 20% (v/v) glycerol, 8% 2-ME, 4% (w/v) LDS, and methyl green as a tracking dye was then added. The gels were run at 40 mA for 4 h at 4°C. The gels were dried and exposed to Kodak XAR-5

x-ray film for autoradiography. Proteins used as molecular weight markers were run in a separate lane on the same gel and stained with Coomassie Blue. There was a linear relationship between log molecular weight and relative mobility on these gels in the range of 20 to 170 kilodaltons.

ATPase Assays - 1. Charcoal adsorption - The reaction mixture contained 50 mM Tris-Cl, pH 7.5, 3 mM [γ-^{32}P]ATP (1-5 cpm/pmol), 5 mM $MgCl_2$ or $CaCl_2$, and sample (2-20 μg protein) in a final volume of 50 μl. 50 mM Tris-MOPS, pH 7.5 was used in experiments to study effects of salts on ATPase activity and did not affect the basal level of ATP hydrolysis. After incubation at 30°C the reaction was stopped by the addition of 0.5 ml of cold perchloric acid. After placing the tubes on ice, 300 μl of a suspension of acid washed charcoal was added. After 10 min on ice, 200 μl of a solution containing 5 mg of bovine serum albumin per ml and 50 mM $NaPP_i$, pH 7, was added. The reaction tubes were vortexed and centrifuged at 2000 rpm in a GLC centrifuge for 5 min. Aliquots (0.5 ml) of the supernatants were removed and counted directly by Cerenkov radiation with an efficiency of 35%. A blank incubation was included to correct for the slight amount of $^{32}P_i$ in the ATP preparation and for any degradation which occurred during the acid treatment. This value was generally 1-3% of the total cpm added to the reaction.

2. Thin layer chromatographic analysis of reaction products - Assays were performed with labeled nucleoside triphosphate as described above but were terminated by the addition of SDS to a final concentration of 1% (w/v). Aliquots (5 μl) of each reaction mixture were spotted on a cellulose thin layer plate which was then developed in n-butanol:acetic acid:H_2O:pyridine, 15:3:12:10. The positions of the nucleotide and P_i were located by autoradiography. The spots were scraped into

scintillation vials for counting.

3. Continuous spectrophotometric assay - ATPase activity was determined by measuring ADP formation in the presence of phosphoenol-pyruvate, NADH, lactate dehydrogenase and pyruvate kinase as described by Barnett (28) in a final volume of 0.5 ml. The disappearance of NADH was monitored by change in absorbance at 340 nm.

NTP-NDP Phosphate Exchange - The phosphate exchange between ATP and ADP was measured as described by Makinose (20). The incubation mixture contained 50 mM Tris-MOPS, pH 7.5, 7 mM $MgCl_2$ or 0.5 mM CDTA, 5 mM ATP, 2 mM [^{14}C]ADP, and membrane sample (2-5 µg of protein) in a final volume of 50 µl. The reaction at 22°C was initiated by the addition of the nucleotides. The reaction was terminated by the addition of 0.5 ml of cold 10% trichloroacetic acid and 50 µl of 5 mg/ml bovine serum albumin. After centrifugation to remove precipitated protein, the supernatant was extracted twice with diethylether to remove the trichloroacetic acid. Aliquots of the supernatant and carrier nucleotides were spotted on PEI-cellulose sheets. After separation in 0.85 M KH_2PO_4, pH 3.4, nucleoside triphosphates were visualized by ultraviolet light and cut out for scintillation counting. Control incubations without unlabeled ATP were included to correct for any adenylate kinase activity in the preparations. Exchange reactions utilizing GTP-ADP and ATP-GDP were performed as described above except that [γ-^{32}P]ATP and [γ-^{32}P]GTP were used at a final concentration of 2 mM with unlabeled GDP or ADP at 2 mM.

ATP-P_i Exchange - The exchange between the terminal phosphate of ATP and P_i was measured according to Ronzani et al. (29). The reaction mixture contained 50 mM Tris-MOPS, pH 7.5, 5 mM ATP, 2 mM ADP, 7 mM $MgCl_2$ or 0.5 mM CDTA, 5 mM [^{32}P]potassium phosphate (200 cpm/pmol) and membrane sample (2-10 µg protein) in a final volume of 50 µl. Incubation was

carried out after the addition of $^{32}P_i$ for various times at 22°C. The reaction was terminated with 0.5 ml of 10% perchloric acid containing 1 mM phosphoric acid. After the addition of 0.5 ml of 2.5% ammonium molybdate, the phosphomolybdate complex was extracted 3 times with 2 ml of water-saturated isobutanol. Radioactivity in the remaining water phase was determined by Cerenkov radiation with a counting efficiency of 35%. To confirm that the radioactivity had been transferred to ATP, aliquots of the water phase were spotted on PEI-cellulose sheets and developed as described for the NTP-NDP exchange reactions.

Protein Determination - Protein was determined by the method of Lowry (30) with bovine serum albumin as the standard.

RESULTS

Substrate Specificity of the Acylphosphorylation Reaction - The time course of acylphosphate formation in lysosomal membranes is shown in Fig. 1. The activity is similar using either [γ-^{32}P]ATP or [γ-^{32}P]GTP as phosphate donor in the presence of 100 μM $CaCl_2$. As demonstrated in Chapter I, this rapidly formed product is an acylphosphate based on its sensitivity to hot acid and pH values above 5. Much greater labeling was observed using [^{35}S]ATPγS as a substrate. Incorporation of ^{35}S reached a maximum by 2 min of incubation at 30°C and remained constant for at least 5 min. The ^{35}S-labeled product showed a pH dependence of hydrolysis similar to the ^{32}P-labeled product (25), with resistance to degradation between pH 0.5 and 6 (data not shown).

Polyacrylamide Gel Analysis of the Acylphosphate - In a previous study (25 and in Chapter I), a ^{32}P-labeled product exhibiting properties of an acylphosphorylated protein was found to migrate at a position corresponding to 14,000 daltons in a cationic detergent gel system. Under similar assay conditions, a ^{32}P-labeled product was found at a molecular weight of 180,000 in this experiment using LDS polyacrylamide gel electrophoresis. This product has also been identified as an acylphosphate based on its rapid turnover and degradation by hydroxylamine (Fig. 2). Quantitative analysis of the labeling of this radioactive band is shown in Table I. Addition of a 100 fold excess of unlabeled ATP to the reaction mixture led to a 64% decrease in radioactivity in 5 s. Incubation of the labeled lysosomal membranes for 15 min with 2% LDS at pH 5.5 caused a slight reduction in radioactivity. Addition of hydroxylamine led to a further decrease in labeling of 36%. No effect of hydroxylamine was observed in the absence of detergent (data not shown).

Figure 1. Nucleotide Specificity of Phosphorylation Reaction.

The phosphorylation of freeze-thaw prepared lysosomal membranes was carried out as in "Experimental Procedures" with the inclusion of 100 μM $CaCl_2$ and the indicated labeled nucleotide in the assay medium. ●-●, [γ-^{32}P]ATP; O-O, [γ-^{32}P]GTP; Δ-Δ, [^{35}S]ATPγS. The results are the mean of 2 experiments.

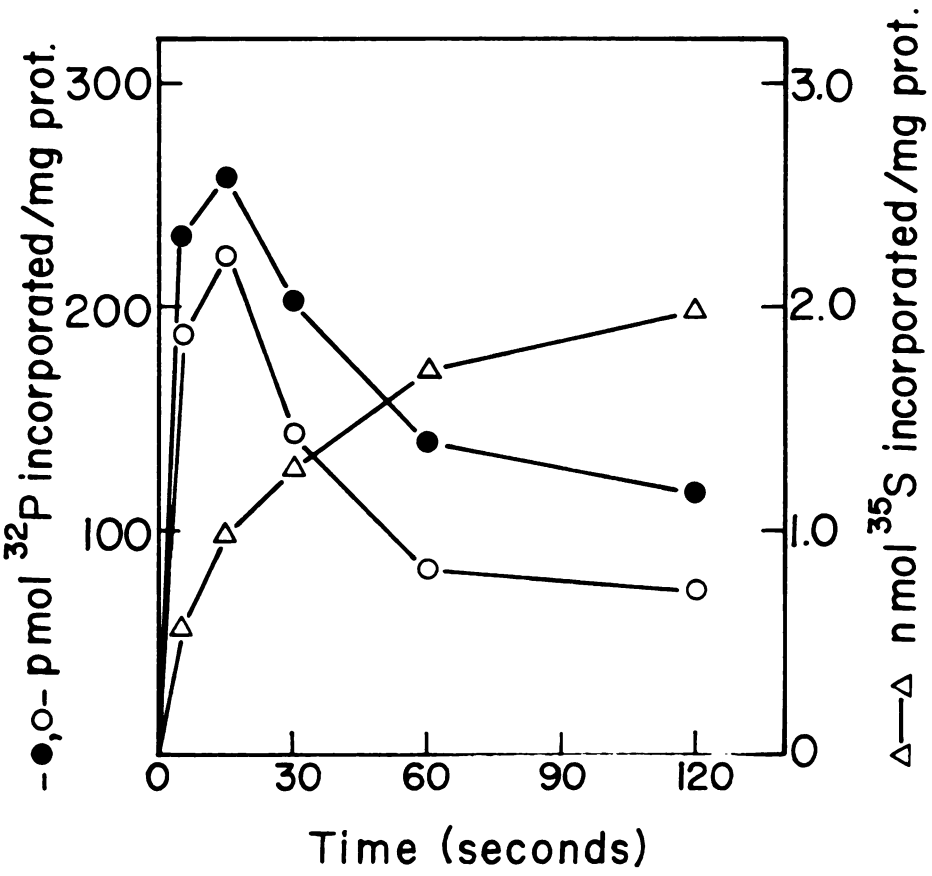
-●,○- p mol 32P incorporated/mg prot.
△—△ n mol 35S incorporated/mg prot.
300
200
100
0
3.0
2.0
1.0
0
0
30
60
90
120
Time (seconds)

Figure 1

Figure 2. Analysis of Acylphosphate on LDS Polyacrylamide Gels. Gel electrophoresis was performed as described under "Experimental Procedures". Lane 1, zero time control; 2, 15 s incubation; 3, 30 s incubation. Lanes 4 and 5, after 15 s of incubation the reactions were terminated by the addition of 250 μl of 10% trichloroacetic acid. After centrifugation the pellets were resuspended and incubated as described in Table I. 4, control incubation with 2% LDS; 5, incubation with 1 M NH_4OH, 2% LDS. After reprecipitation with 10% trichloroacetic acid the pellets were solubilized and subjected to electrophoresis and autoradiography. The arrow indicates the top of the gel and the positions of molecular weight markers are shown.

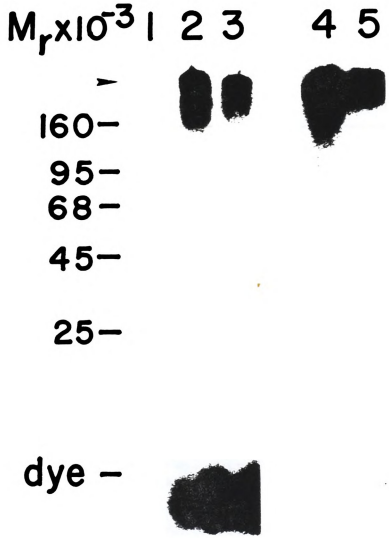

Figure 2

Table I. Acylphosphate Formation in Lysosomal Membranes.[a]

Treatment	cpm Remaining
	%
1. Control	100
2. 5 s chase with 10 mM ATP	35.8
3. 15 min incubation at pH 5.5	87.1
4. 15 min incubation at pH 5.5 in hydroxylamine	51.1

[a]Lysosomal membranes were phosphorylated with [γ-^{32}P]ATP for 15 s at 30°C in the absence of added divalent cation. The assay mixtures were treated as described below and the reaction terminated by the addition of cold 10% trichloroacetic acid. The samples were resuspended in sample buffer and subjected to LDS gel electrophoresis as described in "Experimental Procedures". The radioactive bands were detected by autoradiography, cut out from the gel, rehydrated and counted. 1) Control assays were performed as described above. 2) At 15 s of incubation, 10 mM unlabeled ATP was added for an additional 5 s of incubation. 3,4) After 15 s the samples were precipitated with 0.5 ml cold 10% trichloroacetic acid. After centrifugation, the pellets were resuspended in 0.25 M Na acetate buffer, pH 5.5 containing 2% LDS, and incubated at 30°C for 15 min. The incubation control also contained 1 M NaCl, while 1 M NH_2OH, pH 5, was added to sample 4. The results are expressed as the percent of counts incorporated at 15 s after subtracting a zero time control value.

Cation Requirement for Acylphosphorylation - Previous results indicated that both Ca^{2+} and Mg^{2+} were effective in promoting lysosomal membrane acylphosphate formation (25). This point was examined further (Fig. 3). The results indicate that acylphosphorylation can occur in the absence of a divalent cation (presence of 0.5 mM CDTA). In addition, Mg^{2+} and Ca^{2+} were found to have different effects on phosphorylation when added in concentrations in excess of chelator concentration. When 1 mM $MgCl_2$ was added to the assay mixture, rapid incorporation of ^{32}P occurred followed by dephosphorylation after 30 s of reaction. Ca^{2+} addition resulted in slower ^{32}P incorporation than with chelator alone, even though Ca^{2+} addition in the absence of CDTA led to maximal activity (Fig. 1). At these low concentrations of metal ion, incorporation of ^{32}P due to phosphatidylinositol 4-phosphate formation (Chapter II) was negligible.

Cation Requirement for Dephosphorylation - To examine the possible involvement of divalent metal ions in dephosphorylation, additions were made to the reaction mixture after phosphorylation reached steady state in the presence of CDTA. In Fig. 4A, 5 mM ATP was added to the reaction mixture in the presence or absence of metal ion. With the addition of unlabeled ATP alone or with Ca^{2+}, dephosphorylation occurred rapidly, with an apparent first order rate constant of $0.21 \pm 0.01\ s^{-1}$. Addition of ATP-Mg^{2+} resulted in a slower rate of dephosphorylation. In Fig. 4B, 1 mM Ca^{2+} or Mg^{2+} was added in the absence of unlabeled ATP to the reaction mixture containing 0.5 mM CDTA. The addition of Ca^{2+} resulted in rapid dephosphorylation followed by a return to the previous phosphorylation level. The addition of Mg^{2+} led to a slower and less complete dephosphorylation followed by a rapid increase in labeling which reached twice the incorporation seen in the absence of metal ion within

Figure 3. Metal Ion Specifity for Phosphorylation.

Salt-extracted membranes were phosphorylated with 100 μM [γ-^{32}P]ATP as described under "Experimental Procedures" in the presence of CDTA and metal ions as indicated. The additions to the assay were: 0.5 mM CDTA (●-●), 0.5 mM CDTA and 1 mM $MgCl_2$ (○-○), or 0.5 mM CDTA and 1 mM $CaCl_2$ (Δ-Δ). The results are the mean of 3 experiments.

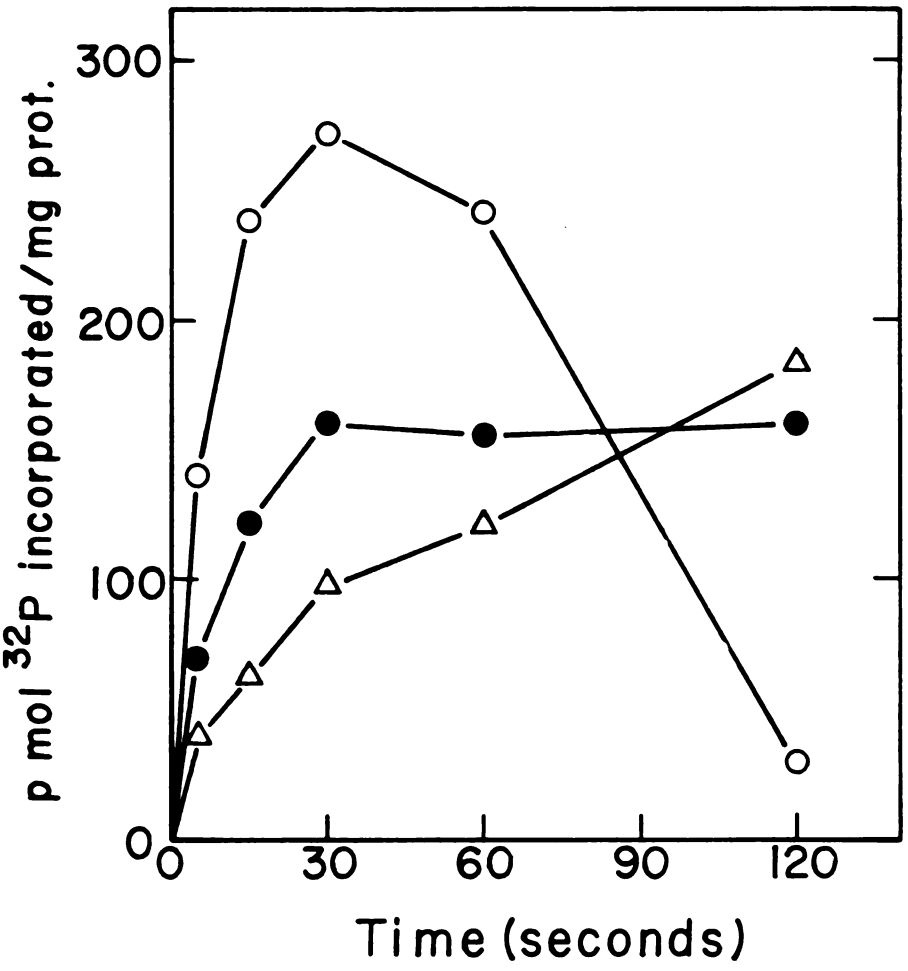
300
200
100
0
p mol ^{32}P incorporated/mg prot.
0
30
60
90
120
Time (seconds)

Figure 3

Figure 4. Metal Ion Requirement for Dephosphorylation.

A. Phosphate turnover in the presence of excess unlabeled ATP. The phosphorylation reaction was carried out as in Fig. 3 in the presence of 0.5 mM CDTA. At the time indicated by the arrow, the following additions were made to the reaction mixture: 5 mM ATP (O-O), 5 mM ATP and 6 mM $CaCl_2$ (Δ-Δ), or 5 mM ATP and 6 mM $MgCl_2$ (□-□).

B. Phosphate turnover in the presence of added metal ion. The phosphorylation reaction was carried out as for part A. At the time indicated, 1 mM $CaCl_2$ (▲-▲), or 1 mM $MgCl_2$ (■-■) was added to the assay medium.

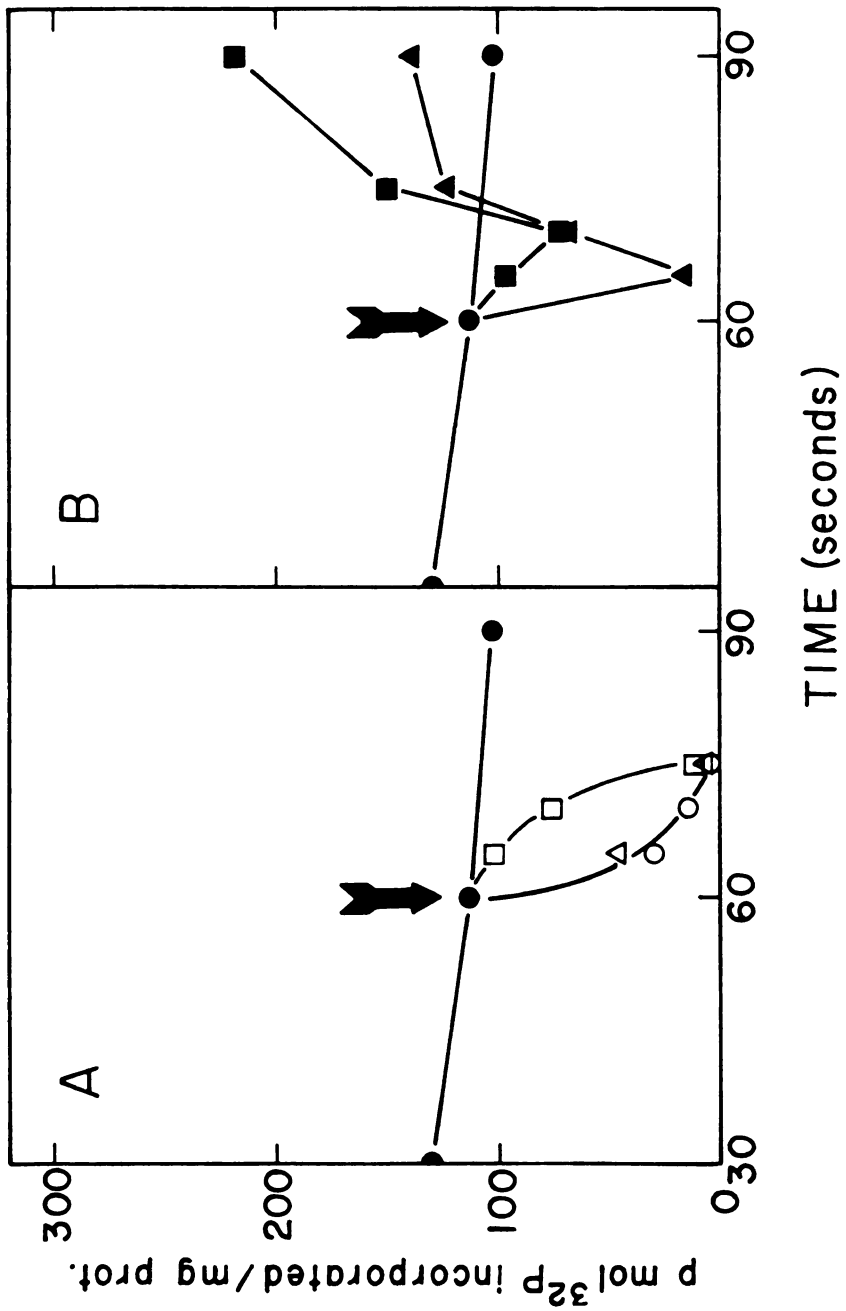
A
B
300
200
100
0
30
60
90
TIME (seconds)
p mol ^{32}P incorporated/mg prot.

Figure 4

30 s.

Substrate Specificity of the Lysosomal Membrane ATPase - ATP hydrolysis was measured by the three methods described under "Experimental Procedures". Each assay method gave comparable results in the determination of ATPase specific activity. GTPase activity was determined by the charcoal adsorption and thin layer chromatography methods which gave identical results. GTP was also a good substrate and either Ca^{2+} or Mg^{2+} was an effective metal cofactor (Table II). Hydrolysis of the ATP analog, ATPγS, was very slow, exhibiting only 1% of the activity observed with ATP. ATPγS hydrolysis was measured by the thin layer separation of ATPγS and thiophosphate and subsequent scintillation counting of these compounds. ATPγS is unstable under acidic conditions, therefore the charcoal adsorption assay could not be used. ADP determination was not as sensitive an assay because of the ADP present in the commercial ATPγS preparations. ATPase activity was also measured under the phosphorylation conditions described in Fig. 3, with 100 μM [γ-^{32}P]ATP. When added in concentrations in excess over CDTA, both Ca^{2+} and Mg^{2+} were effective in stimulating ATP hydrolysis. By 1 min of reaction at 30°C, 80% of the ATP was hydrolyzed. In the presence of 0.5 mM CDTA, ATP hydrolysis was reduced to 2 nmol/min/mg protein at 30°C. Addition of CDTA up to 5 mM did not reduce ATPase activity further (data not shown).

Effect of Inhibitors on Acylphosphate Formation and ATPase Activity - To examine the relationship between ATPase activity and acylphosphate formation, several ATPase inhibitors were examined for their effect on these reactions (Table III). NaN_3 was found to inhibit both activities, but only when the reaction took place in the presence of Mg^{2+}. NaN_3 did not inhibit either the ATPase or acylphosphorylation at up to 20 mM concentration in the presence of Ca^{2+}. Both activities were inhibited by DCCD,

Table II. Substrate Specificity of Lysosomal Membrane ATPase.[a]

Nucleotide	Activity		
	Mg^{2+}	Ca^{2+}	Mn^{2+}
	%		
ATP	100	105	54
GTP	90	89	50
ATPγS	0.26	0.63	1.1

[a]NTP hydrolysis was measured at 30°C in the presence of 3 mM nucleotide and 5 mM of the indicated metal ion as the chloride salt. The reactions were terminated at several time points up to 10 min to ensure linearity of the assay. ATP and GTP hydrolysis were measured by means of the charcoal adsorption assay. ATPγS hydrolysis was determined by separating the reaction products on cellulose thin layer plates as described under "Experimental Procedures". The results are the means of at least 2 experiments and are expressed as a percent of ATP hydrolysis in the presence of Mg^{2+} (1.2 μmol P_i/min/mg protein).

Table III. Effect of Inhibitors on ATPase Activity and Acylphosphate Formation.[a]

Additions	ATPase Activity	Acylphosphate Formation
	%	
None	100	100
NaN_3		
1 mM (Ca^{2+})[b]	102	-
20 mM (Ca^{2+})	89	89
1 mM	76	95
5 mM	65	57
Quercitin, 120 μM	88	62
Diamide, 100 μM	89	93
DCCD		
100 μM	80	-
200 μM	50	64
Ouabain, 1 mM	97	98
NaF, 1 mM	97	88
Na_3VO_4, 1 mM	88	72
ADP[c]	63	62
Trimethyl tin, 100 μM	81	71
FITC, 100 μM	63	46

[a]Lysosomal membrane samples were preincubated with the agents listed above for 30 min at 30°C. The reactions were initiated by the addition of [γ-^{32}P]ATP and assays were carried out as described under "Experimental Procedures". ATPase activity was measured by charcoal adsorption assay after 5 min of incubation at 30°C in the presence of 5 mM $MgCl_2$ except where noted. Acylphosphate formation was measured after 15 s at 30°C by filter assay. The results are the means of at least 4 experiments and are expressed as the percent of the control activity without additions. Control ATPase activity is 950 nmol P_i/min/mg protein and acylphosphate formation is 360 pmol ^{32}P incorporated/mg protein.

[b]These assays were carried out in the presence of 5 mM Ca^{2+}.

[c]1 mM ADP was used for the ATPase assay, 0.1 mM for acylphosphorylation.

ADP, trimethyl tin, and FITC. Acylphosphate formation was more sensitive to quercitin than was the ATPase. The addition of 1 mM Na_3VO_4 resulted in only slight inhibition. Diamide, ouabain, and NaF had no effect. The control activites were not affected by preincubation in the absence of inhibitor, nor by ethanol used as a solvent for some of the agents tested.

The effect of vanadate on ATPase activity was examined further (Fig. 5). The degree of inhibition was found to depend on the pH of the assay. At pH 5, 1 mM Na_3VO_4 inhibited ATP hydrolysis by 63% compared with 20% at pH 7.5. A similar inhibition was observed when ATPase activity was measured with the lower ATP concentration used for the phosphorylation assay. The greater sensitivity to vanadate at pH 5-6.5 cannot be due to inhibition of acid phosphatase or acid pyrophosphatase. The activity of these enzymes with ATP as substrate was measured at pH 5 under the conditions optimal for acid phosphatase (31), and was less than 100 nmol P_i produced/min/mg protein.

Both the ATPase and acylphosphorylation activities were affected by sulfhydryl modifying reagents as shown in Table IV. NEM and pCMB were strong inhibitors, while addition of other agents had mixed results. In particular, the reduced sulfhydryl reagents cysteine, DTT, and 2-ME had little or no effect on ATPase activity byt inhibited acylphosphate formation.

Various monovalent ions were tested for their effect on ATPase and acylphosphate formation in the presence of $MgCl_2$. KCl, NaCl, and NH_4Cl at 100 mM did not affect either activity. The addition of 1 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) also had no effect on the Mg^{2+} stimulated activity (data not shown).

Phosphoryltransfer Reactions - The lysosomal membrane preparation was examined for the presence of phosphate exchange activities (Table V).

Figure 5. Effect of pH on Vanadate Inhibition of ATPase.

ATPase activity was determined by the charcoal adsorption assay in the presence of Na acetate (pH 5), 2-morpholino)ethane-sulfonic acid (pH 6, 6.5), or Tris-Cl (pH 7-8) at a final concentration of 50 mM. The assay was carried out as described under "Experimental Procedures" in the absence (●-●), or presence (○-○), of 1 mM Na_3VO_4.

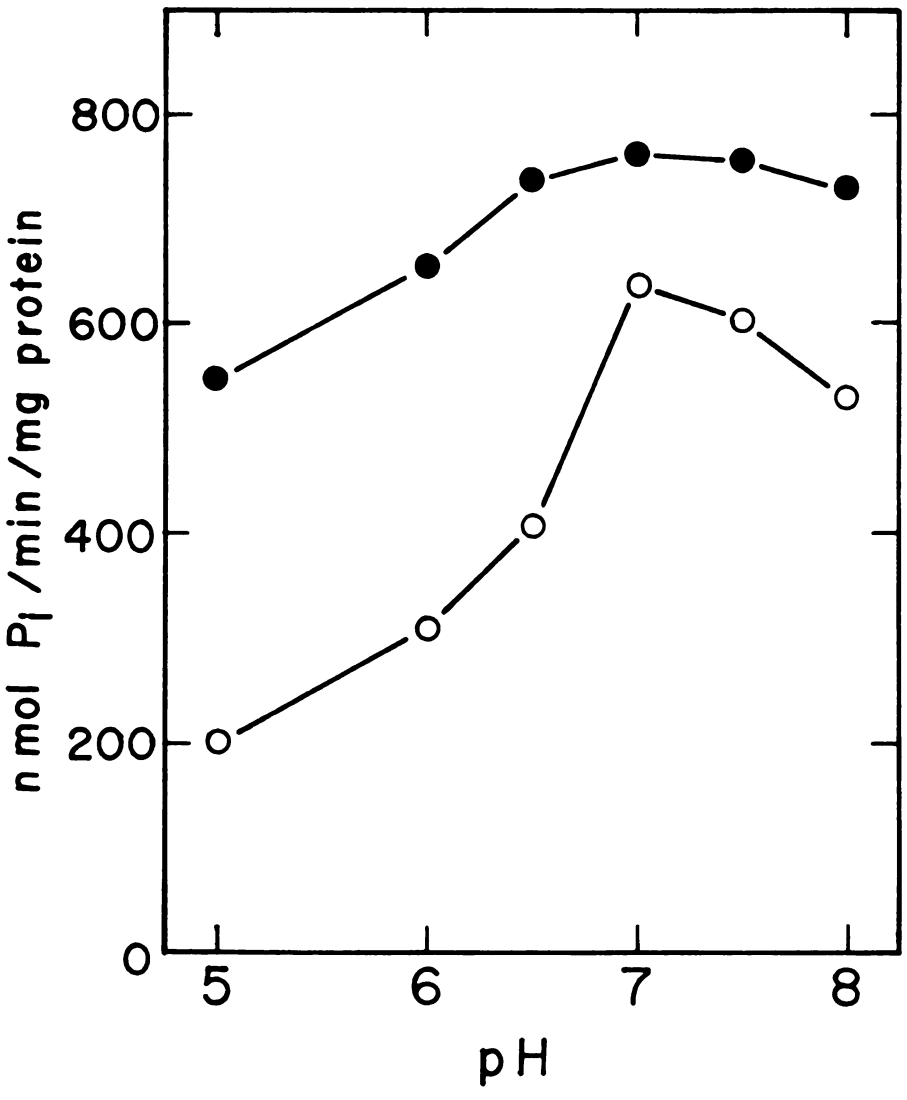

Figure 5

Table IV. Effect of Sulfhydryl Reagents on ATPase Activity and Acylphosphate Formation.[a]

Addition	ATPase Activity	Acylphosphate Formation
	%	
None	100	100
NEM	62	33
pCMB	76	63
GSSG	57	61
GSH	60	59
Cystine	88	113
Cysteine	93	50
DTT	102	69
2-ME	90	41

[a]ATPase assays and acylphosphate formation were carried out as described for Table III. The additions were made to give a final concentration of 1 mM, except to pCMB and 2-ME which were 0.1 mM and 5 mM, respectively. After preincubation for 30 min at 30°C, [γ-^{32}P]ATP was added to start the reaction. The results are the means of 2 experiments and are expressed as percent of control activity.

Phosphoenzyme formation and exchange reactions were performed as described under "Experimental Procedures". ATPase activity was analyzed by the charcoal adsorption assay. Phosphate transfer was demonstrated between NTP and NDP or P_i. Exchange activity between NTP and NDP was less with GTP as the phosphate donor than with ATP. NTP-NDP exchange reactions occurred to almost the same extent in the absence of metal (presence of 0.5 mM CDTA). Phosphate exchange between ATP and $^{32}P_i$ was much lower than NTP-NDP exchange, however the activity was 4 fold higher in the absence of metal ion than in the presence of 7 mM $MgCl_2$.

Table V. Phosphoryl Transfer Reactions of the Lysosomal Membrane ATPase.[a]

Reaction	Transfer Activity	
	Mg^{2+}	CDTA
	nmol/min/mg protein	
Phosphoprotein formation[b] (4)	0.26	0.16
ATPase (4)	1010	7.4
NTP-NDP exchange		
GTP-ADP (4)	29.0	20.0
ATP-GDP (2)	176	76
ATP-ADP (2)	150	116
ATP-P_i exchange (4)	0.42	1.82

[a]The reactions indicated above were carried out in the presence of $MgCl_2$ or 0.5 mM CDTA as described under "Experimental Procedures". The values in parentheses give the number of determinations.

[b]^{32}P incorporation in nmol/mg protein after 30 s of incubation.

DISCUSSION

The acylphosphate product identified in Chapter I has been further characterized with regard to hydroxylamine sensitivity, substrate specificity, metal ion requirement, and effect of inhibitors. Acylphosphate formation appears to be associated with a lysosomal membrane protein since its specific activity is increased in membrane preparations over that observed with intact lysosomes. The acylphosphate is not produced by the action of Na^+/K^+ or Ca^{2+}/Mg^{2+} ATPase since the Triton-filled lysosome preparation contains little contamination by either plasma membrane or microsomes. In addition, acylphosphate formation was not affected by Ca^{2+} or Na^+ and K^+ in a manner consistent with the activity of these other acylphosphate forming ATPases.

Hydroxylamine-dependent loss of label has been accepted as an indication of the presence of an acylphosphate bond (31). It was found that hydroxylamine treatment had no effect unless 2% LDS was included in the incubation mixture. This resistance of an acylphosphate to hydroxylamine has also been found for a phosphoprotein intermediate from Golgi vesicles (33), and suggests that the acylphosphate is not readily accessible to the medium.

The molecular weight derived from LDS gel electrophoresis can be regarded as only approximate. The major difficulty in the gel analysis appears to be the inadequate solubilization of the membrane material under the conditions required to minimize proteolysis and chemical breakdown of the acylphosphate. In most experiments, the radioactivity remained at the top of the gel. Nonionic detergents, urea, and organic solvents have been added to the sample buffer to solubilize the proteins with limited success. In some experiments, however, analysis of the

^{32}P-labeled membranes on LDS gels at pH 3.5 showed a high molecular weight band (Fig. 2). The previously observed 14,000 dalton band (Chapter I) was not detected in this experiment. The kinetics of acylphosphate hydrolysis in this earlier study suggested that only one species was present in the lysosomal membrane. The time course of formation and the pH profile of hydrolysis of the labeled product in the present study were found to be similar to that described in Chapter I, suggesting that the phosphorylated species detected by filter assays in each case were the same. It is possible that the 14,000 and 180,000 dalton species are related and that proteases known to be active in membrane preparations even in the presence of protease inhibitors (3, 34) are responsible for the formation of the lower molecular weight product demonstrated earlier. More recent preparations have utilized 2 additional protease inhibitors, leupeptin and pepstatin, which were not present for the studies shown in Chapter I. The cationic detergent, tetradecyltrimethyl ammonium bromide, may not completely inactivate proteases in the lysosomal membrane during sample preparation and electrophoresis. Lithium dodecylsulfate may reduce proteolytic activity so that the higher molecular weight species can be detected. Attempts to induce the formation of a labeled 14,000 dalton product by the omission of protease inhibitors have been unsuccessful, however.

Acylphosphates of approximately 100 kilodaltons in other membrane systems have been identified as the catalytic intermediate of a cation pump ATPase (16-21). Their identification was facilitated by the requirement for the transported ion for activity of both the ATPase and acylphosphorylation reaction. However, acylphosphate intermediates have also been identified for the proton pump ATPases of yeast and neurospora plasma membranes (12-15). Several studies of the lysosomal proton pump

have been carried out (4, 5, 34, 35), and suggest that a lysosomal membrane ATPase is responsible for this activity (3). The properties of the acylphosphorylation reaction were examined to see if there was a correlation between this activity and the ATPase reaction.

GTP and ATP are good substrates for both acylphosphate formation (Fig. 1) and NTPase (Table II). Both Ca^{2+} and Mg^{2+} will promote lysosomal ATPase or GTPase activity, as has been observed previously (6). GTP has also been found to promote lysosome acidification, although to a lesser extent than ATP (3-5). Ca^{2+}, however, will not substitute for Mg^{2+} in this activity (5).

The ATP analog, ATPγS, was examined as a possible substrate for acylphosphorylation since it is resistant to phosphatase action (36-38). Incorporation of 2 nmol ^{35}S/mg protein into trichloroacetic acid precipitable material was found (Fig. 1). The pH stability of the thiophosphate bond was consistent with this product containing an acyl linkage. To examine whether this analog was hydrolyzed by the lysosomal membrane ATPase, assays were performed as shown in Table II. The low hydrolysis rate and the high level of thiophosphorylation suggest that the turnover of the acyl-intermediate is extremely slow. Therefore, the extent of ^{35}S-incorporation may represent the total number of acyl-phosphorylation sites on the lysosomal membrane.

The experiments described in Figures 3 and 4 were designed to examine the metal ion requirement for acylphosphate formation and turnover. As shown in Fig. 3, acylphosphate formation was not completely dependent on the presence of a divalent cation. The addition of Mg^{2+} increased both the rate and extent of phosphorylation, while Ca^{2+} addition led to a slower ^{32}P incorporation. Similar results have been observed for phosphoenzyme formation in the red blood cell Ca^{2+}

ATPase (39). Acylphosphate formation occurred in the absence of metal with Mg^{2+}-depleted red cell membranes, although the level was only 50% of that with added Mg^{2+}. The dephosphorylation rate was also much slower in the absence of metal. In studies with the Na^+/K^+ ATPase, Ca^{2+} was used to replace Mg^{2+} in order to decrease the rate of phosphoenzyme formation (41). The steady state level of the phosphorylated intermediate was also lower with Ca^{2+}, although Ca^{2+} will replace Mg^{2+} for ATP hydrolysis by this enzyme (42). These results in other systems are similar to what was found here for the lysosomal membrane activity. Although free ATP will act as a substrate for the Ca^{2+} pump ATPase at low concentrations (40), it is likely that the Mg^{2+}-ATP complex is the physiological substrate. Other studies of ATPases which form an acylphosphate intermediate have demonstrated a requirement for a divalent cation, usually Mg^{2+}, which can be met by the low levels of Mg^{2+} bound to membrane and in the assay medium. Acylphosphate formation occurred in the presence of 0.5 mM CDTA in lysosomal membranes which had been prepared in the presence of 1 mM EDTA. Although tightly bound Mg^{2+} may still be present in the membranes under these conditions, this possibility seems unlikely. It is possible, however, that low amounts of Mg^{2+} bound to membrane or in the assay medium in the absence of CDTA account for the increased rate and extent of acylphosphate formation with Ca^{2+} (Fig. 1) compared with Ca^{2+} in the presence of CDTA (Fig. 3). The similar phosphorylation rates observed with either Ca^{2+} or Mg^{2+} in Chapter I can also be explained by the presence of endogenous metal ion. Phosphorylation experiments performed in the absence of chelator and added divalent metal cations indicate that maximal ^{32}P incorporation can be obtained under these conditions, as well.

For the red cell Ca^{2+} ATPase, Mg^{2+} at 0.5 mM increases both the

rate and extent of phosphoenzyme formation, with a concommitant increase in the rate of dephosphorylation (19, 43). Mg^{2+} has been proposed to increase the rate of hydrolysis of the acylphosphate and therefore increase the ATPase activity. Since maximal rates of lysosomal membrane ATP hydrolysis require a metal cation, the effect of Ca^{2+} and Mg^{2+} on the dephosphorylation reaction was examined. Acylphosphate formation was allowed to procede until maximal incorporation of ^{32}P occurred in the presence of 100 μM Ca^{2+} and, presumably, μM amounts of Mg^{2+} as in Fig.1. Addition of 1 mM CDTA at this point stabilized the phosphoenzyme, whereas the control acylphosphate was rapidly dephosphorylated (data not shown). This is consistent with a mechanism requiring metal for rapid turnover to occur. The dephosphorylation reaction was also examined in membranes labeled to steady state in the presence of CDTA. Under these conditions, ATP hydrolysis is very slow, only 2.2 nmol P_i produced/min/mg protein at 30°C. The addition of 5 mM unlabeled ATP or 5 mM ATP and 6 mM Ca^{2+} led to a rapid loss of label from the protein. This rate of dephosphorylation was similar to the rate of ATP hydrolysis (26 pmol/s vs 37 pmol/s) assuming immediate dilution of the ATP label and constant turnover of the acylphosphate. Therefore, the addition of ATP does not stimulate the dephosphorylation reaction. Instead, the loss of label is due to turnover of the enzyme in the presence of unlabeled ATP. Addition of unlabeled ATP with Mg^{2+} had a different effect, however. Dephosphorylation occurred more slowly than with ATP alone or with Ca^{2+}. This is also observed in Fig. 4B, where Mg^{2+} is added without unlabeled ATP. After a slow period of dephosphorylation, increased ^{32}P incorporation was observed. Since unhydrolyzed [γ-^{32}P]ATP is present in the assay medium, Mg^{2+} addition stimulates acylphosphate formation beyond the level observed in the presence of CDTA. Even with an excess of unlabeled ATP as in Fig. 4A,

addition of Mg^{2+} may stimulate acylphosphate formation by [γ-^{32}P]ATP bound by the enzyme which may not be readily exchangeable by added ATP.

The actual metal requirement for dephosphorylation is difficult to assess with these limited number of experiments. However, both Ca^{2+} and Mg^{2+} appear to stimulate the dephosphorylation reaction (Fig. 4B). This suggests that although metal is not absolutely required for acylphosphate formation, rapid ATP hydrolysis will only occur in the presence of a divalent metal cation. The results of experiments examining the effects of Ca^{2+} and Mg^{2+} on acylphosphate formation and hydrolysis are summarized in Table VI. The data are consistent with the dephosphorylation of the acylphosphate intermediate being the rate limiting step for ATP hydrolysis. The inability of Ca^{2+} to replace Mg^{2+} in the H^+ pump activity of the lysosomal ATPase may be related to the different effects these ions have on acylphosphate formation. ATP hydrolysis in the presence of Ca^{2+} may be "uncoupled" from the lysosome acidification observed in the presence of Mg^{2+} and ATP.

In Tables III and IV, the effects of various ATPase inhibitors were tested for their effects on the lysosomal membrane ATPase activity and acylphosphate formation. DCCD has been reported to inhibit ATP-dependent acidification of lysosomes (3, 5, 34, 35) as well as other proton pumps (7-12), but the inhibition of ATPase activity is less than the effect on acidification (3). DCCD and NaN_3 have been found to inhibit the H^+ pump activity of Triton WR-1339 prepared lysosomes but not normal lysosomes (5). Inhibition by NaN_3 was found to be dependent on the metal ion cofactor. Both the ATPase and acylphosphate formation were inhibited by NaN_3 in the presence of Mg^{2+}, but not Ca^{2+}. No other difference between Mg^{2+} and Ca^{2+} stimulated ATPase activity was found. The different effects of Ca^{2+} and Mg^{2+} on acylphosphate formation have

Table VI. Summary of ATPase Partial Reactions under Selected Experimental Conditions.

NTP	Mg^{2+}	Ca^{2+}	Reaction	Rate	E~P	ATPase
100 μM		μM				%
ATP[a]	0	0	$E + ATP \rightarrow E\sim P + ADP$	low	low	
			$E\sim P \rightarrow E + P_i$	low		2
ATP[a]	0	100	$E + ATP \rightarrow E\sim P + ADP$	low	low	
			$E\sim P \rightarrow E + P_i$	high		100
ATP[a]	100	0	$E + ATP \rightarrow E\sim P + ADP$	high	high	
			$E\sim P \rightarrow E + P_i$	high		100
ATPγS[b]	-	100	$E + ATP \rightarrow E\sim P + ADP$	high	high	
			$E\sim P \rightarrow E + P_i$	low		1

[a]Zero indicates that CDTA was present to eliminate divalent metal cations from the incubation mixture. Mg^{2+} or Ca^{2+} was added in excess of CDTA as indicated.

[b]CDTA was not used for this experiment so Mg^{2+} present in the membrane preparation may contribute to the results.

already been discussed.

Quercitin is a strong inhibitor of lysosome acidification (5). Only a slight effect of this reagent on ATPase activity was found. Trialkyl tin has been shown to inhibit the H^+/K^+ exchange of yeast membranes (44) and the proton pump of mitochondria (45). Again, only a slight inhibition was demonstrated in the presence of this compound. FITC has been used to modify the active site of sarcoplasmic reticulum Ca^{2+} ATPase and the Na^+/K^+ ATPase (46). It was shown that FITC dose-dependently inhibited both ATPase activity and acylphosphate formation, indicating a functional relationship between the two reactions. FITC was shown here to inhibit both activities, as well. Inhibition by NEM and pCMB suggest a role for sulfhydryls at the active site, as has been found for other ATPases (47). NEM has also been shown to inhibit the lysosomal proton pump activity (5). On the basis of these results, the lysosomal membrane ATPase cannot be definitively classed with either the H^+ translocating ATPases of mitochondria and yeast membranes, or the cation pump ATPases referred to above. However, the similar degree to which both the ATPase activity and acylphosphate formation are inhibited by these agents suggests that there may be a functional relationship between them.

Inhibition by sodium orthovanadate has been taken as evidence that an ATPase reaction procedes through an acylphosphate intermediate (48). However, the concentration of vanadate required for ATPase inhibition ranges from several micromolar to 1 mM or more (12, 49). In other systems, inhibition by vanadate is influenced by nucleotide concentration, Mg^{2+} concentration, and is strongly dependent on pH (50, 51). Lack of inhibition by vanadate under only one assay condition is not a sufficient criterion for ruling out the possibility of a covalent intermediate in an

ATPase reaction. In at least one case (15), acylphosphate formation was unaffected by 500 μM vanadate even though ATPase activity was inhibited by 70%. Under different assay conditions the acylphosphate formation was found to be inhibited as well (49). It was found here that acylphosphate formation in the lysosomal membrane was inhibited by 28% in the presence of 1 mM Na_3VO_4 at pH 7.5. ATPase activity was examined as a function of pH in the presence of 1 mM vanadate (Fig. 5). As has been previously described for other H^+ pump ATPases (50, 51), the greatest inhibition was observed at slightly acidic pH. Lysosomal acidification was found not to be inhibited by vanadate (4, 5, 35). However, these experiments were conducted at pH 7 where little inhibitory effect on ATPase activity is observed.

The ATPases involved in the transport of Na^+/K^+ and Ca^{2+} across membranes have been shown to procede by a mechanism involving one or more phosphoenzyme intermediates. Under certain conditions, reversal of the reaction sequence can lead to phosphate exchange between nucleotides and inorganic phosphate and from nucleoside triphosphates to nucleoside diphosphates (41, 52, 53). The lysosomal membrane was examined for the presence of these phosphoryltransfer activities. Exchange of the gamma position phosphate from NTP to NDP was found to occur independently of the presence of metal ion. ATP-ADP exchange occurred as rapidly as the ATP-GDP reaction. GTP, however, was only 20% as effective as ATP as a phosphate donor. This may be related to the fact that ATP is three times more effective than GTP in supporting lysosomal acidification, although the hydrolysis rates found here were similar. This substrate specificity has been observed for the exchange reactions of the sarcoplasmic reticulum Ca^{2+} ATPase, even though the hydrolysis rates of ATP and GTP for this enzyme are also similar (29).

Results with the Na^+/K^+ ATPase demonstrate that GTP uncouples hydrolysis from transport, even though GTP is hydrolyzed as well as ATP under normal assay conditions (54).

The NTP-NDP exchange reactions did not require the presence of a divalent cation. This was somewhat expected since the formation of the phosphoenzyme occurred in the presence of CDTA, and the exchange occurs as a result of the reversal of this reaction by the addition of NDP. Nucleoside diphosphate kinase is therefore not responsible for carrying out the exchange activity observed here, since Mg^{2+} would greatly stimulate this enzyme.

The P_i-ATP exchange is due to the reversibility of the last step in the reaction sequence leading to hydrolysis of the acylphosphate and formation of P_i. The rate of this exchange reaction was much lower than that observed for NTP-NDP exchange. The fact that this reaction does occur implies that a phosphoenzyme intermediate may be formed by incubation of the lysosomal membrane with $^{32}P_i$. Preliminary results suggest that this does occur, however, the extent of labeling is very low. ATP-P_i exchange was 4-fold faster in the absence of metal than with 5 mM Mg^{2+}. It is not known whether this reflects a change in affinity of the enzyme for P_i or is a consequence of Mg^{2+} stimulation of the forward reaction, thereby decreasing the ability of the enzyme to catalyze ATP-P_i exchange.

The occurrence of these exchange reactions in the lysosomal membrane suggests that an acylphosphate intermediate is involved in the hydrolysis of nucleoside triphosphates. The ATPase activity of the lysosomal membrane is responsible for catalyzing the transport of H^+ to acidify the interior of the organelle. This H^+ pump may therefore be similar to the H^+ translocating ATPase of fungal membranes in the formation of a covalent phosphoprotein intermediate in the enzyme reaction mechanism.

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SUMMARY

The original purpose of these experiments was to determine whether the known effects of insulin and glucagon on lysosome function in liver cells could be mediated by the phosphorylation of lysosomal membrane proteins. The results presented here do not provide any evidence for this mechanism. The possibility remains that cytosolic protein kinases may interact with membrane substrates, in vivo. The data in Chapter I indicate that lysosomal membrane proteins are not substrates for cyclic AMP-dependent kinase, however other soluble kinases or effectors could be involved.

These studies did reveal the presence of two previously unreported enzyme activities in lysosomal membrane preparations. Phosphatidylinositol kinase had been thought to be located primarily on the plasma membrane. This activity is involved in the polyphosphoinositide cycle, which is stimulated in many different cell types in response to hormones which utilize calcium as a second messenger. The functions of the polyphosphoinositides in hormone action is unknown, although it has been suggested that polyphosphoinositide breakdown is coupled to calcium gating at the plasma membrane. It would be of interest to determine whether lysosomal polyphosphoinositide metabolism is affected by α-adrenergic agonists, or by insulin and glucagon, which also have effects on calcium flux in the cell. The presence of phosphatidylinositol kinase in plasma membrane, endoplasmic reticulum, lysosomes, and chromaffin granules may result from the common origin of these

membranes from the GERL (Golgi-endoplasmic reticulum-lysosomes) complex of the cell. It is not known whether the Golgi apparatus or secretory vesicles also derived from this complex contain polyphosphoinositides.

It is likely that the polyphosphoinositides can exert a large effect on local membrane structure, even though they may be present in low concentrations. The phosphates on the inositol head group may bind divalent metal ions. This greatly affects the hydrophobicity of the lipid, allowing it to move in the plane of the membrane. These lipids may also be clustered in certain regions of the lysosomal membrane, binding tightly to specific proteins such as the ATPase. The regulation of the enyzmes involved in polyphosphoinositide breakdown and synthesis as well as the function of these lipids in the cell remains to be determined.

The second enzymatic activity in the lysosomal membrane catalyzes the incorporation of ^{32}P from [γ-^{32}P]ATP into an acyl linkage. The labeled material migrates with an approximate molecular weight of 180,000. Almost all examples of acylphosphate-containing proteins are cation pump ATPases, the exceptions being acetate kinase from bacteria and ATP citrate lyase from rat liver. Although there is no known Na^+/K^+ or Ca^{2+} ATPase in the lysosomal membrane, there is an ATPase which has been identified as a proton pump. The multisubunit proton pump ATPases of bacteria, chloroplasts, and mitochondria do not procede through an acylphosphate intermediate. The proton translocating ATPases of fungal cells, however, do have covalent intermediates, and have protein subunit compositions similar to those of the cation pumps. A third class of ATP-dependent proton pumps includes those of lysosomes, chromaffin granules, other secretory vesicles, yeast vacuoles, acrosomes, and endosomes. These enzymes have been thought

not to go through a covalent reaction intermediate, but this has not been rigorously tested and the ATPases have not been purified. Although there are multiple phosphatase activities in the lysosomes, the properties of the acylphosphate described in Chapter III are most consistent with those of the proton pump ATPase. More careful analysis of the ATPase and its regulation requires purification and reconstitution of the protein into lipid vesicles in order to demonstrate proton translocation and to determine whether a phosphoenzyme form exists.