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THE Mg^{2+}-DEPENDENT PHOSPHATIDATE PHOSPHOHYDROLASE OF RAT LUNG

By

Paul Albert Walton

Department of Biochemistry

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario, Canada

January, 1986

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ABSTRACT

The Mg^{2+}-dependent phosphatidate phosphohydrolase activity has been studied in rat lung subcellular fractions, and a malignant cell line (A549) which serves as a model of human Type II pneumocytes. Properties of this activity, which used a chemically-defined substrate of equimolar phosphatidylcholine and phosphatidate, were equivalent to those found using membrane-bound substrate.

Microsomes washed in buffers containing high salt concentrations displayed a decrease in the Mg^{2+}-dependent phosphatidate phosphohydrolase which could be quantitatively recovered in the wash supernatant. Mg^{2+}-independent activity remained associated with the microsomes under these conditions. These microsomes displayed a reduced capacity to label glycerolipids from [^{14}C]-glycerol phosphate. Labelling could be returned to normal levels by the addition of Mg^{2+}-dependent phosphatidate phosphohydrolase from cytosol, wash supernatant, or fractionated cytosol, indicating that this activity was required for biosynthesis of glycerolipids.

Determination of the stability constant for magnesium phosphatidate (MgPA) allowed the calculation of the free and bound levels of Mg^{2+} and phosphatidate under assay conditions. The Mg^{2+} concentration at maximum enzyme activity correlated with the intersection of the increasing free Mg^{2+} and decreasing free phosphatidate which was being converted to the MgPA salt.

The MgPA salt appeared to be the required form of the

substrate. Chlorpromazine did not replace the requirement for Mg^{2+} although it stimulated the Mg^{2+}-independent activity.

Triton X-100, Ca^{2+}, and chlorpromazine inhibited the Mg^{2+}-dependent phosphatidate phosphohydrolase activity.

Examination of the substrate requirements, Mg^{2+}-requirements, detergent inhibitions, thermal inactivation, molecular weight, and dissociation and reassociation capabilities led to the conclusion that the Mg^{2+}-dependent phosphatidate phosphohydrolase activity in microsomes and cytosol was the same enzyme in two subcellular locations. Further studies using digitonin permeated A549 cells demonstrated that the intracellular distribution of the Mg^{2+}-dependent phosphatidate phosphohydrolase activity could be shifted from predominately cytosolic to an essentially particulate location upon treatment with 1-4 mM oleate. [^{3}H]-oleate did not accumulate in phosphatidate under these conditions but rapidly accumulated as monoacylglycerol, diacylglycerol, triacylglycerol and phosphatidylcholine. This indicated that the translocation of the Mg^{2+}-dependent phosphatidate phosphohydrolase functioned to achieve greater glycerolipid synthesis and to maintain the concentration of phosphatidate, which is potentially disruptive to membrane integrity, low in times of metabolic flux.

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I am proud to acknowledge that, although my name is on the cover of this thesis, much of which lies between the covers is due to the guidance, assistance, and support of many people.

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I wish to dedicate this thesis to;

my father Robert, who showed me how to dream

my mother Shirley, who taught me how to work

and to my wife Karen,

with whom is shared the labours and the dreams

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NOMENCLATURE

Abbreviation or Trivial Name	Full Name
AMP, ADP, and ATP	adenosine 5′-mono-, di-, and triphosphates
^{o}C	degree Centigrade or Celsius
CDP-choline	cytidine 5′-diphosphocholine
Ci	Curie (=2.22 x 10^{12} disintegrations/minute)
chol.	cholesterol
CoA	coenzyme A
CPCT	cholinephosphate cytidylyltransferase [E.C. 2.7.7.15]
cyclic-AMP, cAMP	adenosine 3′:5′-monophosphate
D-	dextrorotary
DFP	diisopropylflurophosphate
DG, or diacylglycerol	1,2-diacyl-sn-glycerol
DHAP	dihydroxyacetonephosphate
DPPC	1,2-dipalmitoyl-sn-glycerol-3-phosphocholine, or, dipalmitoyl-3-sn-phosphatidylcholine
dpm	distintegrations per minute
E.C.	Enzyme Commission
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid
E.R	endoplasmic reticulum
ESR	electron spin resonance
g	gram
xg	x acceleration of gravity

G.A.	Golgi apparatus
GP	sn-glycerol-3-phosphate
h	hour
Hepes	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
^{3}H-NMR	tritium nuclear magnetic resonance
[illegible]	stability constant
[illegible]	Michaelis constant
L-	levorotary
l	liter
L_f	free ligand
L_t	total ligand
LDH	lactate dehydrogenase [E.C.1.1.1.27]
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
L/S ratio	lecithin/sphingomyelin ratio
M	molar (mol/liter)
M_f	free metal
M_r	relative molecular mass
M_t	total metal
MgPA	magnesium phosphatidate
min	minute
NADH	nicotinamide-adenine dinucleotide, reduced form
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced form
PA, phosphatidate	1,2-diacyl-sn-glycero-3-phosphate
PA_{aq}	aqueous dispersion of 3-sn-phosphatidic acid

PA_{mb}	membrane-bound 3-sn-phosphatidic acid
PAPase	Lα-(3-sn-)-phosphatidate phosphohydrolase [E.C. 3.1.3.4]
PC, or phosphatidylcholine	1,2-diacyl-sn-glycero-3-phosphocholine
PE, or phosphatidylethanolamine	1,2-diacyl-sn-glycero-3-phosphoethanolamine
PG, or phosphatidylglycerol	1-(3-sn-phosphatidyl)sn glycerol, 3-sn-phosphatidylglycerol
P_i	inorganic orthophosphate
PI	phosphatidylinositol
RDS	respiratory distress syndrome
s	second
[S]	substrate concentration
SM	sphingomyelin
sn	stereospecifically numbered
T.M.	tubular myelin
TLC	thin layer chromatography
U	uniformly isotopically labelled
V	reaction velocity
V_{max}	maximum reaction velocity
v/v	volume/volume
w/v	weight/volume

CHAPTER 1

INTRODUCTION

1.1 THE NATURE OF THE PULMONARY SURFACTANT

The alveoli of the lung are lined with a specialized material, the pulmonary surfactant. The function of this material is to decrease the surface tension at the air-liquid interface, thus preventing collapse of the alveoli and/or drainage of interstitial fluid into the lung. Absence or insufficiency of the pulmonary surfactant in the immature lung of the prematurely delivered infant gives rise to a clinical condition known as Respiratory Distress Syndrome (RDS). These infants require mechanical ventilation at high pressures and oxygen tension to maintain adequate pulmonary gas exchange. Indeed, RDS is the most common cause of perinatal morbidity and mortality in the premature infant (Farrell and Avery, 1975).

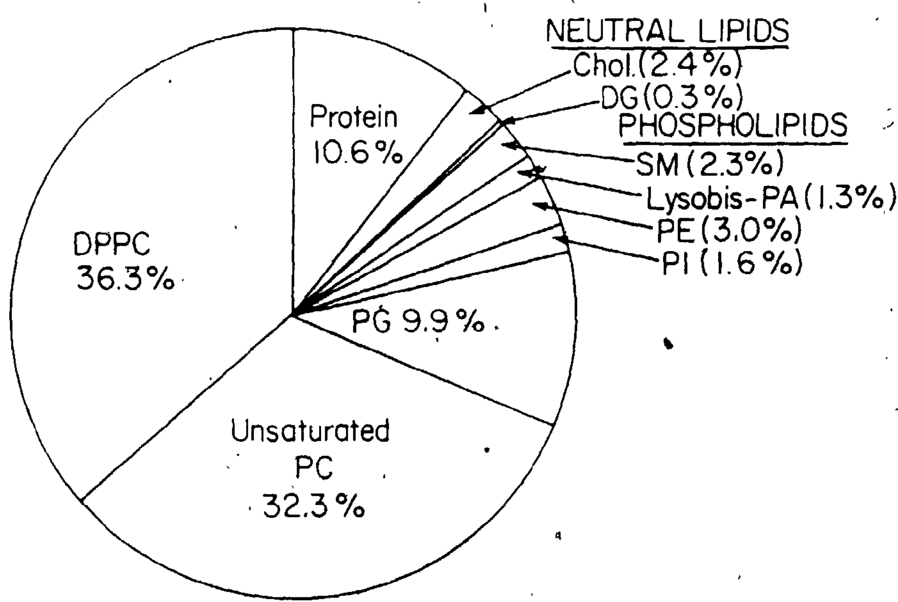
Evidence for a material which decreases the surface tension in the lung was first demonstrated by von Neergaard in 1929. Pressure-volume curves of excised lungs demonstrated that additional pressure was required for comparable inflation volumes in lavaged lungs. This was interpreted to indicate that an extracellular material that could be removed from the alveoli by lavage was acting to decrease the pressure required for pulmonary inflation. This initial discovery went unnoticed for almost 25 years. In 1954, Professor C.C. Macklin, working at the University

of Western Ontario, observed a material he called "mylogenin" being secreted from granular pneumocytes (Macklin, 1954). He concluded that this material may be important in lung function by reducing surface tension at the air-liquid interface. It was the work of Pattle (1955) and Clements (1956) that demonstrated the surface-active properties of materials isolated from the alveolar lining. In the late 1950's, the studies of Avery and Mead (1959) indicated that the pulmonary surfactant was a necessary component of the normal, mature lung and that the lungs of infants with RDS were deficient in this material. It was this connection between RDS and the lack of pulmonary surfactant that has prompted study into many areas of the physico-chemical properties and biosynthesis of this complex material.

The composition of the pulmonary surfactant (Figure 1) is predominately lipidic and the major component is dipalmitoylphosphatidylcholine (DPPC). Although this phospholipid is a minor constituent of most cells (Ohno _et al._, 1978), it accounts for approximately 50% of the phosphatidylcholine in the pulmonary surfactant. DPPC is the major surface-active ingredient in pulmonary surfactant (reviewed by King, 1982; Possmayer, 1983) principally because its small surface area and cylindrical shape allow very tight packing and the binding of water molecules at the surface. However, DPPC is very stable in the bilayer configuration and will form monolayers only very slowly. The presence of the other constituents of the pulmonary

Figure 1.

Composition of bovine pulmonary surfactant. Figure drawn from data of Yu *et al.* (1983).



COMPOSITION OF BOVINE PULMONARY SURFACTANT

surfactant somehow promotes the transfer of DPPC to the air-liquid interface. These other lipids are then excluded from the surface during the expansions and contractions that accompany breathing. For several excellent reviews on the surface chemistry of pulmonary surfactant the reader should consult the book edited by Robertson et al. (1984).

DPPC is synthesized in the endoplasmic reticulum of Type II pneumocytes and passes through the Golgi apparatus to finally reside within the lamellar bodies (Batenberg, 1984). The lamellar bodies appear to be the major intracellular storage location for surfactant material. At the time of surfactant release, the lamellar bodies are discharged into the fluid lining of the alveoli. From there an intermediate structure, called tubular myelin, appears to act as an intermediate and facilitate the formation of the surfactant monolayer.

Because of its principal role in the reduction of surface tension, examination of the biosynthesis of DPPC is paramount in the study of the production of lung surfactant.

1.2 PATHWAY OF GLYCEROLIPID BIOSYNTHESIS

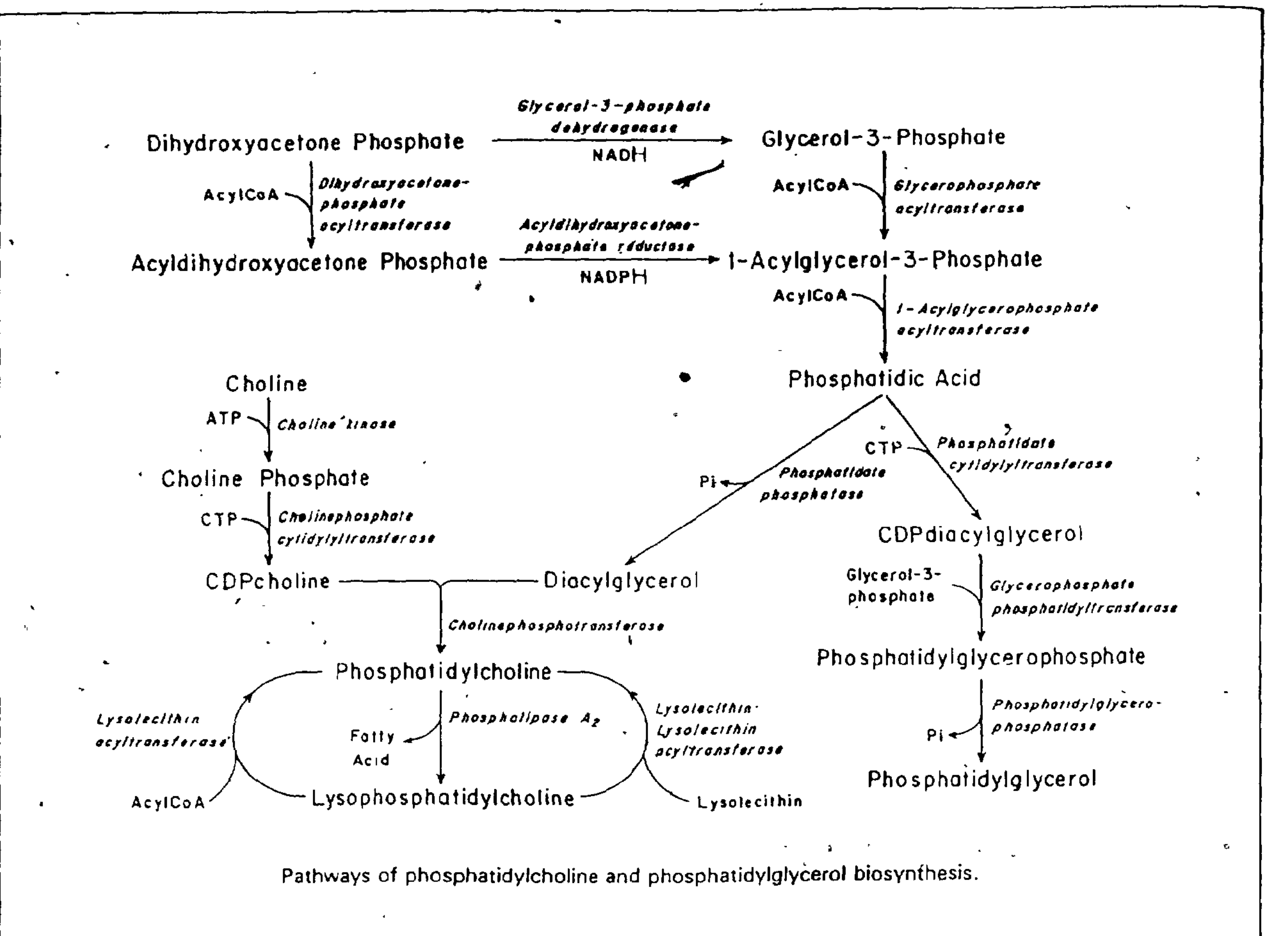
The enzyme activities of pulmonary phosphatidylcholine biosynthesis have been extensively reviewed (Van Golde, 1976; Batenberg and Van Golde, 1979; Possmayer, 1982; Batenberg, 1984; Possmayer, 1984; Rooney, 1985). This introduction is not intended to be a comprehensive review,

but rather an overview of the areas of phosphatidylcholine biosynthesis that formed the basis for initiating work on this thesis.

The route of synthesis of the phosphatidylcholine for lung surfactant follows the *de novo* pathway first described by Kennedy and coworkers (Kennedy and Weiss, 1955; Kennedy, 1961) (Figure 2). Two acyl chains are added to glycerol-3-phosphate (or dihydroxyacetonephosphate) and then the resulting phosphatidate is dephosphorylated to yield diacylglycerol. This is then combined with CDP-choline to produce phosphatidylcholine and CMP. Choline for pulmonary phosphatidylcholine biosynthesis is derived from the diet in mammals, although other organs can make variable amounts via a three-fold N-methylation of phosphatidylethanolamine (Zeisel, 1981). Choline is first phosphorylated and then reacted with CTP to yield the synthetic precursor CDP-choline and pyrophosphate. The acidic phospholipids phosphatidylglycerol and phosphatidylinositol arise from a separate pathway that diverges at the point of phosphatidate utilization. The phosphatidate is combined with CTP to give CDP-diglyceride which is the immediate precursor of phosphatidylinositol and phosphatidylglycerolphosphate which is subsequently dephosphorylated to phosphatidylglycerol. The prominent position of phosphatidate phosphohydrolase at the branch point between the pathways of phosphatidylcholine and acidic phospholipid biosynthesis can be seen in Figure 2.

Figure 2.

Pathways of phosphatidylcholine and phosphatidylglycerol biosynthesis. Figure corrected from Rooney (1978).



Pathways of phosphatidylcholine and phosphatidylglycerol biosynthesis.

1.3 BIOSYNTHESIS OF PHOSPHATIDYLCHOLINE FOR PULMONARY SURFACTANT

The formation of phosphatidate requires the bonding of two acyl chains to a three-carbon phosphorylated backbone. This synthesis can occur via two routes: from glycerol-3-phosphate or from dihydroxyacetonephosphate. Glucose has been shown to be the source of the three-carbon backbone of the phosphoglycerides (Felts, 1964; Scholz et al., 1972; Tierney, 1974; Van Golde, 1976). Electron micrographs of Type II cells show deposits of dark-staining glycogen (Kikkawa et al., 1971; Williams and Mason, 1977; Possmayer, 1984) which decrease as the formation of lamellar bodies progresses. In addition, radioactivity from prelabelled glycogen has been demonstrated to appear in DPPC and phosphatidylcholine in explants of fetal lung (Bourbon et al., 1982). Dihydroxyacetonephosphate is the common metabolic intermediate that links carbohydrate metabolism and phospholipid synthesis. Both glycerol phosphate and dihydroxyacetonephosphate pathways lead to the formation of lysophosphatidate, which is then acylated at the 2-position to yield phosphatidate. The route from dihydroxyacetonephosphate, the glycolytic intermediate, requires both an acylation and a reduction. If acylation occurs first then the DHAP pathway is followed, if reduction to glycerol-3-phosphate is the first step then the glycerol phosphate pathway is used. Glycerol, derived from the bloodstream, can also be used as a phospholipid

precursor provided it is converted to glycerol-3-phosphate. Originally thought to be absent from pulmonary tissue, an appreciable glycerol kinase activity has been demonstrated in Type II cells (Fisher and Chandler, 1982).

The relative contributions of the glycerol phosphate and DHAP pathways is a point of controversy which a number of investigations have attempted to resolve. When subcellular fractions of rabbit lung were incubated with equimolar glycerol phosphate and DHAP, 41% of the resulting products came from DHAP (Fisher and Chandler, 1976). Mason (1978) employed a technique of measuring the $^{3}H/^{14}C$ ratio to determine the contribution of each pathway in isolated Type II cells. This general method had been employed before in other tissues. In those studies, a mixture of [^{14}C-U]-glycerol and [^{3}H-2]-glycerol was used as substrate for glycerolipid synthesis. After being taken up by the cells, the radioactive glycerol is converted to glycerol phosphate with the same isotopic ratio. However, because DHAP possesses a keto group at the 2-position, any glycerol phosphate that was converted to DHAP would lose the ^{3}H at carbon number 2 and the resulting phospholipids would have a $^{3}H/^{14}C$ ratio ranging from the original ratio (all glycerol phosphate pathway) to zero (all via DHAP). A decrease from the original $^{3}H/^{14}C$ ratio would indicate the extent of the DHAP pathway.

Unexpectedly, studies in rat liver (Hill and Lands, 1970) and Clostridium <u>butyricum</u> (Okuyama and Lands, 1970)

found a slight increase in the $^{3}H/^{14}C$ ratio and concluded that the DHAP pathway was unimportant. Workers in E. coli (Benns and Proulx, 1972) observed no change in the $^{3}H/^{14}C$ ratio and came to the same conclusion. However, workers in Mycoplasma (Plackett and Radwell, 1970) and rat (Manning and Brindley, 1972; Bowley et al., 1973) observed that the isotope ratio in the lipid was consistently higher than that of the original glycerol. Further investigation demonstrated a tritium isotope effect upon the enzyme glycerol-3-phosphate dehydrogenase. Tritiated glycerol phosphate was being shunted through the glycerol phosphate pathway because the dehydrogenase was selecting against it. Elevated $^{3}H/^{14}C$ ratios appeared to be the result of loss of [^{14}C]-DHAP via glycolytic pathways. These studies concluded that the $^{3}H/^{14}C$ ratio of glycerol phosphate, not glycerol, was required to calculate the contributions of each pathway (Bowley and Brindley, 1976). Mason measured the $^{3}H/^{14}C$ ratio in phosphatidylglycerol synthesized by Type II cells. His rationale was as follows: the glycerol phosphate in the head group of phosphatidylglycerol should come only from glycerol phosphate and as such be a true measure of the $^{3}H/^{14}C$ ratio of the glycerol phosphate available for phosphatidylglycerol synthesis. The acylglycerol in the tail group of phosphatidylglycerol could have come from both pathways and is the measure of the contribution by each pathway. Using this method, he concluded that the DHAP pathway accounted for 56% of the phosphatidylglycerol synthesized and 64% of the

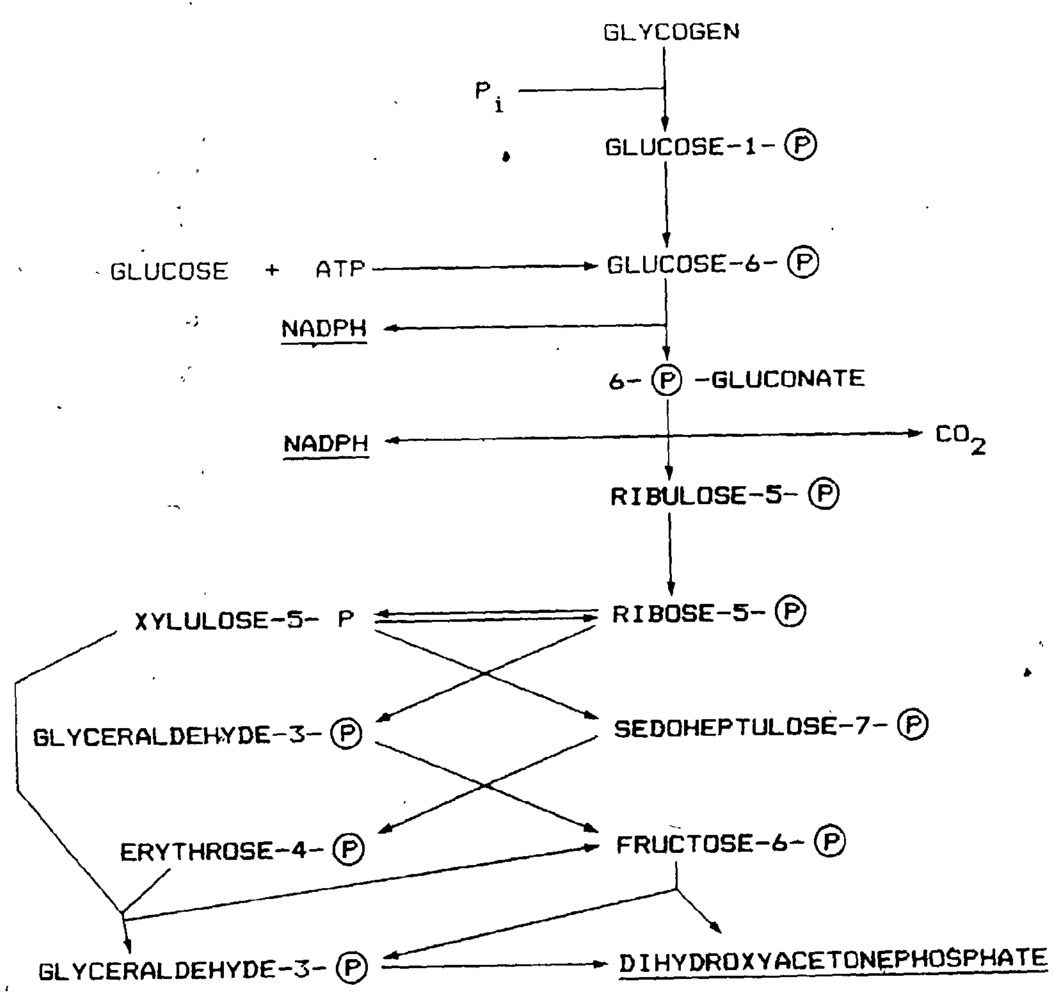
phosphatidylcholine. One argument based on general principles in favour of the DHAP pathway is stated by Brindley and Sturton (1982) who note that most anabolic pathways use NADPH as a reductive cofactor. Reduction of DHAP to glycerol phosphate requires NADH, normally involved in degradative pathways. The conversion of acyl-DHAP to acyl-glycerol phosphate has been shown to require NADPH as a cofactor (Pollock _et al._, 1975). In liver, the glycerol-3-phosphate dehydrogenase is involved in regulation of the cellular redox state. In lung DHAP and reducing equivalents in the form of NADPH could be derived from glycogen via the phosphogluconate and transaldolase-transketolase pathways (Maniscalco _et al._, 1982; Freese and Hallman, 1983) (Figure 3).

Phosphatidate phosphohydrolase, the enzyme that catalyses the conversion of phosphatidate to diacylglycerol, is found in both microsomal and cytosolic fractions in rat lung (Mavis _et al._, 1978; Casola _et al._, 1978; Ravinuthala _et al._, 1978; Casola and Possmayer, 1979; Yeung _et al._, 1979) and rabbit lung (Schultz _et al._, 1974; Brehier _et al._, 1977). Such an "ambiquitous" distribution is unanticipated in light of the exclusive membrane association of phosphatidate.

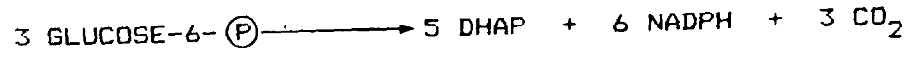
The production of CDP-choline for phosphatidylcholine synthesis occurs via the phosphorylation of choline to cholinephosphate, a reaction catalysed by choline kinase. The conversion of choline phosphate to CDP-choline occurs

Figure 3.

Route of synthesis of DHAP and NADPH from glycogen via the transketolase and transaldolase pathways.



Net Reaction:



via CTP:cholinephosphatecytidylyltransferase. Considerable evidence has accumulated to suggest that this reaction is the rate-limiting step in phosphatidylcholine biosynthesis (Pelech and Vance, 1984). Studies of the pool sizes of choline, cholinephosphate, and CDP-choline show that in the fetal and adult rat and rabbit lung the amount of cholinephosphate was always much greater than that of CDP-choline or choline (Possmayer et al., 1981; Tokmakjian et al., 1981a,b). These results suggest that the CPCT activity in vivo is slower than either of the reactions immediately preceding or following it. These studies measure the pool sizes in whole lung and as such do not differentiate between surfactant-producing cells and other lung cells. However, similar studies on Type II cells (Post et al., 1982) have shown similar results. Further studies on the developmental profiles of CPCT have shown increases in activity just prior to immediately following birth (Rooney et al., 1976; Rooney et al., 1977; Stern et al., 1976; Oldenborg and Van Golde, 1977; Maniscalco et al., 1978; Brehier and Rooney, 1981; Chan et al., 1983). This activity has also been shown to increase under the effects of a number of hormones (cortisol, 17β-estradiol, triiodothyronine) that stimulate lung maturation (reviewed by Rooney, 1985). It has recently been demonstrated that under phosphorylating conditions, preincubation results in an inhibition of the CPCT activity in fetal lung tissue (Radika and Possmayer, 1985). This inhibition could be blocked with an inhibitor of cAMP-dependent protein kinase,

indicating that phosphorylation of a protein may be responsible for control of this enzyme.

The final step in the synthesis of phosphatidylcholine, the transfer of phosphocholine from CDP-choline to diacylglycerol, is catalyzed by cholinephosphotransferase. This enzyme has been extensively studied in lung (Weinhold _et al._, 1973; Oldenborg and Van Golde, 1976; Sarzala, 1976; Rooney and Wai-Lee, 1977; Possmayer _et al._, 1978). Although previously reported to be present in lamellar bodies (Spitzer _et al._, 1976), which implied a potential role of these organelles in the the _de novo_ synthesis of phosphatidylcholine, subsequent studies have shown that this activity could be accounted for by microsomal contamination (Rooney _et al._, 1975; Baranska and Van Golde, 1977; Tsao and Zachman, 1977; Spitzer, 1978). Cholinephosphotransferase activity has been shown to increase during fetal lung development (Farrell _et al._, 1974; Oldenborg and Van Golde, 1976; Farrell _et al._, 1977) and following glucocorticoid-induced fetal lung maturation (Farrell and Zachman, 1973; Oldenborg and Van Golde, 1977). Questions about the method of assaying this enzyme with regard to inappropriate diacylglycerol substrate (Van Golde, 1976; Possmayer, 1977; Rooney and Wai-Lee, 1977; Farrell and Hamosh, 1978; Ohno _et al._, 1978; Possmayer, 1982) have made ambiguous the role of this enzyme in the control of phosphatidylcholine synthesis.

Production of disaturated phosphatidylcholine for

surfactant may follow two pathways: *de novo* synthesis of disaturated phosphatidylcholine or remodelling of 1-saturated / 2-unsaturated phosphatidylcholine (Figure 2). Early work led to the conclusion that 1-saturated / 2-unsaturated phosphatidylcholine was the first synthetic product and that this was then remodelled by replacement of the unsaturated fatty acid at the 2-position with palmitate. Turnover studies *in vivo* demonstrated that radioactive glycerol was found in unsaturated phosphatidylcholine at short time periods and then DPPC at longer times (Vereyken *et al.*, 1972; Kyei-Abogaye *et al.*, 1973; Moriya and Kanoh, 1974). Palmitate, however, was found to enter DPPC at short times, apparently bypassing the *de novo* route (Moriya and Kanoh, 1974).

Both methods of remodelling the 2-unsaturated phosphatidylcholine to DPPC require the initial action of phospholipase A_2. Such an activity has been reported in lung (Ohta and Hasegawa, 1972; Garcia *et al.*, 1975), including a phospholipase A_2 activity that selectively removes unsaturated fatty acids (Longmore *et al.*, 1979). The restoration of an unsaturated fatty acid comes from two sources: palmitoyl-CoA and a reacylation mechanism or a transacylation that results in the production of a disaturated phosphatidylcholine and glycerol phosphate. Both reacylation (Fronsonolo, 1977) and transacylation (Erbland and Marinetti, 1965; Abe *et al.*, 1972; Akino *et al.*, 1972; Okano and Akino, 1978) pathways have been demonstrated in lung tissue. Double-label experiments with

[^{3}H-fatty acid and ^{14}C-choline]-lysophosphatidylcholine have demonstrated that the ratio of $^3H/^{14}C$ remained constant, indicating that the reacylation pathway is important in rat lung (Van Heusden et al., 1981) and Type II cells (Mason and Dobbs, 1980). Type II cells are enriched in lysophosphatidylcholine acyltransferase, but not LPC:LPC transacylase (Battenberg et al., 1979) when compared to whole lung. In an elegant approach, Van Heusden, Vianen, and Van Den Bosch (1980) demonstrated that although the reacylation pathway can only use L-isomers of LPC, the transacylation pathway can use either D- or L-isomers. Making use of the stereochemical specificity of snake venom phospholipase A_2 for L-phosphatidylcholine to assay the percentage of L-phosphatidylcholine, they demonstrated that rats injected with D,L-LPC produced only L-phosphatidylcholine. These results indicated that the reacylation pathway was responsible for the synthesis of phosphatidylcholine.

Early in vivo studies (Vereykin et al., 1972) and studies of CPT (Oldenborg and Van Golde, 1976; Sarzala and Van Golde, 1976; Possmayer et al., 1977; Rooney and Wai Lee, 1977) indicated that disaturated phosphatidylcholine was not generated de novo and that CPT did not appreciably use disaturated diacylglycerol. However, more recent experiments have demonstrated that CPT can use dipalmitoyl as well as dioleoyldiacylglycerols (Miller and Weinhold, 1981; Van Heusden et al., 1981; Ide and Weinhold, 1982; Van

Heusden and Van den Bosch, 1982). In addition, similarities in the disaturated diacylglycerol and phosphatidylcholine pools have been observed upon labelling with ^{3}H-glycerol in vivo (Ishidate, 1981) indicating that de novo synthesis of disaturated phosphatidylcholine does occur. In isolated adult Type II cells, labelling studies and the use of inhibitors of phospholipase A_2 have led to the conclusion that both de novo and remodelling pathways are involved in disaturated phosphatidylcholine synthesis (Post et al., 1983).

1.4 PHOSPHATIDATE PHOSPHOHYDROLASE IN OTHER TISSUES

Phosphatidate phosphohydrolase activity was originally discovered by Kates (1955) in plants. Phosphatidate phosphohydrolase has been found to be present in a great number of mammalian tissues (reviewed by Hubscher, 1970), and was identified and characterized in brain (Agranoff, 1962), kidney (Coleman and Hubscher, 1962), intestine (Johnston and Bearden, 1962) and erythrocytes (Hokin et al., 1963) in the early part of the 1960's. Phosphatidate phosphohydrolase has been extensively studied in liver and adipose tissue and many comprehensive reviews have been published (Fallon et al., 1977; Brindley, 1978; Brindley and Sturton, 1982; Brindley, 1985).

The enzymes of lipid biosynthesis pose a dilemma to their study by conventional means. The insolubility of the substrates in assay mixtures requires that other methods of solubilization be used. These methods fall into three

broad catagories: 1. sonication of substrate into emulsions; 2. solubilization by use of detergents; and 3. utlization of membrane-bound substrates. The use of membrane-bound substrates could be expected to most closely resemble the *in vivo* activity, and this method of substrate preparation has been used for studies of phospholipase A_2 (Longmore *et al.*, 1979), diacylglycerol acyltransferase (Haagsman *et al.*, 1977), cholinephosphotransferase (Sarzala and Van Golde, 1976), and phosphatidate cytidylyltransferase (Sturton and Brindley, 1977; Van Heusden and Van den Bosch, 1978). However, this form of substrate is complicated by unknown levels of other phospholipids, denatured proteins, neutral lipids, competing enzymes, glycolipids, and inorganic ions. Attempts to eliminate these variables make use of substrate emulsions. While these methods result in a chemically-defined system, it is necessary to determine that they relate to the *in vivo* activity.

The original identification and characterization of phosphatidate phosphohydrolase from a number of tissues (Hokin and Hokin, 1959; Agranoff, 1962; Coleman and Hubscher, 1962; Hokin *et al.*, 1963; Wilgram and Kennedy, 1963; Sedgewick and Hubscher, 1965) indicated that the bulk of the activity was found in the mitochondrial and microsomal fractions. When aqueously-dispersed phosphatidate was employed, little activity was detected in cytosol. However, further experiments indicated that there

was a factor present in cytosol which could stimulate triacylglycerol synthesis when membrane-bound phosphatidate was used (Johnston et al., 1967; Smith et al., 1967). This factor was revealed to be a soluble phosphatidate phosphohydrolase that was very active upon phosphatidate generated on microsomes from glycerol phosphate but displayed little activity toward aqueous emulsions of phosphatidate. Further studies in liver (Mitchell et al., 1971; Lamb and Fallon, 1974; Van Heusden and Van den Bosch, 1978; Goldberg et al., 1980; Angelin et al., 1981; Lamb and Dewey, 1981) and adipose tissue (Jamdar and Fallon, 1973; Jamdar et al., 1976; Cheng and Saggerson, 1978a,b) have concentrated on the activity upon membrane-bound substrate. Work in adipose tissue has also employed an aqueously-dispersed substrate, the Mg^{2+}- dependent component of which reflects the activity upon membrane-bound substrate (Jamdar and Fallon, 1973; Lamb and Fallon, 1974; Cheng and Saggerson, 1978a,b). The Mg^{2+}-dependent phosphatidate phosphohydrolase activities differ from the Mg^{2+}-independent activities by a number of parameters including pH optima, kinetic constants, thermal stability, and the effects of EDTA and divalent cations (Jamdar and Fallon, 1973; Jamdar et al., 1984). The Mg^{2+}-dependent phosphatidate phosphohydrolase activity has been demonstrated to decrease in microsomes and increase in the cytosol and mitochondrial fractions in dystrophic human muscle (Kunze et al., 1985). Investigations in liver by Brindley and coworkers (reviewed by Brindley, 1982) have

concentrated on the Mg^{2+}-stimulated phosphatidate phosphohydrolase. Although optimal levels of Mg^{2+} are required for maximal activity, the requirement did not appear to be absolute as the amphiphilic cation chlorpromazine could substitute for Mg^{2+} (Bowley et al., 1977; Sturton and Brindley, 1980).

Phosphatidate phosphohydrolase activity in the liver is regulated by a number of factors including glucocorticoids, which have been demonstrated to increase triacylglycerol synthesis and stimulate phosphatidate phosphohydrolase activity (Lehtonen et al., 1979; Jennings et al., 1981). These increases in phosphatidate phosphohydrolase activity could be blocked by actinomycin D and cycloheximide and the conclusions drawn are that glucocorticoids promote the increased synthesis of phosphatidate phosphohydrolase. These increases could be suppressed by insulin (Lawson et al., 1981a,b; Lawson et al., 1982a,b). Rats fed diets containing fructose, glycerol, or ethanol also displayed an increase in hepatic phosphatidate phosphohydrolase levels (Pritchard et al., 1977; Savolainen, 1977; Sturton et al., 1978; Savolainen and Hassinen, 1978). Phosphatidate phosphohydrolase activity increased 7-fold after ethanol treatment in these rats (Brindley et al., 1979). The increases observed in ethanol-treated rats appear to be due to involvement with glucocorticoids and redox-state of the liver (reviewed by Brindley and Sturton, 1982). The redox change, which

accounts for 15% of the total increase in phosphatidate phosphohydrolase activity could be blocked by pyrazole which inhibits ethanol oxidation (Wood and Lamb, 1979; Savolainen and Hassinen, 1980). In adipocytes, noradrenalin and dibutyryl-cAMP have been demonstrated to inhibit the phosphatidate phosphohydrolase activity (Cheng and Saggerson, 1978a,b; Cheng and Saggerson, 1980; Cheng et al., 1980). Insulin, which has been shown to stimulate the microsomal activity of phosphatidate phosphohydrolase in adipocytes (Roncari et al., 1979), is capable of relieving the inhibition by noradrenalin but not that inhibition produced by dibutyryl-cAMP (Cheng et al., 1980).

In liver, the most thoroughly studied tissue to date, short-term control of Mg^{2+}-stimulated phosphatidate phosphohydrolase activity appears to be regulation of intracellular location (reviewed by Brindley, 1985). The cytosolic phosphatidate phosphohydrolase appears to exist as a metabolically inactive pool that can translocate to an active position on the endoplasmic reticulum under as yet unknown control mechanisms. The most potent agent in causing translocation of phosphatidate phosphohydrolase from cytosol to endoplasmic reticulum is fatty acids (Cascales et al., 1984; Martin-Sanz et al., 1984; Hopewell et al., 1985), although fatty acyl CoA's (Martin-Sanz et al., 1984) and spermine also have this effect (Hopewell et al., 1985; Martin-Sanz et al., 1985). Removal of phosphatidate phosphohydrolase from the microsomes to cytosol can be facilitated by albumin and chlorpromazine

(Hopewell et al., 1985).

Although the investigations of phosphatidate phosphohydrolase in liver and adipose tissue have facilitated the advanced study of this enzyme in lung, it should be noted that liver and adipose tissue are involved in the regulation and control of triacylglycerol and phosphatidylcholine synthesis, whereas the Type II cells of the lung are principally involved in the synthesis of dipalmitoylphosphatidylcholine. Therefore, differences in the metabolic regulation of phosphatidate phosphohydrolase activity between these tissues would be possible.

1.5 PULMONARY PHOSPHATIDATE PHOSPHOHYDROLASE ACTIVITY

Phosphatidate phosphohydrolase activity in lung tissue was first demonstrated using a histochemical approach (Meban, 1972), which localized the activity to within the inclusion bodies of granular pneumocytes. Early studies on phosphatidate phosphohydrolase activity in lung (Jimenez et al., 1974; Schultz et al., 1974; Garcia et al., 1976; Mavis et al., 1978; Spitzer and Johnston, 1978) have concentrated on that activity assayed using aqueously-dispersed phosphatidate, or the amphipathic analog 1-O-hexadecylglycerol-3-phosphate (Bleasdale et al., 1978). Phosphatidate phosphohydrolase has been suggested (Schultz et al., 1974) to be an important regulatory enzyme in the biosynthesis of lung surfactant in the rabbit. Support for this claim rests with the location of phosphatidate

phosphohydrolase at the first branch point in phosphatidylcholine synthesis, a logical and often encountered phenomenon in carbohydrate and cholesterol synthesis. Experimental evidence demonstrates that the rise in pulmonary phosphatidate phosphohydrolase activity precedes by 24 hours the rise in accumulation of phosphatidylcholine (Gluck _et al._, 1967; Schultz _et al._, 1974). Phosphatidate phosphohydrolase activity in hepatic tissues did not increase over this time period. The appearance of phosphatidate phosphohydrolase in amniotic fluid also predates the release of phosphatidylcholine into the alveoli and the increase in the L/S ratio (Jimenez _et al._, 1974; Jimenez _et al._, 1975). The ratio of phosphatidylcholine (lecithin) to sphingomyelin in the amniotic fluid has been used clinically to assess fetal lung maturity (Gluck _et al._, 1974). The conclusion of these authors was that phosphatidate phosphohydrolase was controlling the production of phosphatidylcholine by regulating the supply of substrates for synthesis.

The subcellular distribution of phosphatidate phosphohydrolase in lung indicated that the enzyme activity was associated with particulate fractions (Schultz _et al._, 1974; Mavis _et al_, 1978). Phosphatidate phosphohydrolase was also reported to be found in lamellar bodies (Meban, 1972; Spitzer _et al._, 1975; Garcia _et al._, 1976; Johnston _et al._, 1978; Mavis _et al._, 1978; Spitzer and Johnston, 1978; Okazaki and Johnston, 1980; Casola _et al._, 1982; Crecelius and Longmore, 1983), amniotic fluid (Jimenez _et_

al., 1974; Jimenez, et al. 1975; Johnston et al., 1975; Jimenez and Johnston, 1976; Bleasdale et al., 1978; Rosenfeld et al., 1980), and surfactant from lung lavage (Delahunty et al., 1979; Benson, 1980). The nature of the phosphatidate phosphohydrolase activity associated with lamellar bodies remains unclear. Lamellar bodies lack the full complement of enzymes required to make phosphatidylcholine de novo (Rooney et al., 1975; Garcia et al., 1976; Baranska and Van Golde, 1977). Johnston and coworkers (Johnston et al., 1978) reported that the phosphatidate phosphohydrolase activity of porcine lung lamellar bodies could hydrolyze phosphatidate and phosphatidylglycerolphosphate in a competitive manner and concluded that this activity and the phosphatidylglycerol phosphatase [EC 3.1.3.27] activity were the same protein. The enzyme GP:phosphatidyltransferase [EC 2.7.8.5] was also found in lamellar bodies (Rooney et al., 1975) and it was suggested that lamellar bodies could be involved in the synthesis of phosphatidylglycerol. Further studies (Casola et al., 1982) resolved two distinct activities; a phosphatidate phosphohydrolase and a phosphatidylglycerol phosphatase. The phosphatidate phosphohydrolase activity of lamellar bodies has been suggested to be a non-specific phosphatase (Casola et al., 1982; Crecelius and Longmore, 1983) which may be involved in the degradation of surfactant reabsorbed by Type II cells (Chandler et al., 1983).

The existence of extracellular phosphatidate phosphohydrolase, which can be detected in amniotic fluid or surfactant lavage, has been suggested as a biochemical marker of fetal lung maturity (Jimenez _et al._, 1974; Jimenez _et al._, 1975; Johnston _et al._, 1975; Jimenez and Johnston, 1976; Bleasdale _et al._, 1978; Forman, 1982). From week 31 to week 39 (term 41 weeks), increases in the specific activity of phosphatidate phosphohydrolase activity of approximately 6-fold have been reported (Jimenez _et al._, 1975; Forman, 1982) in human amniotic fluid. These increases predate the rise in L/S ratio by approximately 3 weeks and as such could be the earliest method of measuring the onset of fetal lung maturation.

Studies in lung also reported that the phosphatidate phosphohydrolase activities assayed using aqueous dispersion of phosphatidate in microsomes and cytosol increased prior to the increases in phosphatidylcholine synthesis in the fetal rabbit (Schultz _et al._, 1974), fetal rat (Ravinuthala _et al._, 1978; Brehier and Rooney, 1981), and fetal mouse (Maniscalco _et al._, 1978; Filler and Rhoades, 1979). Additionally, in the rabbit, the PA_{aq}-dependent phosphatidate phosphohydrolase activity has been shown to increase upon administration of glucocorticoids used to accelerate fetal lung development (Brehier _et al._, 1977; Possmayer _et al._, 1979; Rooney _et al._, 1979; Freese and Hallman, 1983).

However, assays using aqueous dispersions of phosphatidate to measure the PA_{aq}-dependent phosphatidate

phosphohydrolase activity in lung are inconsistent with that activity studied in other tissues, studies that concluded that the activity acting upon membrane-bound phosphatidate was important biosynthetically. Studies to resolve this point concluded that there existed four operationally-distinct phosphatidate phosphohydrolase activities (Casola et al., 1978; Yeung et al., 1979; Casola and Possmayer, 1979; Casola and Possmayer, 1981a,b): activities in both the cytosolic and particulate fractions that hydrolyzed either aqueously-dispersed or membrane-bound phosphatidate. Further studies on the developmental patterns of these activities reported increases of 1.5-fold in the PA_{mb}-dependent phosphatidate phosphohydrolase activities in developing fetal rat (Casola and Possmayer, 1981c), or rabbit (Casola and Possmayer, 1981d) lung near the end of gestation. Increases in the phosphatidate phosphohydrolase activity measured using aqueously-dispersed phosphatidate occurred during late gestation in rat lung microsomes and cytosol (Casola and Possmayer, 1981c) and rabbit microsomes (Casola and Possmayer, 1981d). Estradiol-17β or betamethasone did not stimulate the PA_{mb}-dependent phosphatidate phosphohydrolase in fetal rabbit lung (Possmayer et al., 1981), although a significant increase in the phosphatidate phosphohydrolase activity, measured using aqueously-dispersed phosphatidate was observed.

When studies for this thesis were undertaken, the

question of which of the activities previously described was important in pulmonary phosphatidylcholine biosynthesis remained unanswered. In addition, the development of assay conditions that would measure the PA_{mb}-dependent phosphatidate phosphohydrolase activity without the use of microsomal substrates would allow further investigations regarding the nature of this important enzyme.

CHAPTER 2

DEVELOPMENT OF AN ASSAY EMPLOYING A DEFINED CHEMICAL SUBSTRATE WHICH REFLECTS THE PHOSPHOHYDROLASE ACTIVITY MEASURED USING MEMBRANE-BOUND SUBSTRATE

2.1 INTRODUCTION

By virtue of its location at the initial branch point in glycerolipid metabolism, phosphatidate phosphohydrolase [EC 3.1.3.4] has long been considered to be a potentially regulatory enzyme which could influence the production of both acidic and zwitterionic phospholipids and of neutral glycerides (Brindley and Sturton, 1982). Studies in liver and adipose tissue have shown that the Mg^{2+}- dependent form of this enzyme can be affected by a number of agents including: cationic amphiphilic drugs (Bowley et al., 1977; Sturton and Brindley, 1977; Sturton and Brindley, 1980), glucocorticoids (Brehier et al., 1977; Brindley et al., 1979), insulin (Cheng and Saggerson, 1978b), noradrenalin (Cheng and Saggerson, 1978a,b), and cAMP (Moller et al., 1981). Further control may be exerted through reversible phosphorylation (Berglund et al., 1982; Butterworth et al., 1984) and by enzyme translocation between cytosol and endoplasmic reticulum (Butterworth et al., 1984; Cascales et al., 1984). In contrast to the tissues mentioned above, studies on pulmonary phosphatidate phosphohydrolase have concentrated on the Mg^{2+}- independent phosphatase activity observed with aqueous dispersions of phosphatidic acid (PA_{aq}). The observation that this

activity increases in lung during the perinatal period and after glucocorticoid treatment has led to the suggestion that it might be involved in the control of phosphatidylcholine (PC) production for surfactant synthesis (Bleasdale and Johnston, 1982; reviewed in Possmayer, 1982).

Studies in our laboratory have demonstrated that in addition to the phosphatase activity observed with PA_{aq}, lung microsomal and cytosolic fractions contain phosphohydrolase activities acting upon phosphatidate endogenously generated within microsomal membranes (Yeung et al., 1979; Casola and Possmayer, 1981a,b). The activities which utilize membrane-bound phosphatidate (PA_{mb}) differ from those using PA_{aq} by a number of criteria including: thermal stability, pH profile, trypsin resistance, apparent molecular weight, and enzyme kinetics (Casola and Possmayer, 1981a). The single most characteristic factor which distinguishes the PA_{aq}- and PA_{mb}- utilizing phosphohydrolases is a marked requirement for Mg^{2+} by the enzymes utilizing PA_{mb} (Possmayer, 1982). Addition of EDTA virtually abolished the cytosolic PA_{mb}-phosphohydrolase activity and reduced the microsomal activity to less than half of its control value. Addition of EDTA plus Mg^{2+} resulted in a marked stimulation over the activity observed with control incubations without EDTA or Mg^{2+}.

Although previous attempts to demonstrate a Mg^{2+}-dependent phosphatidate phosphohydrolase in lung using pure

phosphatidate as a substrate have been unsuccessful (Yeung *et al.*, 1979; Mavis *et al.*, 1978; Ravinuthala *et al.*, 1978), lipid vesicles prepared from extracts of [^{32}P]-phosphatidate-loaded microsomes appeared to serve as a substrate (Casola and Possmayer, 1981b). These observations indicated that the activity utilizing PA_{mb} was not dependent on the microsomal structure or protein constituents and suggested that the basic difference in the activities utilizing PA_{aq} and PA_{mb} might be due to the presence of Mg^{2+} arising during preparation of the latter substrate. The present report describes studies which demonstrate that a Mg^{2+}-dependent phosphatidate phosphohydrolase can be assayed in lung microsomal and cytosolic fractions using a chemically-defined substrate. The properties of this particular activity reflect the properties of the PA_{mb} rather than the PA_{aq} phosphohydrolase.

2.2 MATERIALS and METHODS

Phospholipid substrates and rat lung subcellular fractions were obtained as described in Chapter 3 (Walton and Possmayer, 1984).

Mg^{2+}-dependent phosphatidate phosphohydrolase activity was assayed using mixed-lipid vesicles of equimolar phosphatidate and phosphatidylcholine. Chemically-defined liposomes were formed by sonication in 0.9% NaCl with a Biosonic Bronwill sonicator fitted with a microprobe.

Reaction mixtures contained: 50 mM HEPES buffer (pH 7.4), 0.2 mM phosphatidate (0.9 mCi/mmol), 0.2 mM phosphatidylcholine for the microsomal assays, 0.5 mM phosphatidate and phosphatidylcholine for the cytosolic assays, 1.25 mM EDTA, 70 µg microsomal or cytosolic protein in a final reaction volume of 0.1 ml. Incubation time was 15 minutes at 37°C. Assays were performed in the absence of $MgCl_2$, or the presence of 3.25 mM $MgCl_2$; giving a free Mg^{2+} concentration of 2 mM. The Mg^{2+}-dependent activity was calculated by difference. Reactions were stopped by the addition of 1.5 ml of chloroform/methanol (5:4). The phases were broken with 0.75 ml of 0.1 M HCl and a sample of the upper aqueous phase taken for scintillation counting to determine [^{32}P]-inorganic phosphate release as a measure of enzymatic activity. Under these conditions addition of Mg^{2+} produces a 2-fold increase in the release of [^{32}P]-inorganic phosphate with the microsomal fraction and a 5-fold increase in the cytosolic activity. All modifications for purposes of characterization were made to this standard assay protocol.

2.3 RESULTS and DISCUSSION

2.3.1 Optimal conditions for the assay of Mg^{2+}-dependent phosphatidate phosphohydrolase

The principle objective of these investigations was to prepare and optimize a substrate for phosphatidate phosphohydrolase that would be representative of the *in vivo* activity upon phosphatidate formed within the

intracellular membranes. Microsomes that are preloaded with phosphatidate, which have been previously employed as substrate (Mitchell et al., 1971; Smith et al., 1967; Johnson et al., 1967; Lamb and Fallon, 1974; Casola et al, 1978; Casola and Possmayer, 1979), have several drawbacks. First, the preparation of such microsomes is time-consuming and variable with regard to the amount of phosphatidate generated. Secondly, the amount of phosphatidate produced as compared to the other phospholipid constituents of the microsomes is small, rarely exceeding a few percent. These small amounts make kinetic experiments difficult and inconclusive because of limiting substrate concentrations. Thirdly, the occurrence of variable and undetermined concentrations of other phospholipids, neutral lipids, glycolipids, cholesterol, and inorganic ions plus denatured proteins complicates experiments into the effects of individual components upon enzyme activity. It was felt that a chemically-defined liposome system would best serve as a substrate model.

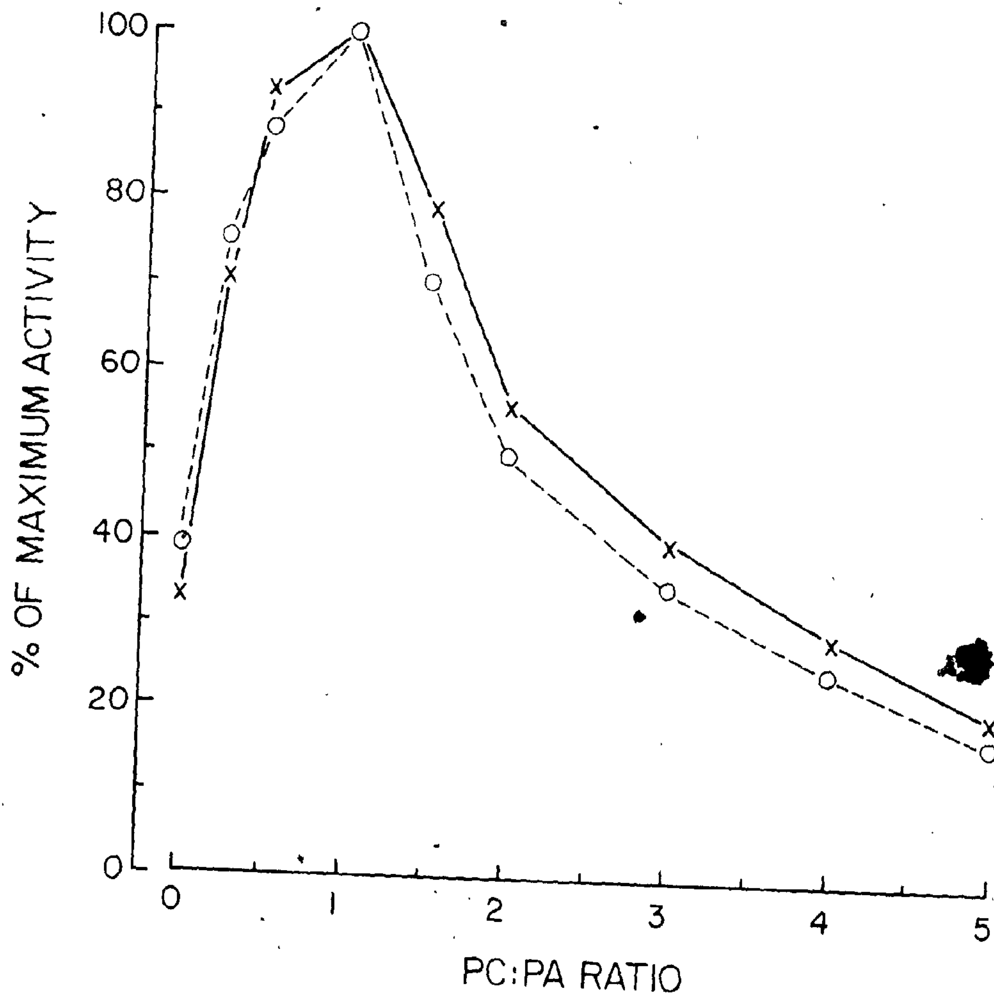
Phosphatidate will form liposomes under sonication but the resulting suspension is often unstable and acts as a poor substrate for the Mg^{2+}-dependent phosphatidate phosphohydrolase. The phosphatidate molecule has a poor shape for the production of closed liposomes. In their review, Cullis and de Kruijff (Cullis and de Kruijff, 1979) likened the shape of phosphatidate to a cone with the small polar head group at the apex. Such a shape would preferentially pack into the inverted hexagonal (H_{II})

phase. Such arrangements have been observed for phosphatidate upon addition of Mg^{2+} and other divalent cations (Verkleij et al., 1982). In the H_{II} phase, the fatty acyl groups extend outwards into the bulk phase while the phosphate groups are sequestered along the inner aqueous pores where they may be less accessible to added enzyme. Assuming that the H_{II} phase would provide an inappropriate physical form to act as a substrate, conditions were sought which would stabilize the bilayer form. Phosphatidylethanolamine also forms H_{II} phase and it has been demonstrated that addition of equimolar phosphotidylcholine will stabilize the bilayer phase (Cullis and de Kruijff, 1978). Mixed emulsions of phosphatidate and phosphatidylcholine have been used to stimulate the Mg^{2+}-dependent phosphatidate phosphohydrolase activity in microsomes and cytosol from adipose tissue (Moller et al, 1977; Jamdar and Osborne, 1983; Hosaka et al., 1975), and liver (Sturton et al., 1978). It is believed that the addition of phosphatidylcholine allows a better interaction between enzyme and substrate, not only by stabilizing the bilayer phase, but by diminishing the negative charge density on the artificial membrane (Brindley and Sturton, 1982).

The present experiments show that the addition of phosphatidylcholine to the phosphatidate vesicles makes them a more suitable substrate (Figure 4). The addition of increasing amounts of phosphatidylcholine initially

Figure 4.

Effects of including phosphatidylcholine in substrate liposomes on the Mg^{2+}-dependent phosphatidate phosphohydrolase activity from rat lung microsomes (X), and cytosol (O). Phosphatidate concentration was 0.2 mM for each point. Results are averages from two separate experiments.


100
80
60
40
20
0
% OF MAXIMUM ACTIVITY
0
1
2
3
4
5
PC:PA RATIO

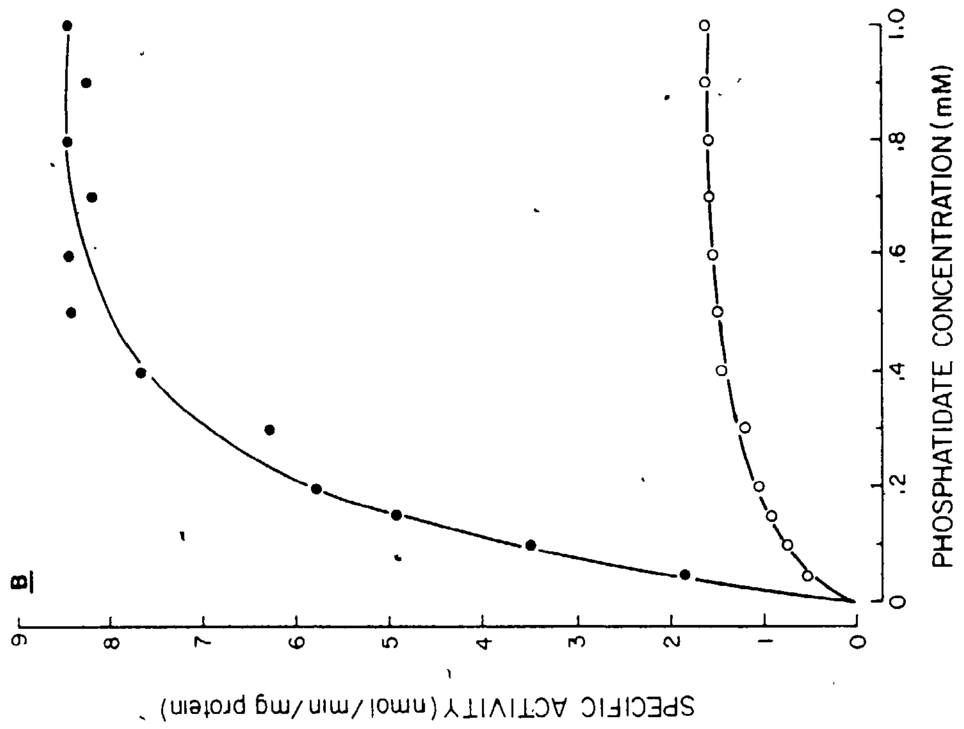
stimulated the Mg^{2+}-dependent phosphatidate phosphohydrolase activity in both microsomes and cytosol. A 3-fold enhancement in activity occurred at an equimolar PC:PA ratio. Greater amounts of phosphatidylcholine in the substrate liposomes caused an increasing depression of the enzyme activity. This depression apparently occurs by simple dilution of substrate. As the ratio of PC:PA was increased beyond 1:1, an "N"-fold increase in PC:PA ratio resulted in a "1/N"-fold decrease in the Mg^{2+}-dependent phosphatidate phosphohydrolase activity.

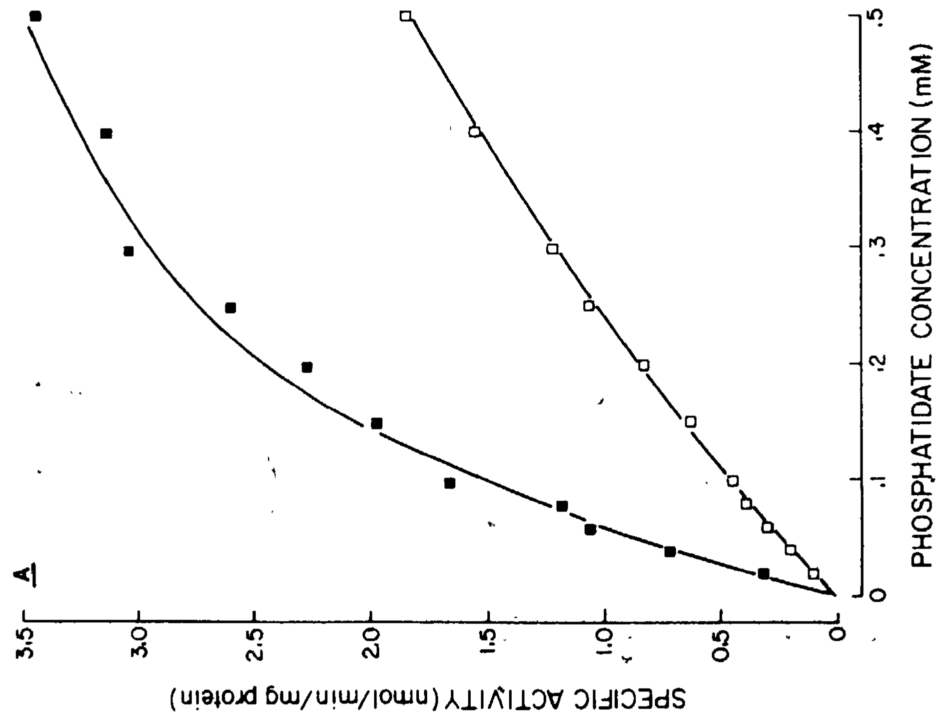
The effects of Mg^{2+} on the activity of the phosphatidate phosphohydrolase associated with glycerolipid metabolism have been extensively studied (reviewed in Brindley and Sturton, 1982). While Mg^{2+} has been shown to inhibit the activity in some tissues and stimulate in others, it is generally accepted that Mg^{2+} is required for maximal activity (Brindley and Sturton, 1982). The phosphatidate phosphohydrolase activities of liver (Mitchell et al., 1971), and adipose tissue (Moller et al., 1981; Jamdar and Fallon, 1973) demonstrate a Mg^{2+}-dependence, provided that endogenous Mg^{2+} is eliminated. Previous work from our laboratory has demonstrated that the phosphatidate phosphohydrolase activity upon membrane-bound substrate is also Mg^{2+}-dependent in lung (Yeung et al., 1979).

Velocity vs. phosphatidate concentration curves in the presence or absence of Mg^{2+} for both microsomes (Figure 5a), and cytosol (Figure 5b) show a marked increase in the

Figure 5.

Velocity versus substrate concentration curves for microsomes (Figure 5a), and cytosol (Figure 5b) in the presence (closed symbols), and absence (open symbols) of 3 mM Mg^{2+}. The Mg^{2+}-dependent phosphatidate phosphohydrolase activity is calculated by difference, and the resulting curves are shown in Figure 6. Results are averages from three separate experiments.

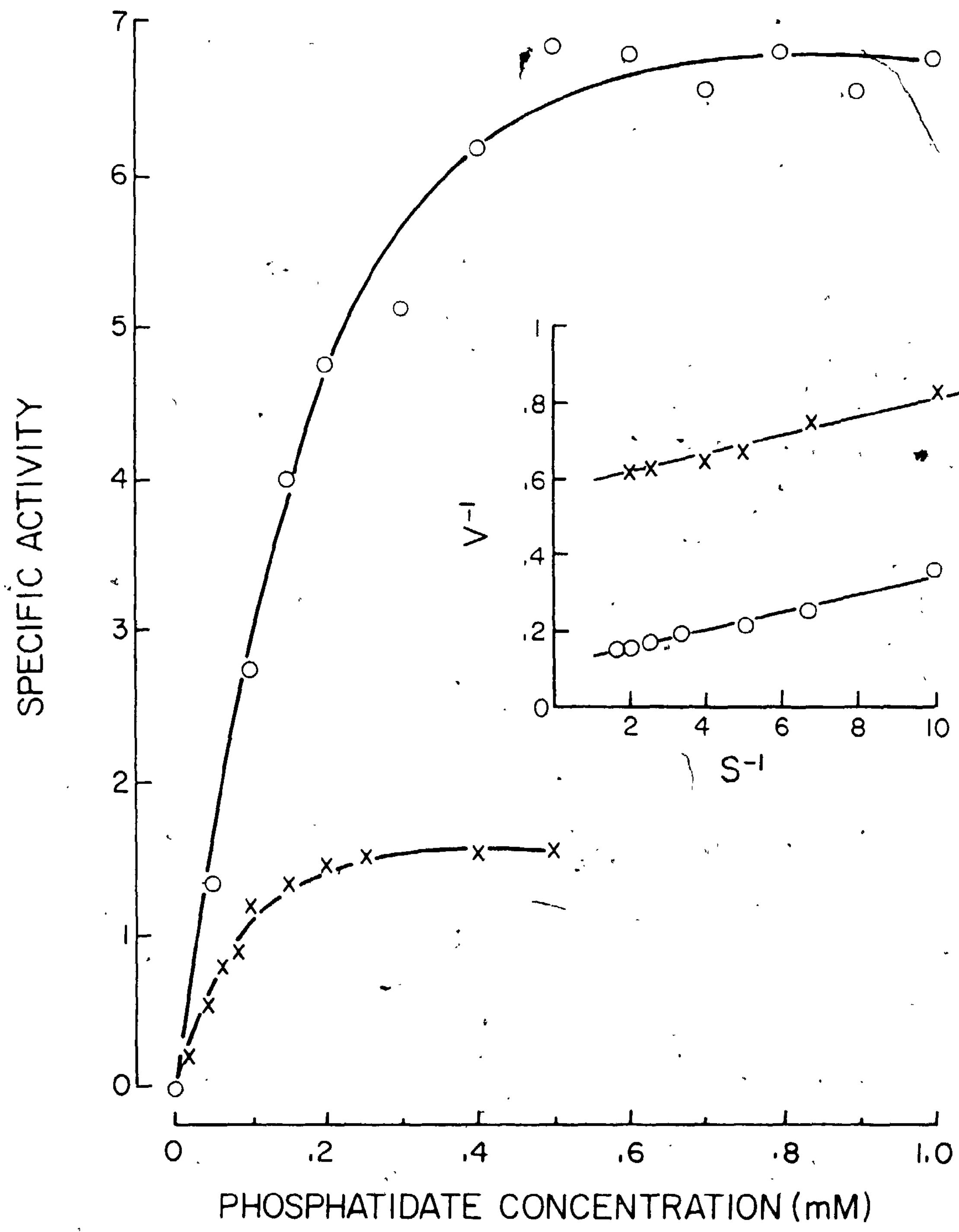

B
SPECIFIC ACTIVITY (nmol/min/mg protein)
PHOSPHATIDATE CONCENTRATION (mM)
0
1
2
3
4
5
6
7
8
9
.2
.4
.6
.8
1.0


A
SPECIFIC ACTIVITY (nmol/min/mg protein)
PHOSPHATIDATE CONCENTRATION (mM)
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
.1
.2
.3
.4
.5

activity in the presence of Mg^{2+}. Estimates of the Mg^{2+}-dependent phosphatidate phosphohydrolase activity were obtained by subtracting the activity remaining in the absense of Mg^{2+} from that measured under optimum Mg^{2+} concentration. Under these conditions, approximately hyperbolic curves were obtained from velocity vs. phosphatidate concentration experiments (Figure 6). The activity was inhibited by high phosphatidate concentrations. Such deviations are often encountered when the substrates are presented as insoluble particulate suspensions (Gatt and Barenholtz, 1973; Verger and de Haas, 1976; Gatt and Bartfai, 1977a,b). Double reciprocal plots of the velocity vs. substrate curves yielded apparent kinetic parameters of: K_m = 55 μM, V_{max} = 1.6 nmol/min/mg protein for the microsomal fraction, K_m = 215 μM, V_{max} = 6.8 nmol/min/mg protein for the cytosolic fraction. Such parameters are consistent with those reported for phosphatidate phosphohydrolase activity using membrane-bound substrate (Casola and Possmayer, 1981b; Mitchell _et al._, 1971; Jamdar and Fallon, 1973; Lamb _et al._, 1980). Kinetic parameters for K_m obtained using aqueously-dispersed substrate are approximately an order of magnitude higher and match those seen in this study for the Mg^{2+}-independent phosphatase (Casola and Possmayer, 1981b). As indicated previously, EDTA virtually abolished the cytosolic activity while the microsomal activity was reduced by 50%. Further studies revealed that the residual

Figure 6.

Velocity versus phosphatidate concentration curves and double reciprical plots (insert) for rat lung microsomes (X) and cytosol (O). Results are from three separate experiments.



SPECIFIC ACTIVITY
PHOSPHATIDATE CONCENTRATION (mM)
0
.2
.4
.6
.8
1.0
1
2
3
4
5
6
7
V^{-1}
S^{-1}
.2
.4
.6
.8
2
4
6
8
10

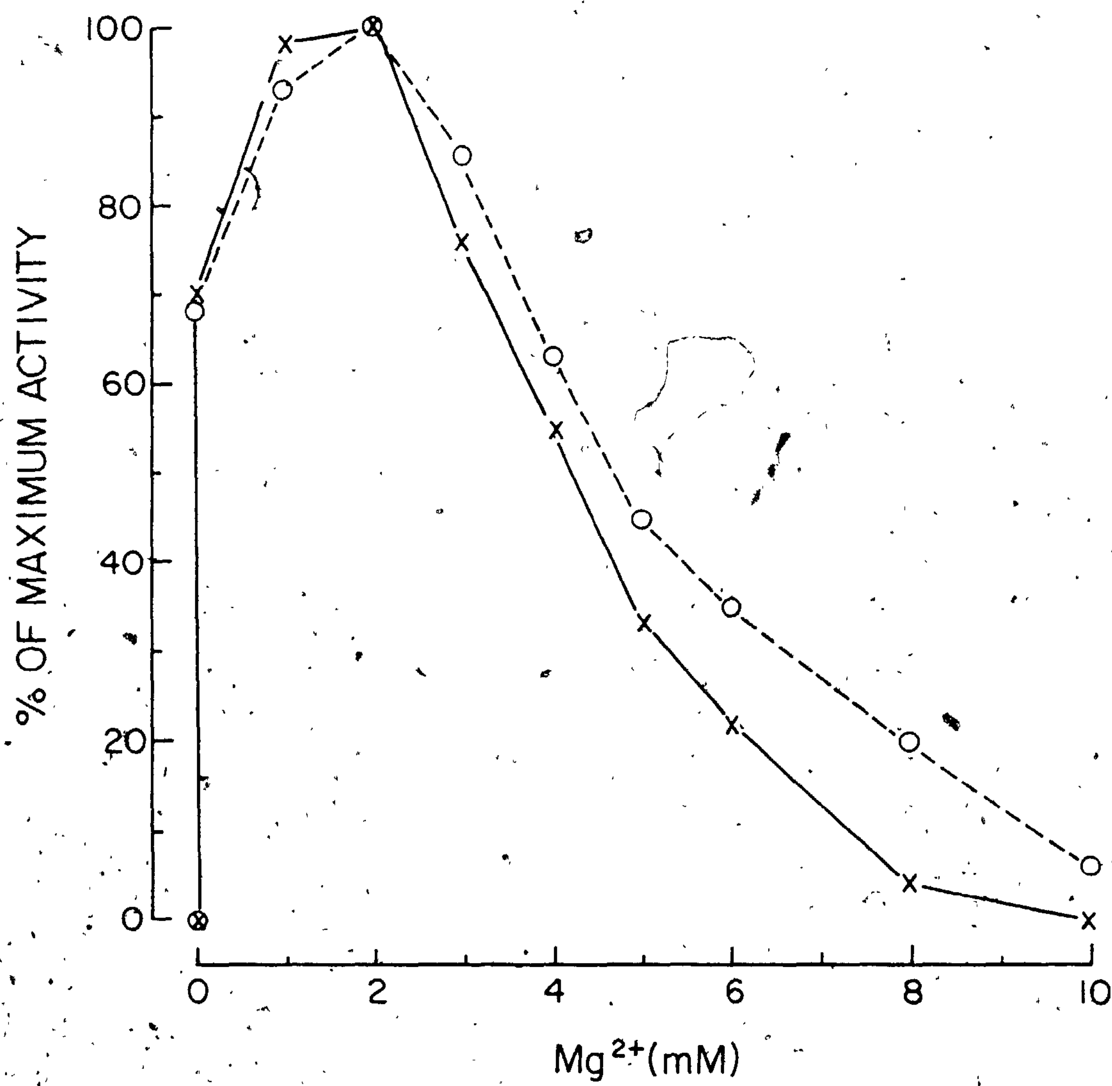
microsomal activity displays the same kinetic properties as the activity upon aqueously-dispersed substrate (Figure 5a).

The phosphohydrolase activity measured with PA:PC liposomes displayed a marked sensitivity to the concentration of Mg^{2+} in the assay mixtures (Figure 7). A free Mg^{2+} concentration of 2 mM gave the greatest stimulation and this final concentration was employed in the standard assay conditions. At higher Mg^{2+} concentrations activity was inhibited. By 10 mM Mg^{2+}, the activity was similar to the control incubations containing only 1.25 mM EDTA. The EDTA was included in the assay mixtures to preferentially chelate other divalent cations (Ca^{2+}, Mn^{2+}) shown to be inhibitory (Yeung et al., 1979).

Mg^{2+}-dependent phosphatidate phosphohydrolase activity from microsomes and cytosol displayed linearity with respect to time of incubation for at least twice the standard assay length of 15 minutes. The addition of microsomal protein showed a proportional decrease from linearity at the higher values. This may be a result of the microsomal phospholipids diluting the substrate, an occurrence seen in the PC:PA ratio experiments (Figure 4). Addition of cytosolic protein showed linearity up to 200 µg. To minimize these problems, assays were performed at a protein concentration of 70 µg microsomal or cytosolic protein per incubation.

Figure 7.

Effects of Mg^{2+} concentration on the Mg^{2+}-dependent phosphatidate phosphohydrolase activity from rat lung microsomes (X), and cytosol (O). Mg^{2+} concentration of point #2 is calculated to be 1.58 µM from the stability constants of MgPA and MgEDTA. Results are average values from three separate experiments.


100
80
60
40
20
0
% OF MAXIMUM ACTIVITY
0
2
4
6
8
10
Mg 2+(mM)

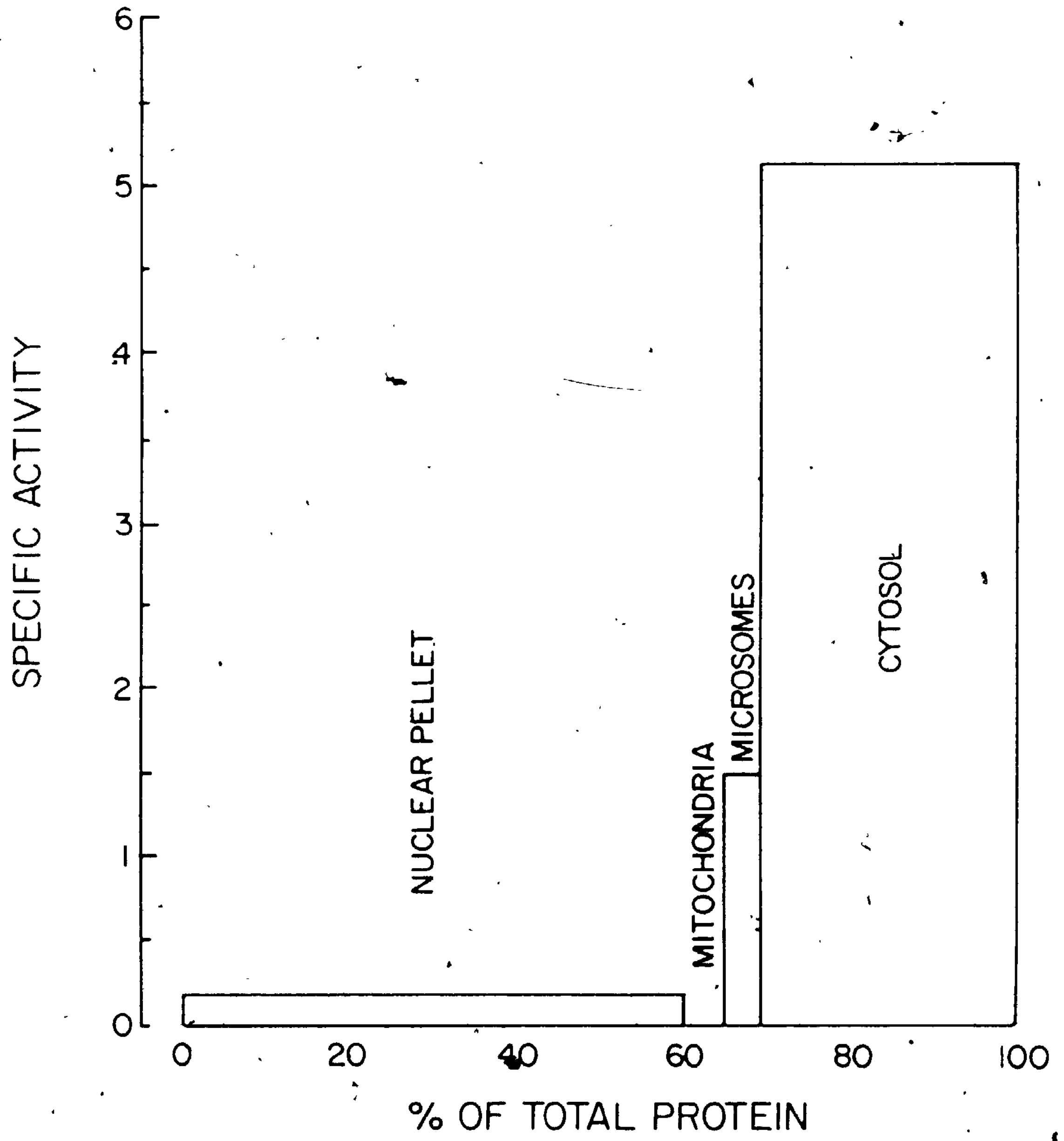
2.3.2 Comparison of the Mg^{2+}-dependent phosphatidate phosphohydrolase of rat lung with the activity acting upon membrane-bound phosphatidate

Mg^{2+}-dependent phosphatidate phosphohydrolase was found predominantly in the cytosolic fraction in amounts equalling approximately 90% of the total activity (Figure 8). The activity isolated in the microsomal fraction amounted to approximately 4% of the total, although the specific activity was some 30% of that found in cytosol. No Mg^{2+}-dependent phosphatidate phosphohydrolase activity was detected in the mitochondrial fraction in the five separate isolations. Mitochondria do, however, contain a phosphatase capable of hydrolysing substrate presented in this form, although this activity appeared to be inhibited by Mg^{2+}. The activity found in the nuclear pellet may be associated with incompletely homogenized cells. Possessing a high amount of connective tissue, lung is difficult to homogenize completely. These observations agree with earlier observations in our laboratory which demonstrated that although the Mg^{2+}-independent phosphatase activity was primarily membrane-associated, the bulk of the PA_{mb}-phosphohydrolase activity was located in the cytosol (Yeung *et al.*, 1979).

The pH profile showed a broad optimum between pH 6.5-8.0 (Figure 9). The microsomal optimum of 7.0 was slightly lower than the cytosolic optimum of 7.5. These values are consistent with previous reports (Yeung *et al.*, 1979;

Figure 8.

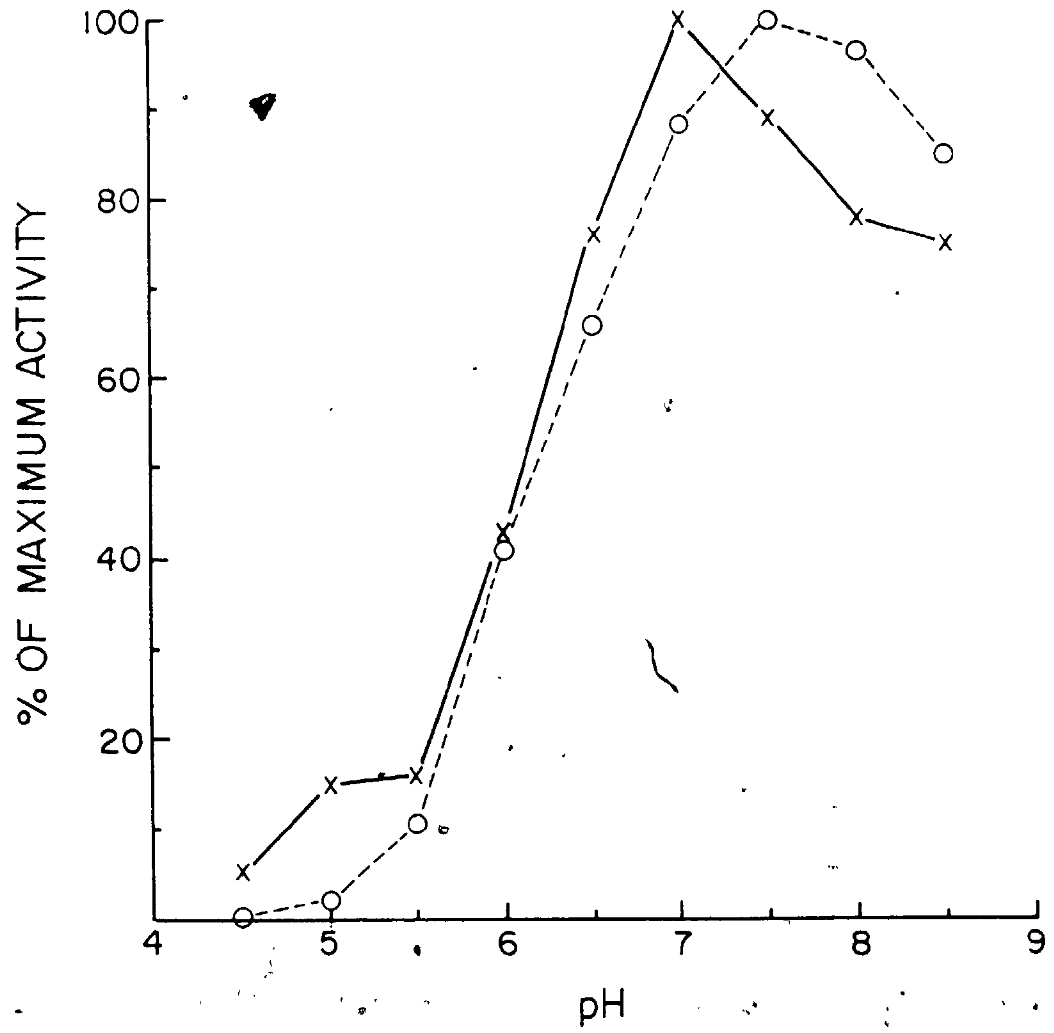
Subcellular distribution of Mg^{2+}-dependent phosphatidate phosphohydrolase from rat lung. Tissue was isolated and subcellular fractions prepared as reported in Materials and Methods. Results presented are of a typical isolation from five preparations.



SPECIFIC ACTIVITY
6
5
4
3
2
1
0
NUCLEAR PELLET
MITOCHONDRIA
MICROSOMES
CYTOSOL
0
20
40
60
80
100
% OF TOTAL PROTEIN

Figure 9.

The pH profile of Mg^{2+}-dependent phosphatidate phosphohydrolase activity for microsomes (X), and cytosol (O). Results are from duplicate experiments.



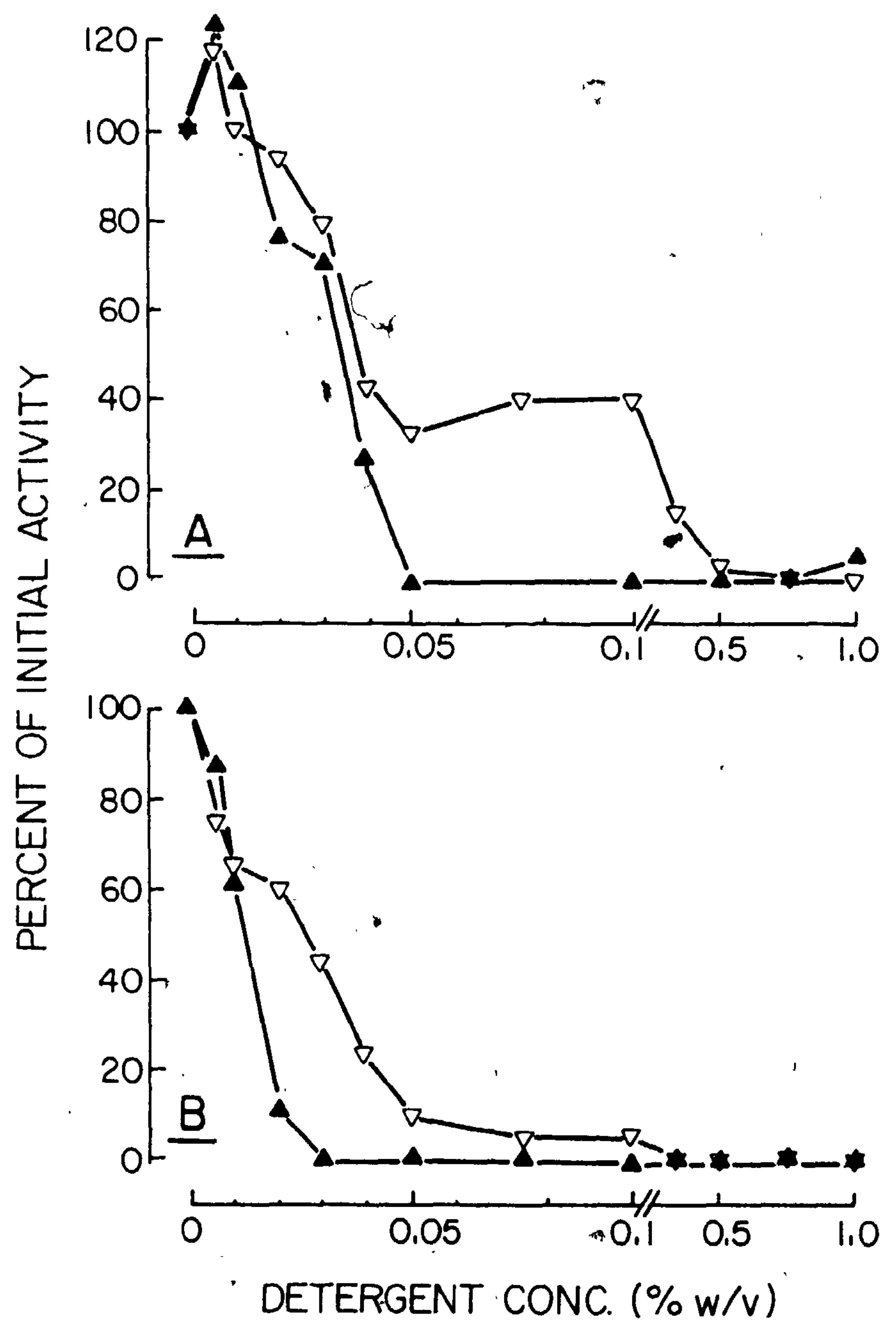
100
80
60
40
20
% OF MAXIMUM ACTIVITY
4
5
6
7
8
9
pH

Casola and Possmayer, 1979) which reported a higher pH optimum with membrane-bound substrate compared to liposomal phosphatidate.

The effects of the non-ionic detergent Triton X-100 and the anionic detergent sodium deoxycholate on the Mg^{2+}-dependent phosphatidate phosphohydrolase activity were examined over a broad range of detergent concentrations (Figure 10 a,b). Previous studies in our laboratory (Casola and Possmayer, 1981a,b) indicated that these two detergents strongly stimulated microsomal activity measured with membrane-bound substrate. Both detergents inhibited the Mg^{2+}-dependent phosphatidate phosphohydrolase activity between 0.01-0.05% (w:v). Complete inhibition of both microsomal and cytosolic activities occurred at detergent concentrations of greater than 0.05%. The phosphatase which is Mg^{2+}-independent was stimulated 11-fold by detergent concentrations in excess of 0.25% (results not shown). It may be that the increase in activity reported using membrane-bound substrate is the stimulation of the Mg^{2+}-independent phosphatase activity found in microsomes and cytosol. The range of 0.01-0.05% corresponds to a ratio of one milligram detergent per milligram phospholipid. It is in this range that solubilization of the liposomes occurs, resulting in the formation of mixed lipid-detergent micelles (Helenius <u>et al.</u>, 1979). At this time it cannot be resolved whether these micelles are an unsuitable substrate for the Mg^{2+}-dependent phosphatidate phosphohydrolase, or the enzyme itself is being

Figure 10.

Effects of detergents Triton X-100 (Figure 10a) and sodium deoxycholate (Figure 10b) on the Mg^{2+}-dependent phosphatidate phosphohydrolase activity from rat lung microsomes (closed symbols) and cytosol (open symbols). Results are averages from two separate experiments.



PERCENT OF INITIAL ACTIVITY
120
100
80
60
40
20
0
A
0
0.05
0.1
0.5
1.0
100
80
60
40
20
0
B
0
0.05
0.1
0.5
1.0
DETERGENT CONC. (% w/v)

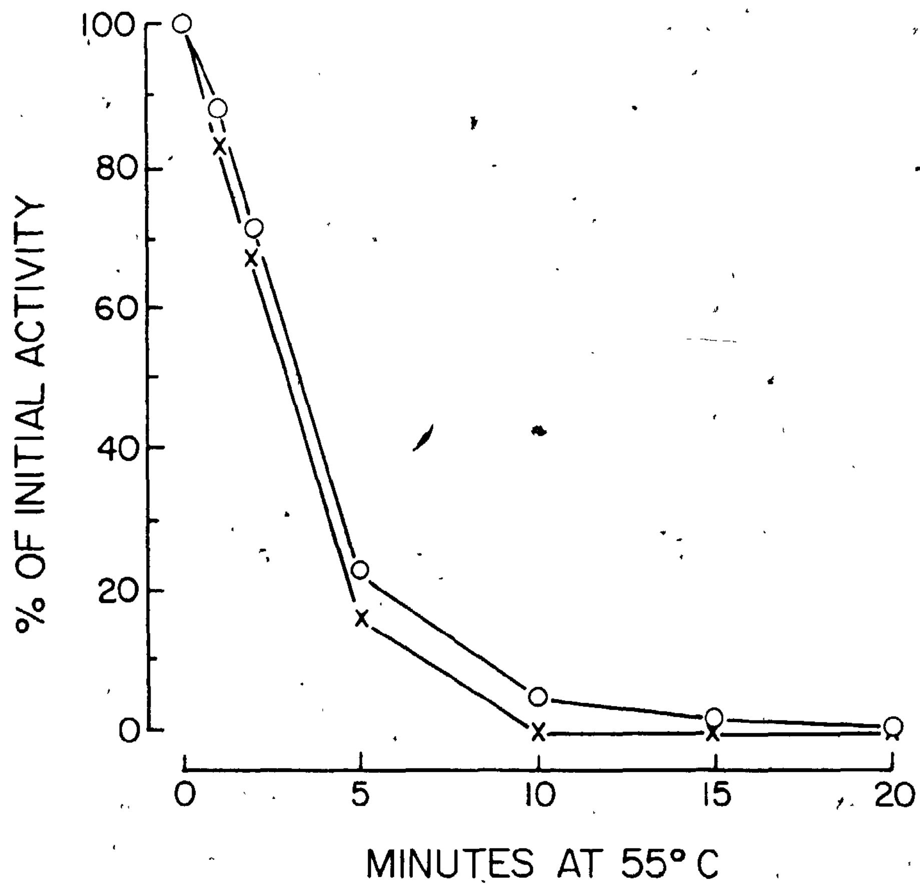
inactivated.

Previous work on the characterization of the phosphatidate phosphohydrolase activity on membrane-bound substrate has shown that the activity is highly susceptible to thermal inactivation; this is in contrast to the activity measured using aqueously-dispersed substrate, which is insensitive to prolonged incubations at 55°C (Casola and Possmayer, 1981). In order to confirm that the Mg^{2+}-dependent phosphatidate phosphohydrolase activity and the activity measured using membrane-bound substrate are equivalent, we examined the thermal stability profiles from both microsomes and cytosol (Figure 11). The half-life of 3 minutes at 55°C is consistent with that observed for the activity using membrane-bound substrate; the activities from both cell fractions were equally susceptible to heating. Thus the microsomal activity appeared to derive no additional stability from its association with the microsomal membrane.

Based on the results presented, the Mg^{2+}-dependent phosphatidate phosphohydrolase activity measured with liposomes containing equimolar amounts of phosphatidate and phosphatidylcholine is representative of that activity measured using membrane-bound substrate on the basis of pH optima, sensitivity to thermal denaturation and detergents, and kinetic parameters. Furthermore, this assay allows discrimination of a Mg^{2+}-independent phosphatase activity which was shown recently not to play a role in the

Figure 11.

Thermal inactivation profile of Mg^{2+}-dependent phosphatidate phosphohydrolase activity from rat lung microsomes (X), and cytosol (O). Results are averages from two separate experiments.


100
80
60
40
20
0
% OF INITIAL ACTIVITY
0
5
10
15
20
MINUTES AT 55° C

synthesis of pulmonary glycerolipids (Walton and Possmayer, 1984). Perhaps because of the difficulty in preparing and characterizing the substrate, studies using PA_{mb} are highly limited. The replacement of the microsomal substrate with a chemically-defined PC/PA liposome system should facilitate future studies on the control of this enzyme.

CHAPTER 3

THE ROLE OF Mg^{2+}-DEPENDENT PHOSPHATIDATE PHOSPHOHYDROLASE IN PULMONARY GLYCEROLIPID BIOSYNTHESIS

3.1 INTRODUCTION

Phosphatidate phosphohydrolase (EC 3.1.3.4), the enzyme which catalyses the conversion of phosphatidic acid to 1,2-diacylglycerol, occupies a central position in the glycerolipid pathway (Bell and Coleman, 1980). Phosphatidic acid, the substrate for this enzyme, may be used in the synthesis of diacylglycerol or the acidic phospholipids. Its product, diacylglycerol, is the immediate precursor for the synthesis of either neutral lipids or the nitrogen-containing phospholipids, phosphatidylcholine and phosphatidylethanolamine (PE). Phosphatidate phosphohydrolase has been observed in the microsomal and in the cytosolic fractions from rat (Casola et al., 1978; Mavis et al., 1978; Ravinuthala et al., 1978; Casola and Possmayer, 1979; Maniscalco et al., 1978; Yeung et al., 1979) and rabbit lung (Schultz et al., 1974; Brehier et al., 1977; Ballard et al., 1977). In addition, phosphatidate phosphohydrolase has been detected in lamellar bodies (Mavis et al., 1978; Spitzer et al., 1975; Johnston et al., 1978) and amniotic fluid (Jimenez et al., 1974; Jimenez et al., 1975; Jimenez and Johnston, 1976; Bleasdale et al, 1978). In these experiments and in most of the previous studies on pulmonary phosphatidate phosphohydrolase, the substrate was presented as an aqueous

dispersion of phosphatidic acid (Ravinuthala et al., 1979; Maniscalco et al., 1978; Schultz et al. 1974; Brehier et al., 1977; Filler and Rhoades, 1982). Studies by Casola and coworkers (Casola and Possmayer, 1979; Yeung et al., 1979; Casola and Possmayer, 1981a,b) revealed that, in addition to the phosphohydrolase activity directed towards aqueously-dispersed phosphatidate, lung microsomes and cytosol contain an apparently distinct enzymatic activity capable of hydrolyzing membrane-bound phosphatidic acid. The cytosolic activities could be separated by gel chromatography. Differences between the activities acting on membrane-associated and aqueously-dispersed phosphatidic acid were noted during pulmonary development and after glucocorticoid treatment (Possmayer et al., 1979). Although these authors argued that membrane-bound phosphatidic acid more likely represents the biosynthetic substrate, it was not possible to determine whether the activity observed with membrane-bound substrate or that observed with aqueous dispersions of phosphatidic acid functions in glycerolipid synthesis.

Further experiments indicated that the activities observed with membrane-bound substrate may be representative of the Mg^{2+}-dependent activities (Smith et al., 1967; Mitchell et al., 1971; Jamdar and Fallon, 1973; Lamb and Fallon, 1974; Moller et al., 1977). The Mg^{2+}-dependent phosphatidate phosphohydrolase activities of liver (Brindley, 1978) and adipose tissue (Cheng and

Saggerson, 1978a,b; Moller *et al.*, 1981; Jamdar and Osborne, 1983) also show characteristics similar to the activities observed with membrane-bound phosphatidic acid, presumably because the substrate is produced in the presence of Mg^{2+}. In these latter tissues, changes in glycerolipid metabolism are reflected by alterations in the Mg^{2+}-dependent cytosolic activity. However, decisive experiments which unequivocally demonstrate that either the Mg^{2+}-dependent or the Mg^{2+}-independent phosphohydrolase activities are involved in glycerolipid synthesis have not been reported for any tissue. The present studies were undertaken to resolve this question.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Phosphatidylcholine was isolated and purified from chicken yolks by the method of Ansell and Hawthorne (1964). Phosphatidic acid was prepared from phosphatidylcholine via the action of phospholipase D (EC 3.1.4.4) by the method of Kates and Sastry (1969), and converted to the disodium salt by passage through a Chelex 100 column (Bio-Rad) (Renkonen, 1968). The method of Pieringer and Kunnes (1965) was used to prepare [^{32}P]phosphatidic acid from diolein (Serdary Research Laboratories, London) and [γ^{32}P]ATP (New England Nuclear, Boston). The labelled lipid was isolated using a TLC system of oxalate-treated plates and a solvent of petroleum ether/acetone/formic acid (154:46:0.5, v/v) (Yeung *et al.*, 1979). [U-^{14}C]-glycerol-3-phosphate was

purchased from NEN. Biochemicals were purchased from Sigma (St. Louis) and reagent grade chemicals obtained from Fisher Scientific (Toronto). Whatman thin-layer chromatography plates were obtained from Terochem (Toronto).

3.2.2 Subcellular fractions

Microsomes were isolated from the lungs of 200-300 g male Sprague-Dawley rats. Animals were killed by decapitation or chloroform suffocation, the thorax was opened and the lungs perfused with ice-cold saline via the right ventricle with the left atrium bisected to provide drainage. Once the lungs were white, they were excised, trimmed of the large vessels and airways, weighed and chopped with a McIlwain tissue chopper (1 mm^3). The tissue was homogenized in 9 volumes of ice-cold buffer containing 0.25 M sucrose, 0.1 mM EDTA, 1.0 mM Hepes (pH 7.4) (hereafter referred to as buffered sucrose). The homogenate was serially centrifuged at 1450 x g for 5 minutes, 10 000 x g for 15 minutes, and 100 000 x g for 60 minutes, the final pellet being the microsomes and the final supernatant being the cytosolic fraction. For salt-washing, the microsomes were resuspended in buffered sucrose of appropriate NaCl concentration in a glass homogenizer and homogenized with a Teflon pestle by hand. Microsomes were isolated by centrifugation as above and resuspended in buffered sucrose without NaCl at a protein

concentration of 3-6 mg/ml. The microsomes were snap-frozen at -80°C. Protein was measured by a modification of the Lowry procedure (Hendry and Possmayer, 1974) using bovine serum albumin as the standard.

3.2.3 Enzyme Assays

The [^{14}C]-glycerol-3-phosphate incorporation assay was based on that described by Germershausen et al. (1980), and contained 22.5 mM Tris-HCl (pH 7.0), 0.1 mM potassium oleate, 8 mM $MgCl_2$, 4 mM ATP, 0.1 mM CoA, 1.25 mM dithiothreitol and 2 mM [U-^{14}C]-glycerol-3-phosphate (1mCi/mmol) in a final volume of 0.2 ml. The reaction was initiated by the addition of 120 µg of microsomal protein and incubated at 25°C for 20 minutes. The reaction was terminated by the method of Bligh and Dyer (1959) with the addition of 10 ml of chloroform/methanol (1:1, v/v) and the phases separated with 5 ml of 0.1 M HCl. The chloroform phase was washed, concentrated under a stream of nitrogen, and the reaction products were isolated along with pure marker compounds on oxalate-treated TLC plates developed in petroleum ether/acetone/formic acid (160:40:0.5, v/v). Lipids were visualized with a light spraying of Rhodamine 6-G, the bands scraped into scintillation vials, and the radioactivity was measured using ACS scintillant.

Mg^{2+}-independent phosphatidate phosphohydrolase activity was assayed by the method of Casola and Possmayer (1981a), using aqueously-dispersed substrate. Mg^{2+}-dependent phosphatidate phosphohydrolase was measured using

mixed-lipid vesicles containing equimolar amounts of phosphatidylcholine and phosphatidic acid. Chemically-defined liposomes were formed by sonication in 0.9% NaCl with a Biosonic Bronwill sonicator fitted with a micro-probe. Reaction mixtures contained 50 mM Hepes buffer (pH 7.4), 0.2 mM phosphatidic acid (0.9 mCi/mmol), 0.2 mM PC, 1.25 mM EDTA and 5-20 µg of wash supernatant protein or 50-150 µg of microsomal or cytosolic protein in a final volume of 0.1 ml. Assays were performed in the presence and absence of 6 mM $MgCl_2$ and the Mg^{2+}-dependent activity was calculated by difference. Reactions were stopped by the addition of 1.5 ml of chloroform/methanol (5:4, v/v). The phases were broken with 0.75 ml of 0.1 M HCl and a sample of the upper aqueous phase was taken for scintillation counting to measure [^{32}P]-inorganic phosphate release as a measure of enzymatic activity.

Phosphatidylcholine formation from [^{14}C]-glycerol-3-phosphate was measured by an adaptation of the method of Fox and Zilversmit (1982). Microsomes were preincubated for 10 minutes at 37°C in 50 mM Hepes (pH 7.0), 5 mM diisopropylfluorophosphate, 5 mM $MgCl_2$, 0.1 mM EGTA, 3.5 mM ATP, 0.17 mM CoA, 10 mM dithiothreitol, 0.33 mM CDPcholine, 0.13 mM fatty acid (palmitate and oleate) and 1.33 mg/ml bovine serum albumin. At time zero, [^{14}C]-glycerol-3-phosphate (0.16 mCi/mmol) was added to a concentration of 4 mM. The final reaction volume was 0.6 ml. The reactants were incubated for 45 minutes at 37°C and the reaction

terminated by the addition of 4 ml of chloroform/methanol (1:1, v/v). The products were isolated using the two-step TLC system of Ide and Weinhold (1983). The plate was first developed to one half its length in chloroform/methanol/NH_4OH/H_2O (65:30:5:1, v/v), dried for 1 h under a stream of nitrogen and developed to its full length in petroleum ether/diethyl ether/acetic acid (50:50:1, v/v). Lipid products were visualized with Rhodamine 6-G, scraped into vials and the radioactivity was determined in NEN scintillant.

Cholinephosphotransferase (EC 2.7.8.2) activity in microsomes was measured as previously described (Possmayer et al., 1977). Gel filtration of rat lung cytosol was performed by the method of Casola and Possmayer (1981a), except that a 2.5 x 76 cm column of Sephacryl S-400, at a flow rate of 30 ml/h, was employed.

3.2.4 Statistical analysis

Analysis of variance was employed to measure difference between treatments with the Scheffe Multiple Range test use to evaluate difference between pairs. The Pearson Product-Moment Coefficient was employed to determine the coefficient of determination between the formation of neutral lipid and the Mg^{2+}-dependent phosphatidate phosphohydrolase activity.

3.3 RESULTS

3.3.1 Effects of saline washing of microsomes on the lipid products of glycerol-3-phosphate

Washing rat lung microsomes in buffered sucrose with the addition of NaCl at various concentrations was shown to alter the location of microsomal protein (Table I). Approximately 20% of the microsomally-associated protein was removed by washing in buffered sucrose and this increased to 35% with the addition of 1.0 M NaCl. Because the changes in protein would change the specific activities, the results were expressed as activity per g lung tissue.

When these microsomes were assayed for the ability to make neutral lipids from the radioactive precursor, [^{14}C]-glycerol-3-phosphate, an alteration in the relative amounts of the products was observed (Figure 12). Microsomes washed in increasing concentrations of NaCl demonstrated a sigmoidal increase in the radiolabelling of phosphatidic acid, with a decreased labelling of neutral lipid. In microsomes washed with greater than 0.4 M NaCl, phosphatidic acid accounted for 90% of the products, compared with 64% in the case of unwashed microsomes.

3.3.2 Effects of saline washing of microsomes on phosphatidic acid phosphohydrolase activities

In order to determine which, if either, of the two phosphatidate phosphohydrolase activities could account for the increased labelling of phosphatidic acid relative to the neutral lipid levels, assays of the Mg^{2+}-independent

TABLE I

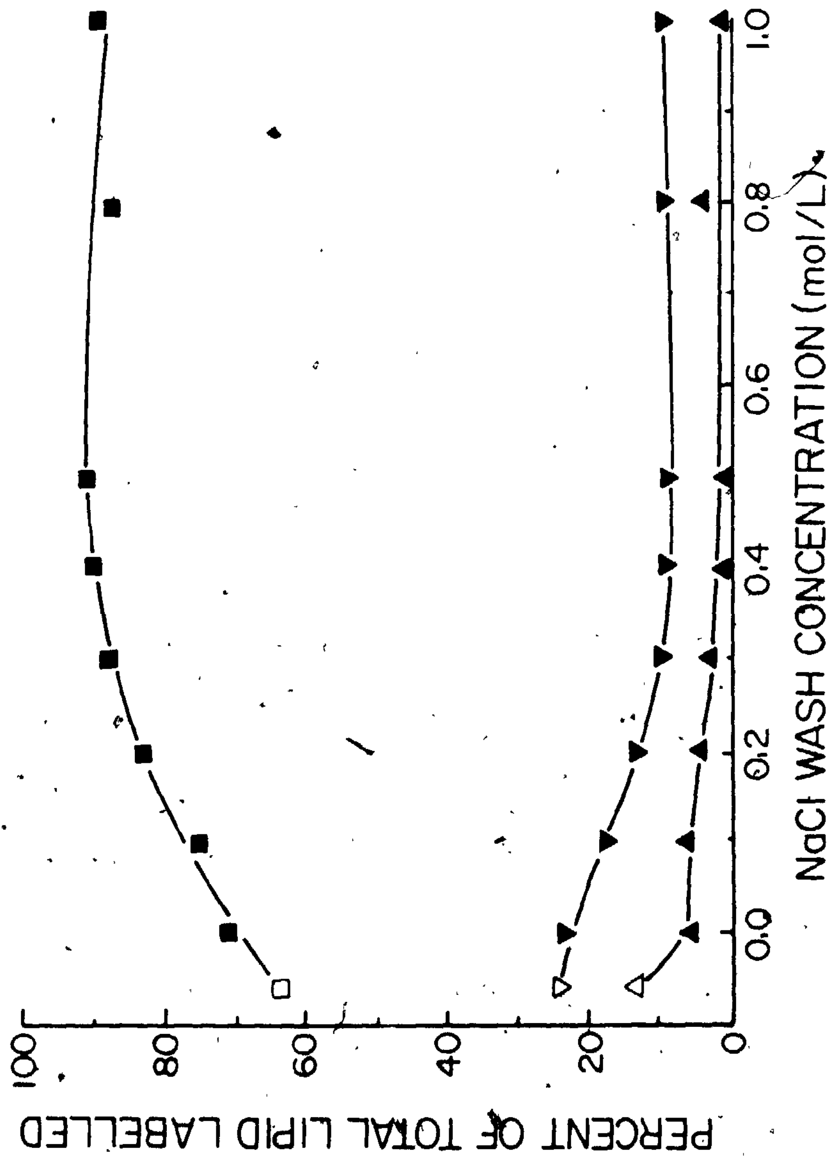
PROTEIN CONTENT OF MICROSOMES AND WASH SUPERNATANTS FOLLOWING WASHING IN BUFFERED SUCROSE AT INDICATED NaCl CONCENTRATIONS

Results from a typical isolation are shown.

Wash NaCl concn. (mol/l)	Protein content				
	Microsomes		Wash supernatant		Total
	mg/g lung	% of total	mg/g lung	% of total	mg/g lung
0.0	3.85	79	1.04	21	4.89
0.1	3.98	77	1.22	23	5.20
0.2	3.75	71	1.50	29	5.25
0.5	3.41	66	1.76	34	5.17
1.0	3.03	64	1.73	36	4.76

Figure 12.

Effects of washing microsomes with NaCl on the radioactive profile of the lipids produced from [^{14}C]-glycerol-3-phosphate. Isolated microsomes (open symbols) were washed in buffered sucrose of indicated NaCl concentrations. Reisolated microsomes (closed symbols) were assayed for the production of labelled glycerolipids as described in Materials and Methods. Results expressed as percent of total lipid labelled as either phosphatidic acid (■), diacylglycerol (▼) or triacylglycerol (▲).


PERCENT OF TOTAL LIPID LABELLED
100
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
1.0
NaCl WASH CONCENTRATION (mol/L)

and Mg^{2+}-dependent phosphatidic acid phosphohydrolase activities were undertaken. The results (Figure 13 a,b) demonstrate that the Mg^{2+}-independent phosphatidic acid phosphohydrolase activity was unaffected by the washing in NaCl solutions up to 0.5 M. An increase of approximately 20% was observed in microsomes washed in 1.0 M NaCl. The reason for the increase is not known, but could be related to the removal of peripheral proteins from the microsomal surface, thus allowing easier access to the substrate.

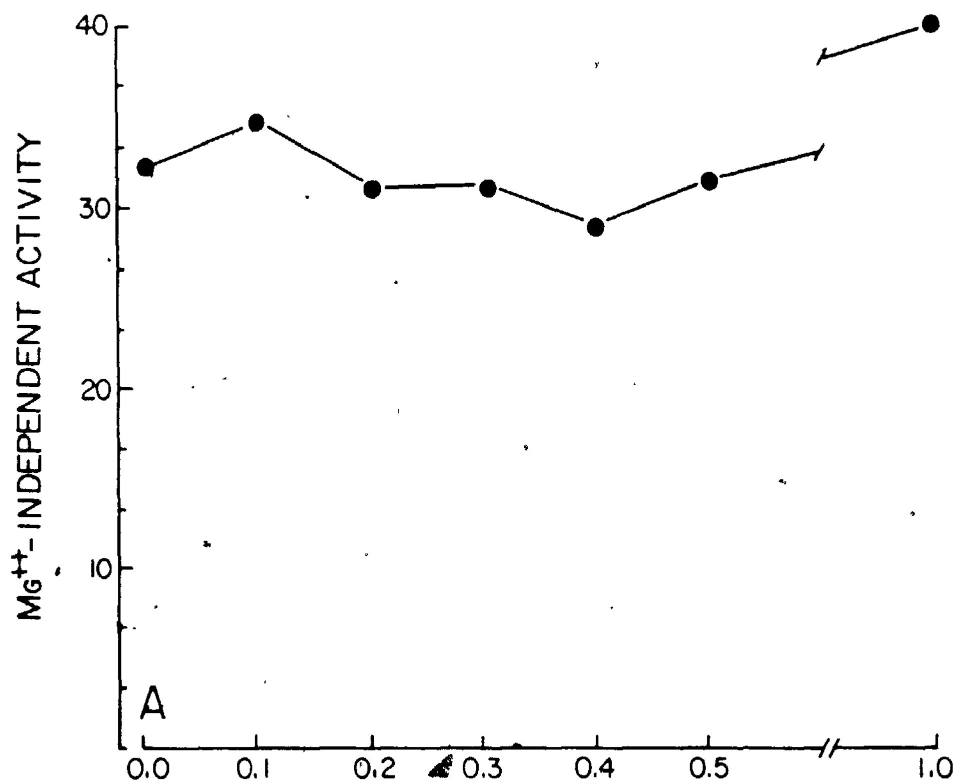
In contrast, the Mg^{2+}-dependent phosphatidic acid phosphohydrolase activity decreased in a sigmoidal manner with increasing concentrations of salt in the washes. At a salt concentration of 0.2 M, half of the Mg^{2+}-dependent phosphatidic acid phosphohydrolase activity remained and, by 1.0 M NaCl, little detectable activity remained on the microsomes. The major decrease in the Mg^{2+}-dependent phosphatidic acid phosphohydrolase activity occurred between 0.1 and 0.3 M NaCl, the salt concentrations at which the greatest increase in the relative labelling of phosphatidic acid from [^{14}C]-glycerol-3-phosphate was observed (Figure 12).

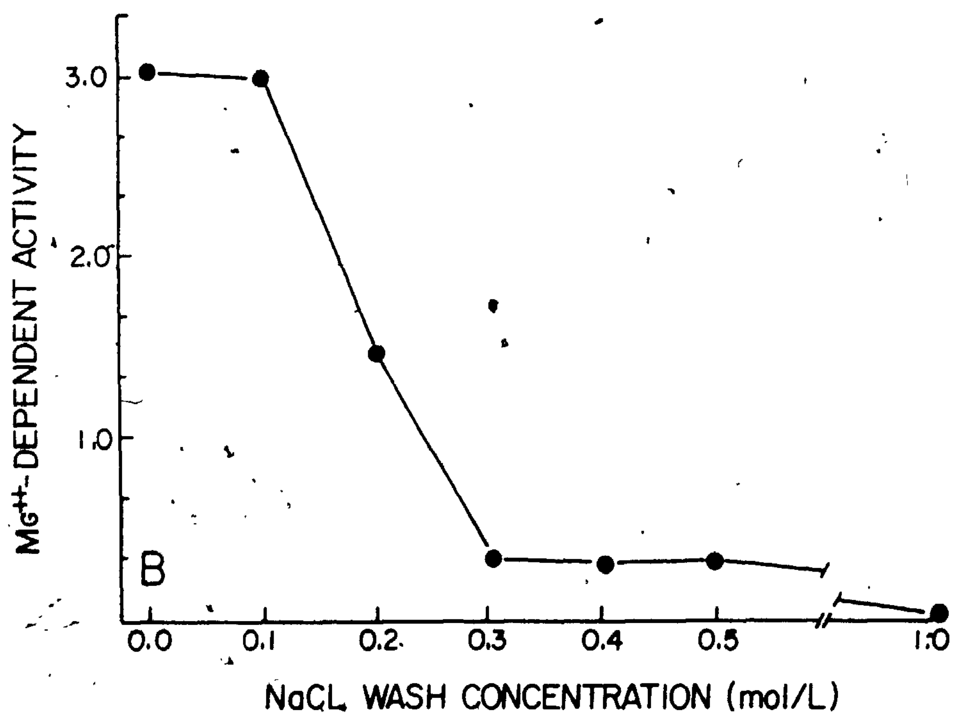
The next series of experiments were undertaken to determine whether the Mg^{2+}-dependent phosphatidate phosphohydrolase activity removed from the microsomes could be detected in the wash supernatants. Wash concentrations of 0.05 M and 0.50 M NaCl were chosen. Low salt is reported (Moller and Hough, 1982) to assist in the binding

Figure 13.

Mg^{2+}-independent (A), and Mg^{2+}-dependent (B) phosphatidate phosphohydrolase activity in microsomes, following NaCl washing. Microsomes were washed in buffered sucrose with the addition of NaCl at the indicated concentrations and reisolated. The phosphatidate phosphohydrolase activity was measured using the systems to quantify the Mg^{2+}-independent and Mg^{2+}-dependent activities as indicated in Materials and Methods. Results are expressed as nmol P_i released/minute per gram lung.

PHOSPHATIDATE PHOSPHOHYDROLASE





of Mg^{2+}-dependent phosphatidate phosphohydrolase to the microsomal membrane. High salt was shown in the previous experiment to remove over 90% of this activity. The Mg^{2+}-dependent phosphatidate phosphohydrolase activity was assayed in microsomes and wash supernatants to determine whether the lost microsomal activity was demonstrable in the wash supernatents, and whether the recovery was quantitative. The distribution of these activities (Table IIa) show that the bulk of the Mg^{2+}-dependent activity (85%) remained associated with the microsomes when washed with 0.05 M NaCl, but was translocated to the wash supernatant (81%) when the NaCl concentration was raised to 0.50 M. The sum of the activities contained in the microsomes plus the wash supernatants was essentially the same in both treatments ($P > 0.25$), suggesting that the Mg^{2+}-dependent phosphatidate phosphohydrolase activity could be quantitatively assayed irrespective of location. The presence of the Mg^{2+}-dependent phosphatidate phosphohydrolase activity in the wash supernatant is indicative of an intact enzyme and not of the removal of a factor that confers activity or Mg^{2+}-dependency to an integral protein. Essentially all of the Mg^{2+}-independent phosphatidate phosphohydrolase activity remained associated with the microsomes at both NaCl concentrations (Table IIb).

TABLE II

DISTRIBUTION OF Mg^{2+}-DEPENDENT AND Mg^{2+}-INDEPENDENT PHOSPHATIDATE PHOSPHOHYDROLASE ACTIVITIES BETWEEN MICROSOMES AND WASH SUPERNATANTS

Freshly isolated microsomes were washed in low-salt (50 mM) or high-salt (500 mM) isolation buffers. Microsomes were reisolated by centrifugation, and microsomes and wash supernatants were assayed for A, Mg^{2+}-dependent, or B, Mg^{2+}-independent phosphatidate phosphohydrolase activity as described in the Materials and Methods. Results are from a typical isolation and are given as nmol/min per g lung.

NaCl concn. of washes (mM)	Microsomes	Wash Supernatants
A. Mg^{2+}-dep. phosphatidate phosphohydrolase		
50	13.42 ± 0.32	2.45 ± 0.26
500	2.74 ± 0.37	[illegible].93 $\pm$ 1.17
B. Mg^{2+}-indep. phosphatidate phosphohydrolase		
50	61.41 ± 2.89	2.80 ± 0.10
500	76.05 ± 2.73	6.30 ± 0.06

3.3.3 Phosphatidylcholine biosynthesis in saline-washed microsomes

The ability of the 0.50 M NaCl-washed microsomes to produce phosphatidylcholine from [^{14}C]-glycerol-3-phosphate was measured using the 0.05 M NaCl-washed microsomes as a control (Table IIIa). Statistically significant differences existed between the two treatments. Phosphatidylcholine synthesis decreased by 60% and the neutral lipids by 42% in the 0.50 M NaCl-washed microsomes. The accumulation of labelled phosphatidic acid, the level of which increased by 84%, was indicative of a block at the level of phosphatidate phosphohydrolase. To determine whether the decrease in phosphatidylcholine synthesis could be explained by a decrease in the cholinephosphotransferase activity, this activity was measured as indicated in Materials and Methods. No significant difference was observed between microsomes washed with 0.05 and 0.50 M NaCl (525 ± 19 vs. 487 ± 10 nmol/min per gram lung). The slight decrease may be due to an incomplete recovery of the microsomes by centrifugation.

Since the decrease in the Mg^{2+}-dependent phosphatidate phosphohydrolase is the only apparent cause for the alterations in the neutral lipid and phosphatidylcholine synthesis, studies were undertaken to determine whether the addition of isolated Mg^{2+}-dependent phosphatidate phosphohydrolase could restore the normal product profile to the 0.50 M NaCl-washed microsomes. When rat lung cytosol was passed through a gel filtration column, three

2

MICROCOPY RESOLUTION TEST CHART
NBS 1010a
ANSI and ISO TEST CHART No 2

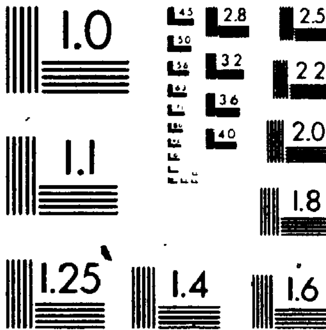


TABLE III

EFFECT OF VARIOUS FRACTIONS IN THE RESTORATION OF THE SYNTHESIS OF LABELLED GLYCEROLIPIDS

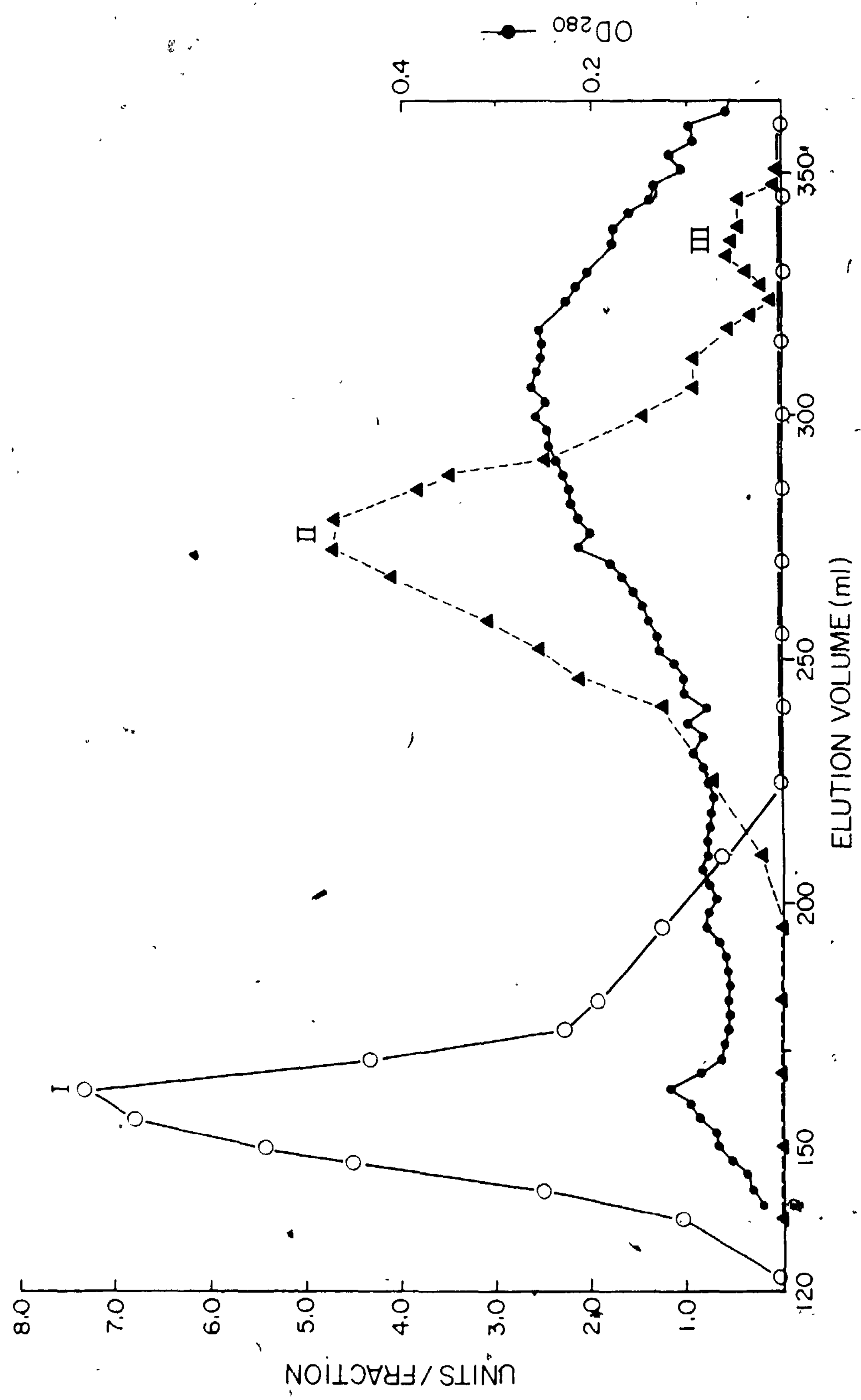
Control microsomes (microsomes 50), salt-washed microsomes (microsomes 500) and salt-washed microsomes plus various fractions were assayed for the production of labelled products from [^{14}C]glycerol 3-phosphate fatty acids, and CDPcholine as described in Materials and Methods. Results are expressed as specific activity ($nmol \cdot min^{-1} \cdot mg^{-1}$) and as the percent of total glycerolipids labelled. Results are expressed as means and S.E. of four determinations except for peaks I-III which are from two determinations. PA, phosphatidic acid; NL, neutral lipids.

Condition	Products labelled						
	PA		NL		PC		Total
	spec. act.	% of total	spec. act.	% of total	spec. act.	% of total	spec. act.
A. Microsomes (50)	0.48 ± 0.09	47	0.33 ± 0.01	32	0.22 ± 0.07	21	1.03 ± 0.18
Microsomes (500)	0.90 ± 0.13	76	0.19 ± 0.05	16	0.09 ± 0.02	08	1.18 ± 0.20
B. Microsomes (500)							
+ Cytosol	0.41 ± 0.10	27	0.72 ± 0.01	48	0.37 ± 0.02	25	1.49 ± 0.12
+ Wash supernatant	0.74 ± 0.02	45	0.64 ± 0.04	39	0.26 ± 0.01	16	1.64 ± 0.07
+ Peak I	1.19 ± 0.35	73	0.31 ± 0.02	18	0.14 ± 0.02	09	1.64 ± 0.39
+ Peak II	1.10 ± 0.04	55	0.62 ± 0.05	31	0.27 ± 0.04	14	1.99 ± 0.13
+ Peak III	1.55 ± 0.15	72	0.39 ± 0.06	18	0.22 ± 0.02	10	2.16 ± 0.23

major peaks of phosphatidate phosphohydrolase activity were recovered (Figure 14). These include a Mg^{2+}-independent peak at the void volume ($M_r > 8 \times 10^6$), followed by two Mg^{2+}-dependent peaks, the first at 275 ml ($M_r \approx 4 \times 10^5$), the other at 335 ml ($M_r \approx 1 \times 10^5$). Of these two Mg^{2+}-dependent peaks, the first accounted for 95% of the total activity, the second for only 5%. To determine whether these activities could stimulate phosphatidylcholine production, salt-washed microsomes were incubated with either cytosol, wash supernatant, or each of the three peaks. Microsomes isolated from 80 mg of lung were incubated with cytosolic activity recovered from 40 mg of lung in the case of peaks I and II, and 80 mg of lung in the case of peak III. Table XIIb shows that the decrease in phosphatidylcholine production seen in 0.50 M NaCl-washed microsomes can be reversed by cytosol, wash supernatant or peak II, all of which contain the Mg^{2+}-dependent phosphatidate phosphohydrolase activity. Addition of protein from peak I, the Mg^{2+}-independent phosphatidate phosphohydrolase activity or the minor Mg^{2+}-dependent peak III had no effect on the relative labelling of phosphatidic acid, neutral lipids or phosphatidylcholine. It was observed, however, that the addition of protein from cytosol, wash supernatant or cytosolic peaks I-III increased the overall incorporation of glycerol-3-phosphate into the total lipids. This might be expected in the case of wash supernatant or peak II, should the accumulation of phosphatidate on the microsomal

Figure 14.

Separation of cytosolic Mg^{2+}-independent and Mg^{2+}-dependent phosphatidate phosphohydrolase activities using a Sephacryl S-400 gel filtration column. 85 mg of cytosolic protein was brought to 55% saturation (320 g/l) with ammonium sulfate. Precipitated proteins were isolated by centrifugation at 12 000 x g for 20 minutes and resuspended in 5 ml of buffer containing 50 mM Tricine-KOH (pH 7.4), 0.1 M KCl, 2 mM 2-mercaptoethanol and 0.02% sodium azide. Resuspended proteins were applied to the column and eluded at a flow rate of 30 ml/h. 3.0-ml fractions were collected. Results are expressed as nmol P_i released/minute per 3 ml fraction for the Mg^{2+}-independent (○) and Mg^{2+}-dependent (▲) phosphatidate phosphohydrolase activity.



UNITS / FRACTION
ELUTION VOLUME (ml)
OD_{280}
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
120
150
200
250
300
350
0.4
0.2
I
II
III

membrane act to regulate the production of more phosphatidate. However, Lawson et al. (1982b) have shown that the addition of semi-purified phosphatidate phosphohydrolase from rat liver did not increase the total esterification of glycerol phosphate with homologous microsomes. Furthermore, the increase in total lipid synthesis observed with the addition of peaks I and III cannot be readily explained by feedback inhibition and this phenomenon requires further investigation.

3.4 DISCUSSION

Although the existence of both Mg^{2+}-independent and Mg^{2+}-dependent phosphatidate phosphohydrolase activities in mammalian systems has been known for over 25 years, no direct evidence has been reported as to which of these activities is responsible for glycerolipid biosynthesis. Germershausen et al. (1980), in reporting a method for preparing radiolabelled phosphatidic acid on mouse liver microsomes, found that the yield of radioactive phosphatidic acid could be increased and that of radioactive neutral lipid decreased by first washing the microsomes in 0.5 M NaCl. These authors suggested that salt washing either inhibited or dislodged the microsomal phosphatidate phosphohydrolase. Since less than 20% of the activity was recovered in the wash supernatant, they concluded that inactivation was the most likely cause. In studies with adipocytes, Moller and Hough (1982) observed

that the Mg^{2+}-dependent phosphatidate phosphohydrolase could be recovered in greater amounts by isolation of microsomes in homogenization buffers containing low amounts of KCl (54 mM). These reports indicated that microsomal phosphatidate phosphohydrolase activity could be translocated between microsomes and cytosol by varying the ionic strength of the isolation buffers.

Experiments using rat lung microsomes, presented here, have confirmed these previous findings. In addition, these studies revealed that rat lung microsomes washed with buffers of increasing ionic strength displayed a decreased capacity to synthesize neutral lipid from fatty acids and [^{14}C]-glycerol-3-phosphate, with a corresponding increased labelling of phosphatidic acid. These findings are indicative of an inactivation or progressive removal of the microsomal phosphatidate phosphohydrolase.

The quantitative recovery of the Mg^{2+}-dependent phosphatidate phosphohydrolase in wash supernatants reported here is in contrast with the original report by Germershausen et al. (1980). It is interesting to note that, in our initial experiments, we also failed to recover all the Mg^{2+}-dependent phosphatidate phosphohydrolase activity removed from the microsomes and therefore postulated the presence of a membrane-bound protein cofactor which confers Mg^{2+}-dependency on the integral Mg^{2+}-independent activity (Possmayer and Walton, 1983). However, by lowering the volume of washing buffer, it became apparent that the Mg^{2+}-dependent activity that

was removed from the microsomes could be quantitatively recovered in the washes. This does not dismiss the possibility of such a protein cofactor as postulated by Roncari and Mack (1981). The increase in neutral lipid and phosphatidylcholine labelling observed in Table III, especially with the addition of the nonfunctionally active peak III, could be due to the presence of low molecular weight proteins (Mishkin and Turcotte, 1974).

Most of the enzymes catalyzing reactions in the pathway between glycerol-3-phosphate and phosphatidylcholine are believed to be integral proteins on the cytoplasmic surface of the endoplasmic reticulum. The exception to this rule is phosphatidate phosphohydrolase which exists in both cytoplasmic and microsomal domains (Bell and Coleman, 1980; Ballas and Bell, 1980; Chan _et al._, 1983; Harding _et al._ 1983). Since phospholipids, such as phosphatidic acid, never exist in monomer form, phosphatidate phosphohydrolase must associate with membranes in order to act. The Mg^{2+}-dependency of the phosphatidate phosphohydrolase may reflect its requirement in the binding of enzyme to its substrate, as in the case of mitochondrial hexokinase (Rose and Warms, 1967), the first such ambiquitous enzyme discovered.

The decreased Mg^{2+}-dependent phosphatidate phosphohydrolase activity observed after washing is consistent with the hypothesis that a peripheral enzyme is being washed off the membrane by the high ionic strength

buffers. The decrease was sigmoidal and correlated highly ($r^2 = 0.97$) with the decrease in neutral lipid production observed in the glycerol-3-phosphate incorporation experiments (Figure 12). It is interesting to note that the point where the removal of the Mg^{2+}-dependent phosphatidate phosphohydrolase activity was half complete is approximately the same as the internal cationic concentration of the cell (Guyton, 1981).

In liver, cytosolic phosphatidate phosphohydrolase appears to show the greatest response to metabolic stimuli, including divalent cations and amphiphilic cationic drugs (Sturton and Brindley, 1980), vasopressin and glucocorticoids (Pollard and Brindley, 1984), and dietary influences (Brindley, 1978; Brindley and Sturton, 1982). In addition, this reviewer (Brindley and Sturton, 1982) reported that microsomes may contain a phosphatidate phosphohydrolase that is under metabolic control but is masked by a nonspecific phosphohydrolase. Reports on adipose tissue have stated that it is the Mg^{2+}-dependent phosphatidate phosphohydrolase activity that is affected by a number of hormones and drugs including insulin, propranolol, noradrenalin (Cheng and Saggerson, 1978a,b), epinephrine, isoproterenol (Moller et al., 1981) and polyamines (Jamdar and Osborne, 1983).

The quantitative recovery of the Mg^{2+}-dependent phosphatidate phosphohydrolase activity in the wash supernatants is suggestive of an ambiquitous enzyme as described by Wilson (1978). The activity of such an enzyme

could be identical irrespective of location and its control effected by its translocation on or off the membrane. Although no evidence of such a control is presented here, studies in adipocytes (Moller *et al.*, 1981) have shown an increase in microsomal Mg^{2+}-dependent phosphatidate phosphohydrolase and a decrease in the cytosolic activity following exposure to the lipolytic agents, epinephrine and isoproterenol. Recently, studies in hepatocytes (Cascales *et al.*, 1984) have shown a translocation from cytosol to endoplasmic reticulum, following exposure to oleic acid.

Casola and Possmayer (1979) reported that when membrane-bound [^{14}C]-phosphatidic acid-labelled microsomes were incubated with CDPcholine, the addition of cytosol was required for the production of labelled phosphatidylcholine. These results could be interpreted as indicating that, during the process of labelling the microsomes with membrane-bound [^{14}C]-phosphatidic acid and the subsequent reisolation, Mg^{2+}-dependent phosphatidate phosphohydrolase was removed, an occurrence also observed in the process of these experiments (data not shown). The addition of cytosol would thus restore the ability to make phosphatidylcholine. The possible removal of phosphatidate phosphohydrolase normally associated with the endoplasmic reticulum *in vivo* during standard cell fractionations must also be considered.

In order to demonstrate fully that the Mg^{2+}-dependent phosphatidate phosphohydrolase is responsible for

glycerolipid synthesis, it was necessary to show not only that its absence would affect synthesis, but that its return would restore a normal pattern to the lipid products of the salt-washed microsomes. A functional pathway of phosphatidylcholine synthesis in salt-washed microsomes could be re-established by the addition of whole cytosol, wash supernatant, or peak II from cytosol. Peak II is a Mg^{2+}-dependent phosphatidate phosphohydrolase activity with a molecular weight of approximately 4×10^5. The Mg^{2+}-independent phosphatidate phosphohydrolase activity seen at the void volume (peak I) could represent small vesicles unsedimented from cytosol, or aggregates of enzymes. This Mg^{2+}-independent activity was incapable of restoring the inhibited phosphatidylcholine synthetic pathway, as was the minor Mg^{2+}-dependent phosphatidate phosphohydrolase activity, peak III. These experiments demonstrate both that the removal of Mg^{2+}-dependent phosphatidate phosphohydrolase impedes the *de novo* pattern for the synthesis of triacylglycerols and phosphatidylcholine and that the restoration of this activity removes this interference. Thus, using two separate approaches, the importance of the Mg^{2+}-dependent phosphatidate phosphohydrolase activity in the biosynthetic pathway for pulmonary glycerolipids is confirmed.

CHAPTER 4

THE REQUIREMENT FOR MAGNESIUM PHOSPHATIDATE AS THE SUBSTRATE FOR THE Mg^{2+}-DEPENDENT PHOSPHATIDATE PHOSPHOHYDROLASE

4.1 INTRODUCTION

Phosphatidate phosphohydrolase [E.C.3.1.3.4] catalyzes the conversion of phosphatidate to diacylglycerol and orthophosphate. The reaction catalyzed by this enzyme is located at the first branch point in glycerolipid synthesis. Consequently, regulation of this enzyme represents a potential control point in the production of phosphatidylcholine for surfactant synthesis. A number of studies have demonstrated that Mg^{2+} stimulates the phosphatidate phosphohydrolase activity at low concentrations but is inhibitory at high levels (Jamdar and Fallon, 1973; Casola and Possmayer, 1979; Yeung et al., 1979; Sturton and Brindley, 1980; Casola and Possmayer, 1981; Ide and Nakazawa, 1985; Walton and Possmayer, 1985).

Using optimum Mg^{2+} levels, researchers studying adipose tissue have described a Mg^{2+}-dependent phosphatidate phosphohydrolase activity (Jamdar and Fallon, 1973; Lamb and Fallon, 1974; Moller et al., 1977; Cheng and Saggerson, 1978a,b; Moller et al., 1981; Moller and Hough, 1982; Jamdar and Osborne, 1984; Hall et al., 1985).

Recently we have demonstrated that the Mg^{2+}- dependent phosphatidate phosphohydrolase is essential for neutral

lipid and phosphatidylcholine synthesis in lung tissue (Walton and Possmayer, 1984). The use of a chemically-defined mixed-lipid substrate allows controlled manipulation of such parameters as ion requirements for the reaction. In these investigations, the effects of Mg^{2+} on the Mg^{2+}-dependent phosphatidate phosphohydrolase have been studied. In addition, the results clarify some of the observed effects of Ca^{2+}, F^{-}, and EDTA on the Mg^{2+}-dependent phosphatidate phosphohydrolase.

4.2, MATERIALS AND METHODS

4.2.1 Materials

Phosphatidylcholine was isolated and purified from egg yolks by the method of Ansell and Hawthorne (1964). Phosphatidate was prepared from phosphatidylcholine via the action of phospholipase D (EC 3.1.4.4) by the method of Kates and Sastry (1969) and converted to the disodium salt by 6 passes through a Chelex-100 column (Bio-Rad) (Renkonin, 1968). The method of Pieringer and Kunnes (1965) was used to prepare [^{32}P]-phosphatidate from diolein (Serdary Research Laboratories, London) and [γ^{32}P]-ATP (New England Nuclear, Lachine). The labelled lipid was isolated using TLC on oxalate-treated plates with a solvent of petroleum ether/acetone/formic acid (154:46:0.5) (Yeung et al., 1979). Biochemicals were purchased from Sigma (St. Louis) and reagent grade chemicals were obtained from Fisher Scientific (Toronto). Millex-GS filter units were

purchased from Millipore (Bedford, Massachusetts).

Rat lung cytosol was obtained as described previously (Walton and Possmayer, 1984). Mg^{2+}-dependent phosphatidate phosphohydrolase activity was assayed using a mixed-lipid vesicle system of equimolar phosphatidate and phosphatidylcholine (Walton and Possmayer, 1985). All modifications for purposes of characterization were made to this standard assay protocol.

4.2.2 Determination of stability constants

The stability constant of the MgPA complex was determined by incubating various concentrations of phosphatidate-phosphatidylcholine vesicles with $MgCl_2$ in a 50 mM Hepes buffer, pH 7.4. After incubation for 10 minutes at 37°C, 1 ml of the 3 ml total volume was passed through a Millex-GS filter unit and collected as 4 x 0.25 ml aliquots. Although a 0.2 µm filter would not be expected to pass any vesicles, control experiments employing radioactive phosphatidate were undertaken. No radioactivity was detected in the ultrafiltrate (results not shown). The Mg^{2+} concentration of the four aliquots was measured using a Jarrell Ash model 810 Atomic Absorption Spectrophotometer. The free Mg^{2+} concentration, $[Mg^{2+}]_f$, was determined by extrapolating the four measured values back to the point of zero volume, which represents the value before the equilibrium between free and bound Mg^{2+} was perturbed by the process of filtration. The value of the stability constant 'K' was calculated, knowing the

initial $[Mg^{2+}]_t$, $[PA]_t$, and final $[Mg^{2+}]_f$ concentrations, and using the formula:

$$1. \quad K = \frac{[MgPA]}{[Mg^{2+}]_f \; [PA]_f} = \frac{[Mg^{2+}]_t - [Mg^{2+}]_f}{[Mg^{2+}]_f \; ([PA]_t - ([Mg^{2+}]_t - [Mg^{2+}]_f))}$$

By convention, the log of K was taken from that point where the substrate concentration was extrapolated to zero (Morrison, 1979). Using this stability constant and the $[Mg^{2+}]_t$ and $[PA]_t$ concentrations, the $[PA]_f$, [MgPA], and $[Mg^{2+}]_f$ concentrations at each point of a Mg^{2+} concentration curve were determined. These values were computed from the formula:

$$2. \quad K = \frac{[M\,L]}{[M]_f \; [L]_f} = \frac{X}{(M_t - X)(L_t - X)}$$

which reduces to:

$$3. \quad KX^2 - X(1 + KM_t + KL_t) + KM_tL_t = 0$$

The value of 'X', the MgPA concentration, can be obtained by solving the quadratic equation #3, and the concentrations of $[Mg^{2+}]_f$ and $[PA]_f$ calculated by subtracting that amount which was converted to MgPA from

the amount initially present.

The stability constant of the CaPA complex was determined in the same manner as outlined above for the MgPA complex.

The equilibrium concentrations of the Ca^{2+}, Mg^{2+}, EDTA, and phosphatidate incubations were calculated using the NCOMICS computer program of Perrin and Sayce (1967).

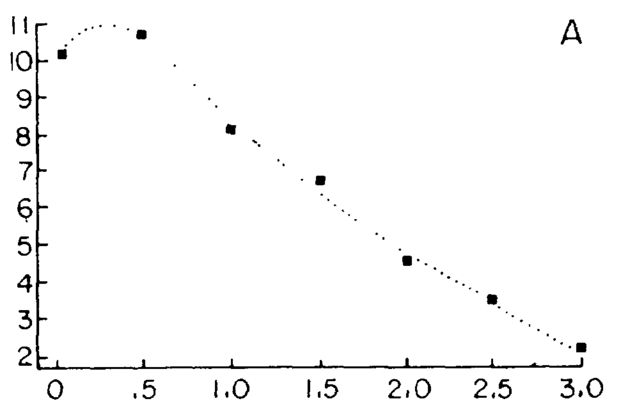
4.3 RESULTS

4.3.1 Effects of Mg^{2+} concentration on the Mg^{2+}-dependent phosphatidate phosphohydrolase activity

Figure 15 shows the optimum Mg^{2+} concentration at various phosphatidate concentrations for the cytosolic form of the Mg^{2+}-dependent phosphatidate phosphohydrolase using the mixed-lipid assay of phosphatidate phosphohydrolase. Similar requirements have been demonstrated for the microsomal form of the enzyme (Walton and Possmayer, 1985). The requirement for Mg^{2+} was related to the concentration of substrate rather than the enzyme. As the substrate concentration increased, the optimum Mg^{2+} concentration increased as a linear relationship (coefficient of determination = 0.98) (Figure 16). The slope of this line was 0.49 which indicates that the optimum Mg^{2+} concentration was equal to one-half that of the phosphatidate. These results assume that PA:PC liposomes have an even distribution of phosphatidate and phosphatidylcholine on both the interior and exterior surfaces (Mille and Vanderkooi, 1977), that Mg^{2+} can

Figure 15.

Mg^{2+} concentration versus enzyme activity curves. Substrate concentration was: A; 0.5 mM, B; 1.0 mM, C; 1.5 mM, D; 2.0 mM. Mg^{2+}-dependent phosphatidate phosphohydrolase was assayed as described in Materials and Methods. Specific activity reported in nmol/min/mg protein. Curves of best-fit were obtained from Curve-Fit computer program. Results from two separate determinations.


A
11
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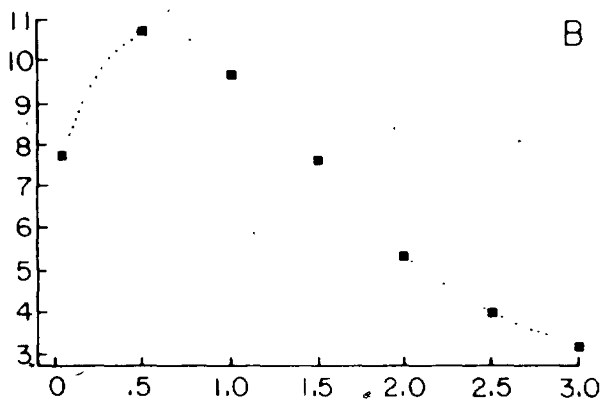
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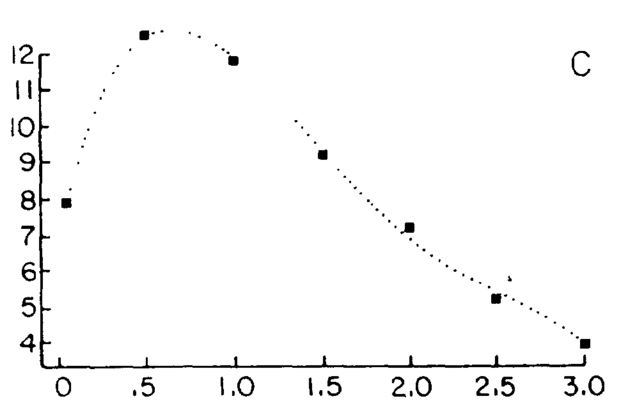
C
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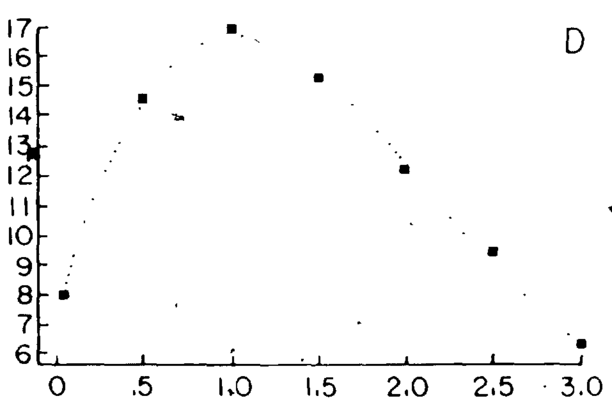
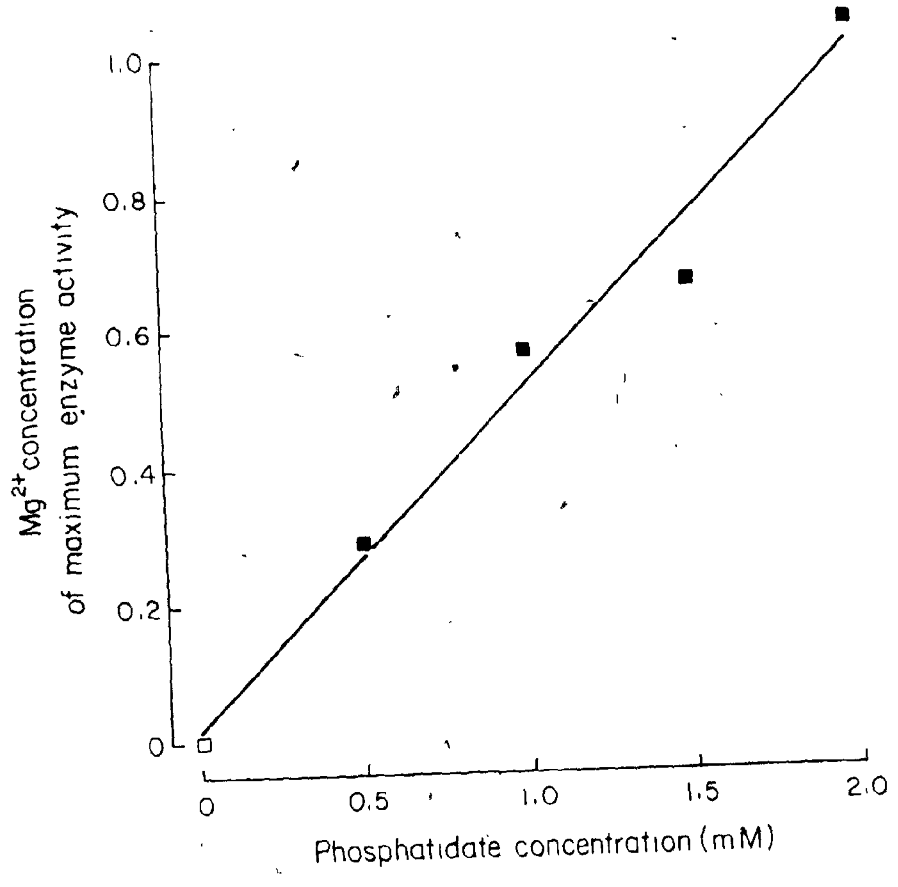
2.5
3.0


D
17
16
15
14
13
12
11
10
9
8
7
6
0
.5
1.0
1.5
2.0
2.5
3.0

SPECIFIC ACTIVITY
$MgCL_2$ (mM)

Figure 16.

Mg^{2+} concentration of maximal activity versus phosphatidate concentration. Mg^{2+} concentration derived from data in Figure 15.


Mg2+ concentration
of maximum enzyme activity
1.0
0.8
0.6
0.4
0.2
0
0
0.5
1.0
1.5
2.0
Phosphatidate concentration (mM)

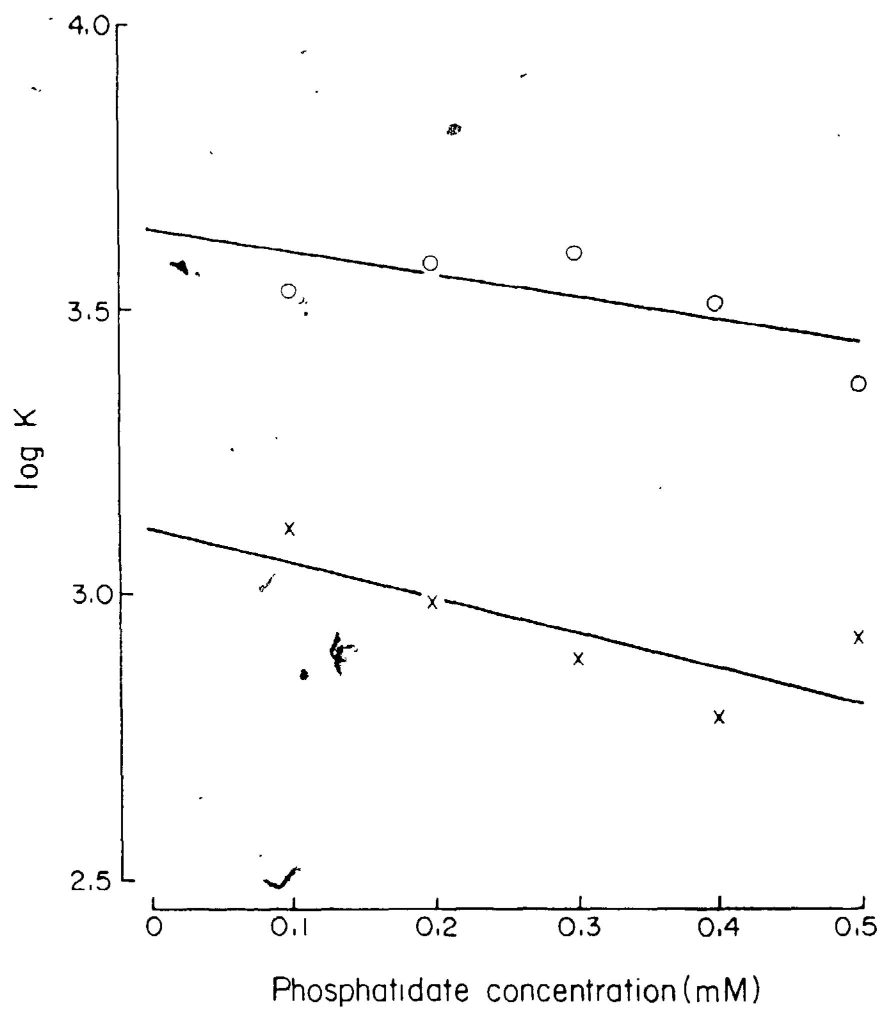
interact with phosphatidate on both sides of the bilayers, and that only the exterior surface is accessible to enzyme action. Since additional sources of Mg^{2+} binding, such as phosphate buffers, would complicate this relationship, it was deemed advisable to employ buffers such as Hepes which show little interaction with divalent cations (Good et al., 1966).

4.3.2 Stability constants of MgPA and CaPA

In order to examine the mechanism whereby Mg^{2+} stimulates phosphatidate phosphohydrolase activity, it was necessary to determine the concentration of Mg^{2+}_{free}, PA_{free}, and MgPA in the assay mixtures. Establishing the stability constant between the substrate liposomes and Mg^{2+} would permit the calculation of the various ionic species. On the basis of published reports (Hauser and Dawson, 1967), it appears reasonable to assume that the binding between divalent cations and the zwitterionic phosphatidylcholine is negligible when compared to that between divalent cations and phosphatidate. The stability constant of the MgPA and CaPA complexes at various phosphatidate concentrations are shown in Figure 17. Extrapolation back to the point of zero concentration indicates that the stability constant of MgPA is 1259 M^{-1}. This is equivalent to a dissociation constant of 0.79 mM. The stability constant of CaPA is 3981 M^{-1}, which corresponds to a dissociation constant of 0.25 mM. These results demonstrate that phosphatidate has a 3-fold greater

Figure 17.

Log of stability constant versus phosphatidate concentration. Various concentrations of phosphatidate were incubated with Mg^{2+} (X) or Ca^{2+} (O), and the stability constant determined as described in Materials and Methods. Results from a typical determination of four.


4.0
3.5
3.0
2.5
log K
0
0.1
0.2
0.3
0.4
0.5
Phosphatidate concentration (mM)

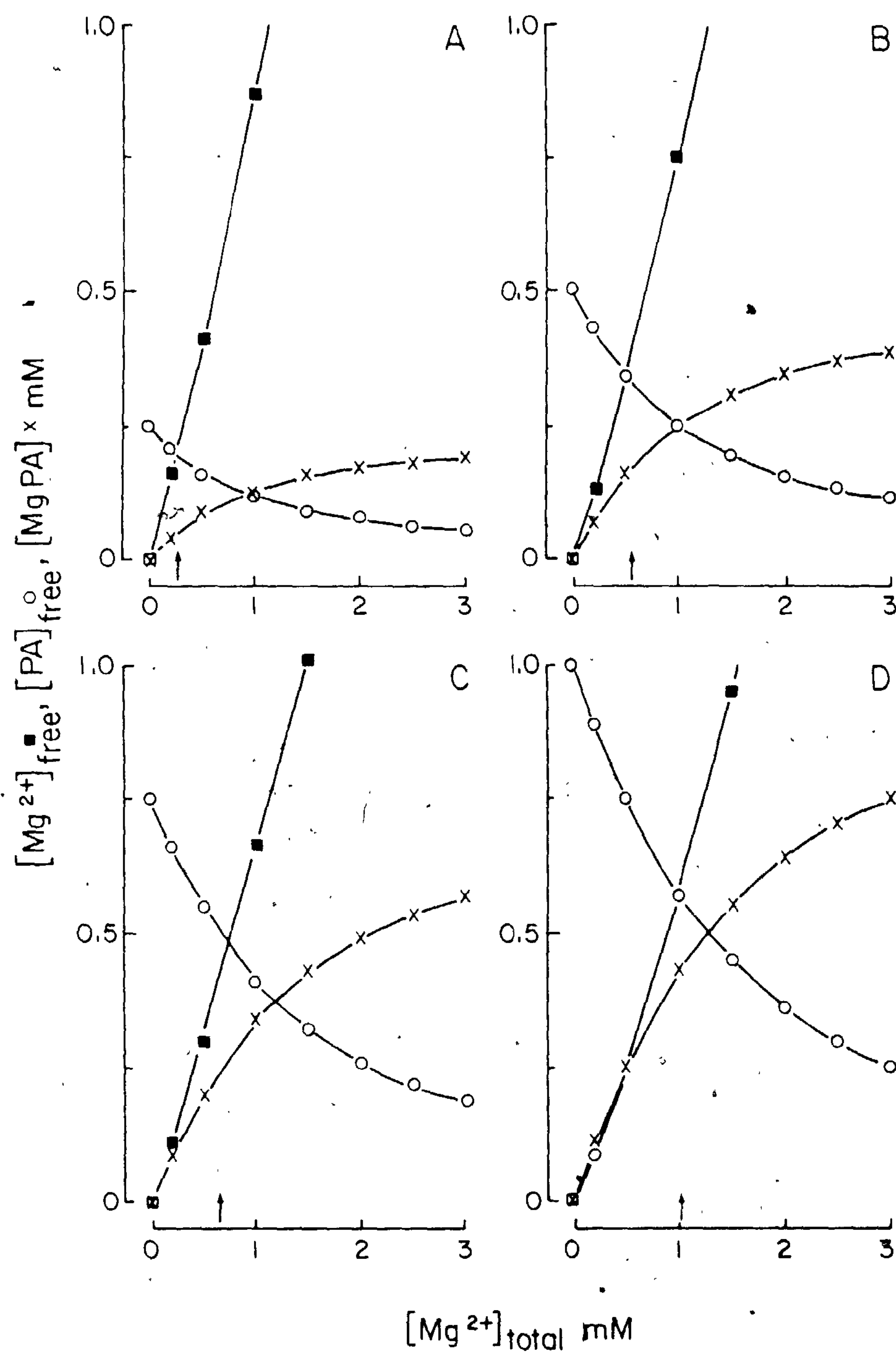
affinity for Ca^{2+} than Mg^{2+}. The parallel nature of the lines in Figure 17 indicates that this increased affinity of CaPA over MgPA exists at all substrate concentrations examined. The experimental value of the stability constant displayed some variability depending upon the preparation of the PA/PC liposomes. However the relative difference between the two values remained constant. The stability constants determined in this report are within the same order of magnitude but lower than those reported by Barton (1968). Whether this difference is due to the method of preparing the liposomes or in calculating the stability constants remains unknown.

4.3.3 Calculation of the free and bound levels of Mg^{2+} and phosphatidate

Employing the experimentally-derived stability constant for MgPA, it was possible to calculate the bound and free levels of Mg^{2+} and PA. Figure 18 shows the concentration of Mg^{2+}_{free}, PA_{free}, and MgPA at the four substrate concentrations shown in Figure 15. The optimum enzymatic activity appeared to coincide with the intersection of the falling PA_{free}, which appears not to be a suitable substrate, and the rising Mg^{2+}_{free} concentrations, which are inhibitory. The maximum enzyme activity occurred at this saddle-point for all four experimental substrate concentrations. The inhibition by Mg^{2+}_{free} was investigated by replotting the Mg^{2+} concentration curves (Figure 15) as velocity versus MgPA

Figure 18.

Concentrations of Mg^{2+}_{free} (■), PA_{free} (O), and MgPA (X) at various Mg^{2+}_{total} concentrations. Values were calculated using the experimentally-derived stability constant and equation #3 in Materials and Methods. The total PA concentrations are as indicated in Figure 15. Arrows indicate Mg^{2+}_{total} concentration of maximum enzyme activity.


A
B
C
D
1.0
0.5
0
0
1
2
3
$[Mg^{2+}]_{free}$ ■, $[PA]_{free}$ ○, [MgPA] × mM
$[Mg^{2+}]_{total}$ mM

concentration at two different Mg^{2+}_{free} concentrations. MgPA concentrations at 0.5 mM and 2.0 mM Mg^{2+}_{free} were obtained from the data in Figure 18 and the Mg^{2+}_{total} that gave this pair of concentrations was recorded. The velocity at this Mg^{2+}_{total} was determined from the computer-derived best fit graphs (Figure 15). Double reciprocal plots of this data indicate that Mg^{2+}_{free} acts as a mixed-type inhibitor of the Mg^{2+}-dependent phosphatidate phosphohydrolase (Figure 19).

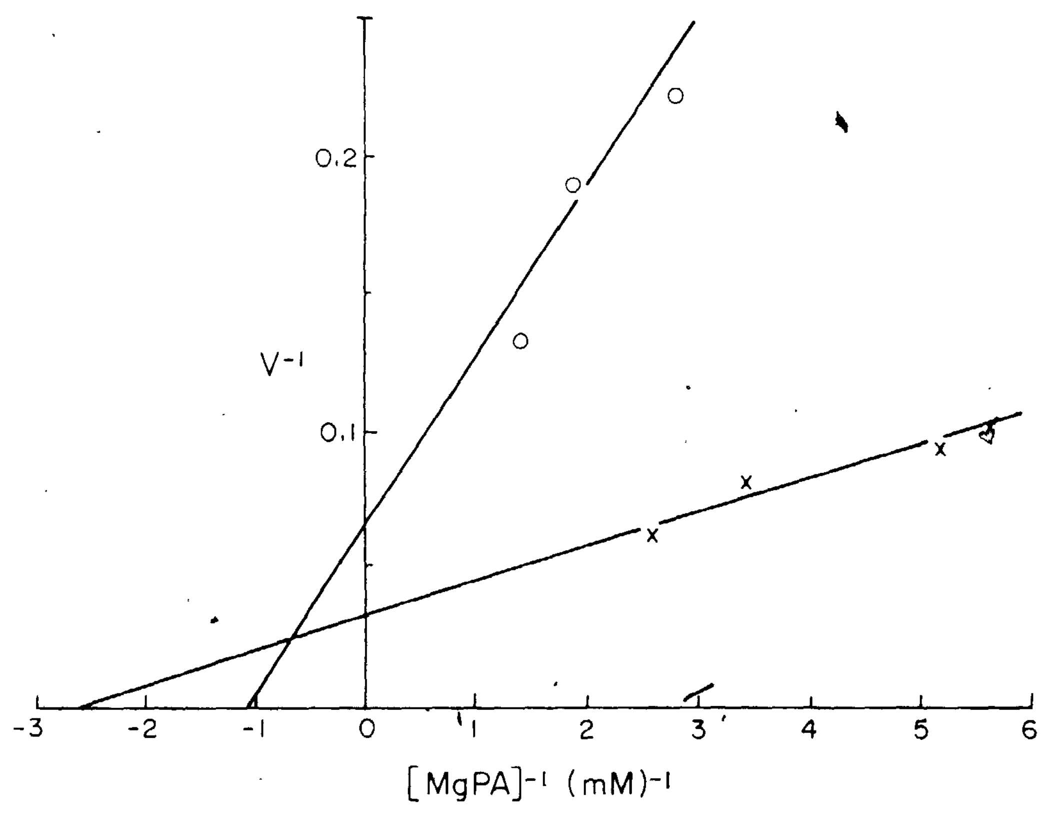
Double reciprocal plots of the MgPA concentration at saddle-point optima versus velocity (Figure 20) reveal a K_m = 0.187 mM MgPA and a theoretical V_{max} = 23 nmol/min/mg protein.

4.3.4 Inhibition of the Mg^{2+}-dependent phosphatidate phosphohydrolase by Ca^{2+}

Ca^{2+} has also been shown to be an inhibitor of the Mg^{2+}-dependent phosphatidate phosphohydrolase in various tissues (Lamb and Fallon, 1974; Caras and Shapiro, 1975; Bowley et al., 1977; Yeung et al., 1979; Sturton and Brindley, 1980; Abdel-Latif and Smith, 1984; Hosaka and Yamashita, 1984). Figure 21 demonstrates that the fall in activity observed upon addition of Ca^{2+} occurred as the MgPA concentration decreased while that of CaPA increased. The initial plateau of activity at low Ca^{2+} levels was due to the EDTA in the assay mixtures preferentially chelating the Ca^{2+}. At equimolar MgPA and CaPA, the enzymatic

Figure 17.

Double reciprocal plot of velocity versus MgPA concentration at 0.5 mM Mg^{2+}_{free} (**X**), and 2.0 mM Mg^{2+}_{free} (**O**). Results are derived from data in Figure 15. Values from 0.5 mM PA were omitted because of difficulty in assessing the greatest velocity.



0.2
0.1
V^{-1}
-3
-2
-1
0
1
2
3
4
5
6
$[MgPA]^{-1}$ $(mM)^{-1}$

Figure 20.

Double reciprocal plot of velocity versus MgPA concentration at optimum $Mg^{2+}_{total}:PA_{total}$ ratios. Results are derived from data in Figure 15. Values from 0.5 mM PA were omitted because of difficulty in assessing the greatest velocity.

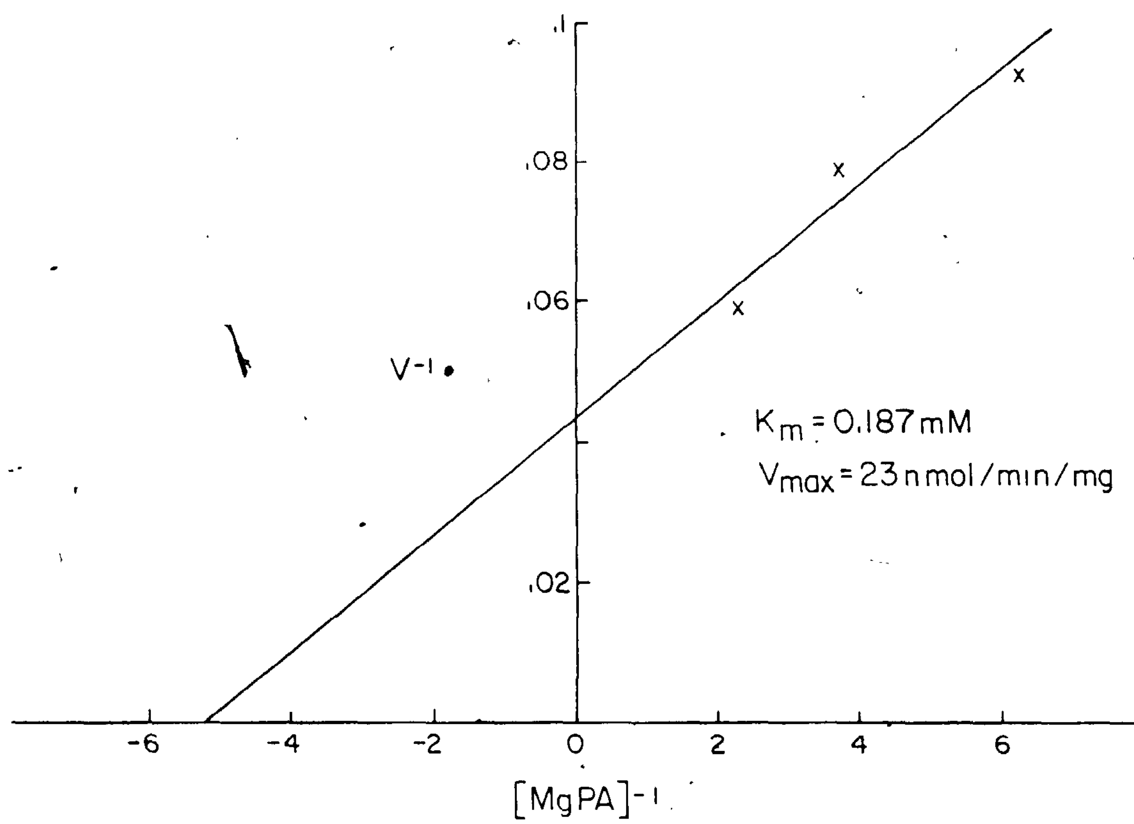
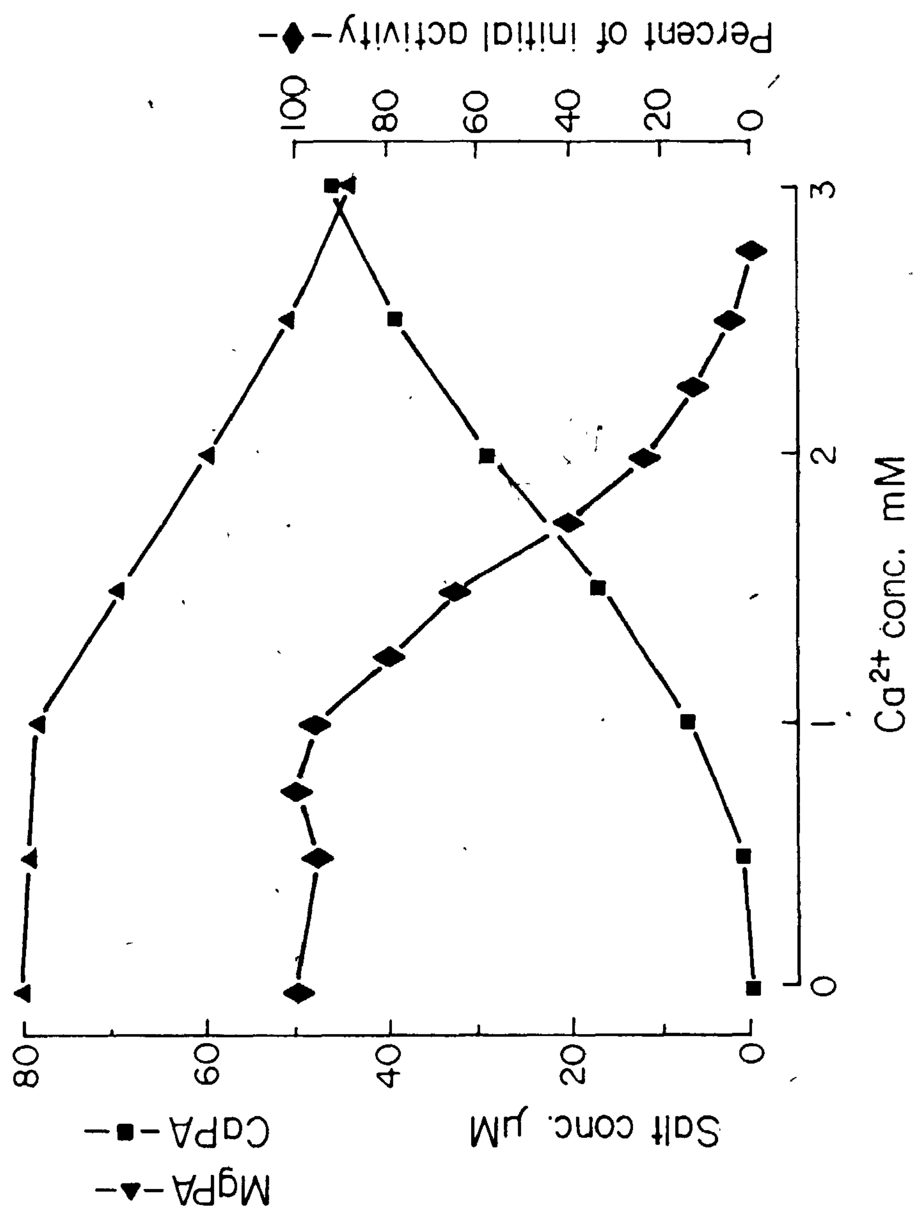

.1
.08
.06
.02
V-1
Km = 0.187 mM
Vmax = 23 nmol/min/mg
-6
-4
-2
0
2
4
6
[MgPA]-1

Figure 21.

Effects of Ca^{2+} on the Mg^{2+}-dependent phosphatidate phosphohydrolase activity (◆) and the levels of MgPA (▲) and CaPA (■). Results are from three separate determinations. MgPA and CaPA levels were estimated using experimentally-derived stability constants and the computer program NCOMICS as described in Materials and Methods.


MgPA
CaPA
Salt conc. μM
Percent of initial activity
Ca2+ conc. mM
0
1
2
3
0
20
40
60
80
0
20
40
60
80
100

activity was zero. This data indicates that CaPA is not an acceptable substrate and suggests that Ca^{2+} acts as a competitive inhibitor by changing the salt form of the substrate.

4.4 DISCUSSION

The term "Mg^{2+}-dependent phosphatidate phosphohydrolase" was used originally by Hokin, Hokin, and Matheson (1963) in erythrocytes. It described an activity that was the difference between the activities at optimal Mg^{2+} concentration and the activity in the presence of EDTA.

The requirement for Mg^{2+} in phosphatidate phosphohydrolase was first demonstrated in liver by Mitchell, Brindley, and Hubscher in 1971. Prior to that point, Mg^{2+} had been shown to stimulate activity in aqueous emulsions and inhibit activity using membrane-bound substrates (Smith *et al.*, 1967). The requirement was demonstrable only after exhaustive treatment with EDTA, to remove endogenous Mg^{2+}. The phosphatidate generated on microsomes was synthesized as the Mg^{2+} salt, as the acyltransferase enzymes both require Mg^{2+} for activity (Monroy *et al.*, 1972; Liu and Kako, 1974; Dodds *et al.*, 1976; Schlossman and Bell, 1976; Bates and Saggerson, 1979). As Mg^{2+} is the predominant intracellular divalent cation (Guyton, 1981), the Mg^{2+} salt of compounds like phosphatidate is the most likely *in vivo* form.

This Mg^{2+}-dependent phosphatidate phosphohydrolase

activity has been extensively studied in adipose tissue (Jamdar and Fallon, 1973; Lamb and Fallon, 1974; Moller *et al.*, 1977; Cheng and Saggerson, 1978a,b; Moller *et al.*; 1981; Moller and Hough, 1982; Jamdar and Osborne, 1984; Hall *et al.*, 1985), and the Mg^{2+}-stimulated activity studied in liver (reviewed in Brindley and Sturton, 1982). However, much of the previous work in lung has focussed on a Mg^{2+}-independent phosphatase. Recent work in our laboratory has shown the Mg^{2+}-dependent phosphatidate phosphohydrolase in lung subcellular fractions to be responsible for glycerolipid synthesis (Walton and Possmayer, 1984). In addition, phosphatidate phosphohydrolase from *S. cerevisiae*, purified 600-fold (Hosaka and Yamashita, 1984), demonstrates an almost absolute magnesium requirement for activity. Using the mixed-lipid vesicle assay of Mg^{2+}-dependent phosphatidate phosphohydrolase (Walton and Possmayer, 1985), it has been possible to carefully control the assay conditions and study the effects of divalent cations.

The effects of Mg^{2+} on this enzyme are clearly more complex than a simple stimulation or inhibition. Studies in liver (Sturton and Brindley, 1980), adipose tissue (Jamdar and Fallon, 1973) and lung (Walton and Possmayer, 1985) show that increasing concentrations of Mg^{2+} first stimulates and then inhibits the phosphatidate phosphohydrolase activity. The general shape of the Mg^{2+} concentration versus activity curves appear to be products

of two hyperbolic functions, one increasing and the other decreasing. High levels of Mg^{2+} are inhibitory and low levels do not produce the stimulation. Determination of the stability constant of MgPA has enabled us to assess the relative contribution of each species. The Mg^{2+}-dependent phosphatidate phosphohydrolase utilizes MgPA as its substrate. This is to be expected as this salt form of phosphatidate is synthesized by the acyltransferases. Thus under low Mg^{2+} concentrations the enzyme activity is limited by the low MgPA levels. As the concentration of Mg^{2+} increases the amount of PA_{free} decreases with the formation of MgPA. At a certain point, and under these conditions, it is at a $Mg^{2+}{}_{total}$ concentration equal to one-half the PA_{total} concentration, the stimulation in activity from the formation of MgPA is counterbalanced by the inhibition of the $Mg^{2+}{}_{free}$. This saddle-point is the $Mg^{2+}{}_{total}$ that gives the greatest enzyme activity. Beyond this point the increasing $Mg^{2+}{}_{free}$ inhibits the enzyme activity. The basis of this inhibition is not known. But one potential explanation makes use of the observation by Verkleij et al. (1982) that high levels of Mg^{2+}, levels in the range of 2 moles of Mg^{2+} to 1 mole of phosphatidate, can induce the formation of the H_{II} phase in equimolar PA/PC vesicles. Phosphatidate in this form would be an unacceptable substrate, as the phosphate head groups are clustered together in the core of the tubes with the acyl chains projecting outward.

The observed kinetics of velocity versus MgPA

concentration are in accord with other published values for the cytosolic form of phosphatidate phosphohydrolase which set the K_m at approximately 200 µM. However, the V_{max} is increased 3-fold by utilizing optimal Mg^{2+} to phosphatidate ratios.

The inhibition by Mg^{2+}_{free} explains some seemingly contradictory observations on the effects of EDTA on the Mg^{2+}-dependent phosphatidate phosphohydrolase. EDTA has been shown to stimulate at low levels (Sturton and Brindley, 1977; Casola and Possmayer, 1979; Sturton and Brindley, 1980; Casola and Possmayer, 1981) and inhibit at higher concentrations (Mitchell et al., 1971; Jamdar and Fallon, 1973; Sturton and Brindley, 1977; Casola and Possmayer, 1979; Yeung et al., 1979; Sturton and Brindley, 1980; Casola and Possmayer, 1981; Ide and Nakazawa, 1985). Previous results in lung have shown that EDTA can provide a 2-fold stimulation over the maximum activity at optimal Mg^{2+} concentrations (Casola and Possmayer, 1979; Casola and Possmayer, 1981). As the Ca^{2+} would be bound by the EDTA, its inhibitory effects on the Mg^{2+}-dependent phosphatidate phosphohydrolase would be lessened. At all EDTA concentrations the chelator would compete with phosphatidate for magnesium. As the stability constant of Mg-EDTA (log K = 8.7) is approximately 10^6-fold higher (O'Sullivan, 1969) than that for MgPA, the inhibition observed could result from the loss of the MgPA substrate. Fluoride ions (Mitchel et al., 1971; Jamdar and Fallon,

1984) have revealed that Ca^{2+} can readily induce phase transitions in a number of anionic phospholipids including phosphatidate. A phase transition from bilayer to H_{II} phase would place the phosphate head groups on the inside of a tube, separated from the enzyme by the hydrophobic acyl chains. This explanation is more likely under high concentrations (5 mM) of Ca^{2+}. The Ca^{2+} concentration at which the Mg^{2+}-dependent phosphatidate phosphohydrolase activity becomes zero is the point of equimolar MgPA and CaPA. It has been suggested that Ca^{2+} is able to bind two anionic phospholipids by chelating their two polar headgroups (Papahadjopoulos, 1968; Scimiya and Ohki, 1973; Ohki, 1982; Ohki and Oshima, 1984). Evidence for this manner of binding includes an increased surface tension and the formation of the H_{II} phase that occurs upon addition of Ca^{2+}. In this manner, a situation of equimolar CaPA and MgPA would be better represented as $Ca_{1/2}$ - PA - $Mg_{1/2}$, with each phosphatidate molecule bound to a Ca^{2+} and a Mg^{2+}.

The requirement of Mg^{2+}-dependent phosphatidate phosphohydrolase for Mg^{2+} is less of a coincidence in light of these observations. MgPA appears to be the _in vivo_ substrate for the reason that this salt-form more readily retains the bilayer configuration and prevents the disruptions that non-bilayer forms would produce. The Mg^{2+} requirements of the acyltransferase enzymes that produce phosphatidate, and the phosphatidate phosphohydrolase and

1973) may inhibit in a similar manner. Having a solubility product of 6.4 x 10^{-9} at 27°C (Handbook of Chemistry and Physics, 36th edition, 1955), fluoride removes Mg^{2+} by precipitation.

Calcium and other divalent cations such as Fe^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} have been shown to be inhibitory (Jamdar and Fallon, 1973; Caras and Shapiro, 1975; Yeung et al., 1979; Abdel-Latif and Smith, 1984; Hosaka and Yamashita, 1984). Employing the stability constants for CaPA, Ca-EDTA, MgPA, and Mg-EDTA determined in the present studies and using the computer program NCOMICS (Perrin and Sayce, 1967), the free and bound levels of all species could be estimated. The inhibitory effects of Ca^{2+} became evident as phosphatidate switched from MgPA to the CaPA salt. Because phosphatidate has a 3-fold higher affinity for Ca^{2+} then Mg^{2+}, it requires correspondingly less Ca^{2+} to abolish the enzyme activity. The calcium salt is not a suitable substrate for two possible reasons. First, the binding of the bulkier Ca^{2+} ion (ionic diameter, 1.98 Å) may sterically hinder the active site which accepts the smaller Mg^{2+} ion (ionic diameter, 1.30 Å). A second mechanism of inhibition may be a change in the membrane configuration that the CaPA can adopt. Numerous studies (reviewed in Cullis and de Kruijff, 1979) including electron microscopy (Verkleij et al., 1982), membrane fusion (Ohki and Oshima, 1984), ^{31}P-NMR (Miner and Prestegeard, 1984), ^{3}H-NMR (Liao and Prestegeard, 1980), ESR (Ito and Ohnishi, 1974), and X-ray diffraction (Miner et al., 1983; Caffery and Feigenson,

CTP:PA cytidylyltransferase (Brindley and Sturton, 1982) which utilize it as substrate, can be viewed as accommodations made to retain phosphatidate in a form that maintains membrane integrity.

CHAPTER FIVE

THE EFFECTS OF TRITON X-100 AND CHLORPROMAZINE ON THE Mg^{2+}-DEPENDENT PHOSPHATIDATE PHOSPHOHYDROLASE

5.1 INTRODUCTION

The Mg^{2+}-dependent phosphatidate phosphohydrolase lies at the first branch point in the biosynthesis of phosphatidylcholine for lung surfactant. This enzyme catalyzes the conversion of phosphatidate to diacylglycerol. The amphipathic and membrane-bound nature of the substrate demands that the enzyme activity be assayed under artificial conditions that mimic the *in vivo* substrate. In assaying phosphatidate phosphohydrolase, addition of exogenous compounds has been shown to stimulate or inhibit the activity. Lung also contains a Mg^{2+}-independent phosphatase activity which does not appear to have a role in glycerolipid synthesis. Assays which do not distinguish between the Mg^{2+}-dependent and independent activities could conceivably attribute a stimulation in phosphatidate phosphohydrolase that could be accounted for by the Mg^{2+}-independent activity. Such a stimulation would not exist *in vivo* and must be considered artifactual and misleading. It is important, therefore, to ensure that the stimulation or inhibition of phosphatidate phosphohydrolase activity attributed to a particular compound is acting via the Mg^{2+}-dependent activity before an effect of these agents on glycerolipid synthesis can be inferred. In this

paper, we report results for two such exogenous compounds: the detergent Triton X-100, and the amphiphilic cation chlorpromazine, and their effects upon the Mg^{2+}-dependent phosphatidate phosphohydrolase.

Studies in a number of tissues have shown that Triton X-100 can either inhibit (McCaman et al., 1965; Casola and Possmayer, 1981; Jamdar et al., 1984; Walton and Possmayer, 1985), stimulate (Casola and Possmayer, 1981a,b), or have no effect (Sedgewick and Hubscher, 1965; Jamdar et al., 1984) on the activity of phosphatidate phosphohydrolase. As the enzymatic activities have been measured at different substrate concentrations, in a number of different tissues, and both with and without Mg^{2+}, the true effect of Triton X-100 is difficult to interpret. Other studies in lung (Johnston et al., 1978) and isolated Type II cells (Douglas et al., 1983) have employed phosphatidate solubilized with Triton X-100 as the substrate. Reports from our laboratory have demonstrated that, in rat lung, Triton X-100 stimulates the PA_{mb}-dependent phosphatidate phosphohydrolase in microsomes and cytosol (Casola and Possmayer, 1981), but inhibits the Mg^{2+}-dependent phosphatidate phosphohydrolase in both cell fractions (Walton and Possmayer, 1985). Since the Mg^{2+}-dependent activity was designed to measure the phosphohydrolase activity which acts upon membrane-bound phosphatidate *in vivo*, these results were perplexing. The present investigations were initiated to define the effects of

Triton X-100 and to resolve the apparent differences between PA_{mb} and Mg^{2+}-dependent phosphatidate phosphohydrolase activities with regard to Triton X-100.

Chlorpromazine and other amphiphilic cationic drugs such as propranolol and methacholine appear to be able to redirect the synthesis of glycerolipids (Brindley _et al._, 1975; Eichberg _et al._, 1979). In the presence of amphiphilic cations, the synthesis of triacylglycerol and phosphatidylcholine is decreased and the accumulation of the acidic phospholipids, phosphatidate, CDP-DG, phosphatidylinositol, and phosphatidylglycerol is increased (reviewed by Brindley _et al._, 1975; Brindley, 1978; Brindley and Sturton, 1982). These drugs have also been shown to inhibit phosphatidate phosphohydrolase in a number of tissues (Giusto _et al._, 1983; Weiss _et al._, 1983), and it is generally felt that this inhibition may reflect control in the direction of glycerolipid synthesis at the level of the first branch point. Recent reports suggest that chlorpromazine may exert its effects by inhibiting the cytosolic phosphatidate phosphohydrolase from translocating to the microsomal surface (Hopewell _et al._, 1985). It has also been suggested that chlorpromazine can replace the Mg^{2+} requirement in the phosphatidate phosphohydrolase of rat liver (Bowley _et al._, 1977; Sturton and Brindley, 1980). These investigators described experiments whereby the stimulations produced by chlorpromazine could be masked by addition of Mg^{2+} but could not be reversed by EDTA. They concluded that the requirement for Mg^{2+} was not

absolute and that the positively-charged amine group of chlorpromazine was an acceptable substitute. However, these experiments are not unambiguous, and could be interpreted to indicate that the effect of chlorpromazine is to stimulate a Mg^{2+}-independent phosphatase while not affecting or even inhibiting the Mg^{2+}-dependent phosphatidate phosphohydrolase activity. It was to resolve this question that the effect of chlorpromazine was studied in this report.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Chlorpromazine hydrochloride was obtained from Sigma Chemical (St. Louis). All other materials were obtained as described previously (Chapter 2, 3).

5.2.2 Methods

Assays of the Mg^{2+}-dependent phosphatidate phosphohydrolase were performed as described previously (Chapter 3).

Heat inactivation of microsomes and cytosols was performed in a 55°C water bath. At the end of 15 minutes, tubes containing microsomes and cytosols were placed on ice until the enzyme activity was assayed.

5.3 RESULTS AND DISCUSSION

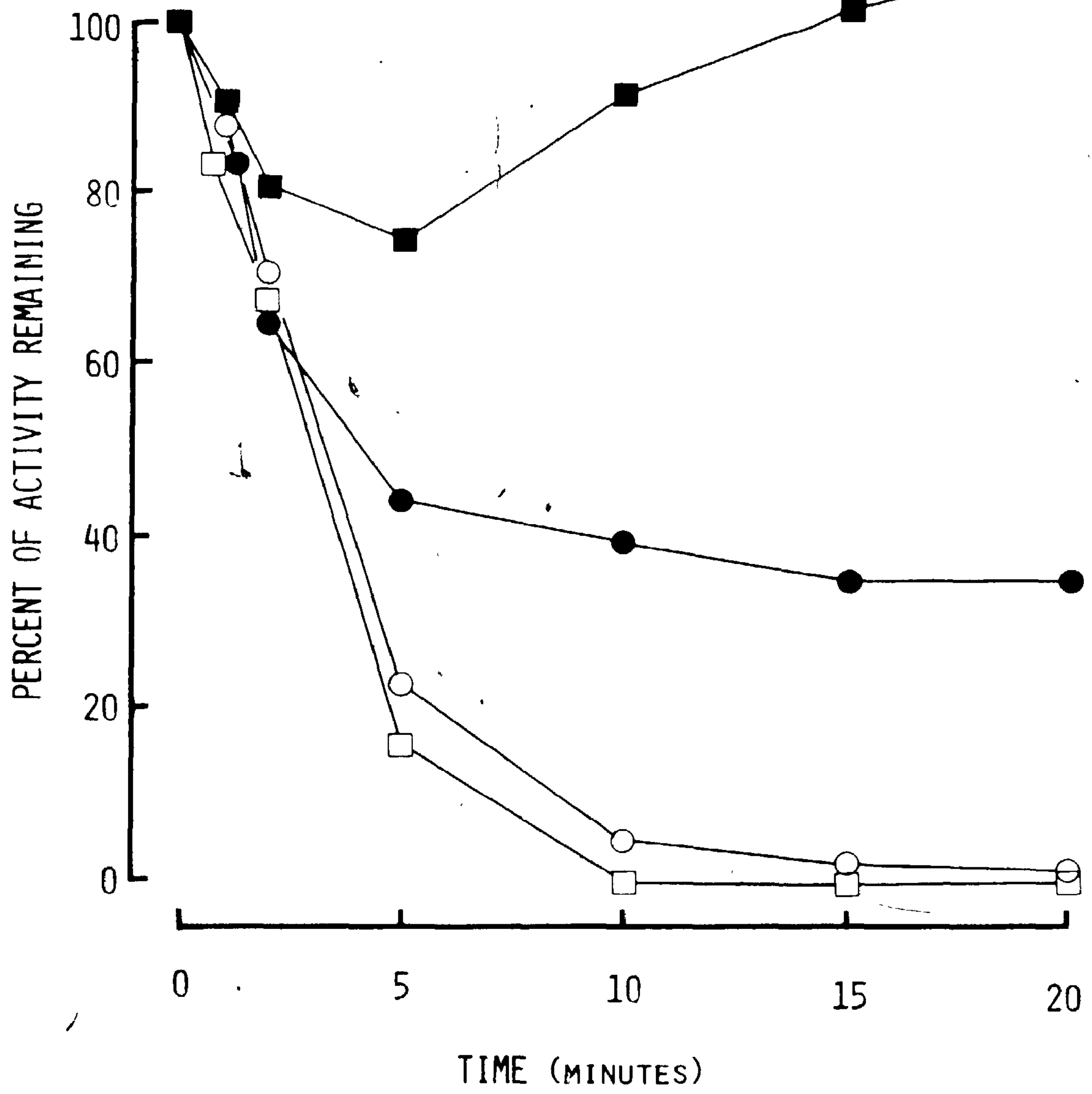
5.3.1 Thermal inactivation of Mg^{2+}-dependent

phosphatidate phosphohydrolase and Mg^{2+}-independent phosphatase activities

The Mg^{2+}-dependent phosphatidate phosphohydrolase activities of microsomes and cytosol were quite susceptible to thermal inactivation (Figure 22). By 10 minutes at 55°C, virtually all of the Mg^{2+}-dependent activity was abolished. The intracellular location of the activity made no difference in the thermal inactivation and the microsomal activity appeared to derive no additional stability from its membrane association. The Mg^{2+}-independent phosphatase activities were more resistant to thermal inactivation. This resistance varied with the subcellular location of the Mg^{2+}-independent phosphatase. The cytosolic activity was the more susceptible. The activity dropped to approximately 40% of the control value after 5 minutes at 55°C, and remained at this level for the duration of the time course. The microsomal activity was originally reduced to approximately 75% of the control activity after 5 minutes at 55°C. The activity returned to control levels at longer incubation times. The basis for this recovery in activity is unknown but may be due to an increase in the access between a non-specific, heat-stable phosphatase and the phosphatidate liposomes as thermally-denatured proteins are removed from the microsomal surface. Increases in activity of the microsomal Mg^{2+}-independent phosphatase activity have been observed previously with high salt washing (Walton and Possmayer, 1984). The differences between the microsomal and cytosolic thermal

Figure 22.

Thermal inactivation profiles of the Mg^{2+}-dependent (○, □) and Mg^{2+}-independent (●, ■) phosphatidate phosphohydrolase activities in microsomes (□, ■) and cytosols (○, ●). Subcellular fractions were preincubated at 55°C for the time indicated and then chilled on ice. Activities reported are from four separate determinations each done in triplicate.



100
80
60
40
20
0
PERCENT OF ACTIVITY REMAINING
0
5
10
15
20
TIME (MINUTES)

stability profiles are indicative of more than one, and perhaps several, Mg^{2+}-independent phosphatase activities that can hydrolyse phosphatidate in an opportunistic manner.

5.3.2 Triton X-100

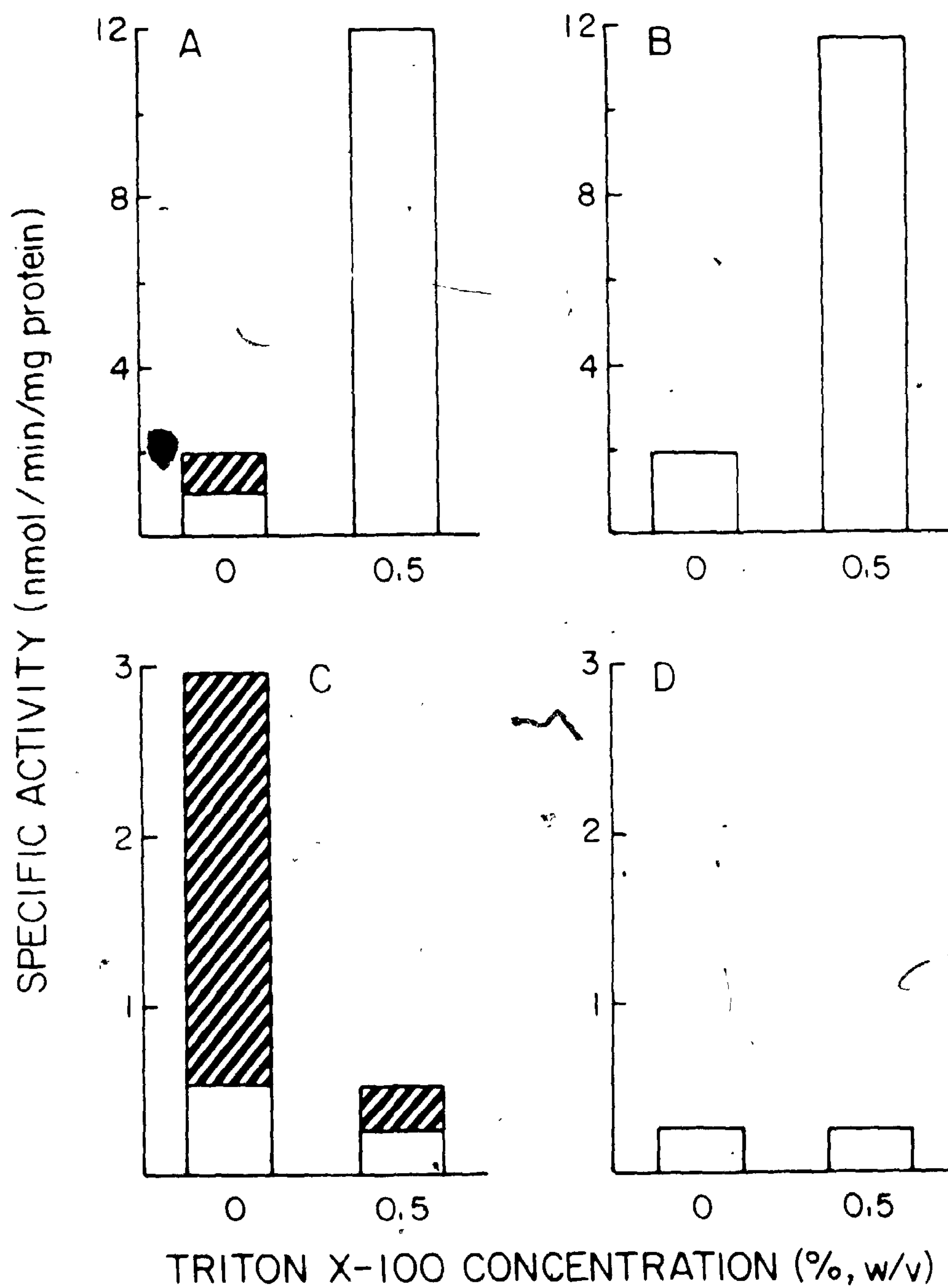
Previous results (Walton and Possmayer, 1985) have demonstrated that the Mg^{2+}-dependent phosphatidate phosphohydrolase activity in rat lung is inhibited by Triton X-100 at concentrations exceeding 0.05% (w/v). Present results show that the total phosphatidate phosphohydrolase activity in microsomes is stimulated 6-fold by 0.5% Triton X-100 (Figure 23a), although the Mg^{2+}-dependent component is reduced to zero. In cytosol (Figure 23c), 0.5% Triton X-100 inhibits the total phosphatidate phosphohydrolase by 80% and the Mg^{2+}-dependent activity by almost 90%. It is possible, however, that in the presence of Triton X-100 the requirement for Mg^{2+} in the microsomal phosphatidate phosphohydrolase is lost. In this case, the resulting increases in total phosphatidate phosphohydrolase activity observed in microsomes could be due to the phosphatidate/Triton X-100 emulsion acting as a better substrate. To test this possibility, we took advantage of the differences in the thermal inactivation profiles of the Mg^{2+}-dependent phosphatidate phosphohydrolase and the Mg^{2+}-independent phosphatase. The Mg^{2+}-dependent phosphatidate phosphohydrolase activity is essentially abolished by

heating to 55°C for 15 minutes, whereas the Mg^{2+}-independent phosphatase is only slightly affected in microsomes and reduced by approximately 60% in cytosol (Figure 22). If the stimulation in total phosphatidate phosphohydrolase activity in microsomes was due to the loss of the Mg^{2+} requirement, the large stimulation seen at 0.5% Triton X-100 should not be observed in the heat-inactivated microsomes. As can be seen in Figure 23b, heat-inactivated microsomes retain the 13-fold stimulation in activity at 0.5% Triton X-100. This result is indicative of a stimulation of the Mg^{2+}-independent phosphatase by Triton X-100 and is consistent with the view that the Mg^{2+}-dependent phosphatidate phosphohydrolase is inhibited by the detergent.

The previous results from our laboratory (Casola and Possmayer, 1981) can be explained in light of these findings. The increases in the PA_{mb}-dependent phosphatidate phosphohydrolase activity at high levels of Triton X-100 were due to the solubilization of the microsomal substrate (Helenius and Simons, 1975; Helenius et al., 1979) with the resulting increase in the PA_{aq}-dependent activity. Assays which employ Triton X-100-solubilized phosphatidate as the substrate (Johnston et al., 1978; Douglas et al., 1983) should be interpreted with caution, since under such conditions the PA_{aq}-dependent phosphatase activity, which is not responsible for glycerolipid synthesis (Walton and Possmayer, 1984), is being measured.

Figure 23.

The effects of Triton X-100 on phosphatidate phosphohydrolase activity of control and heat-inactivated microsomes and cytosols. Control (A,C) or heat-inactivated (B,D) microsomes (A,B) and cytosols (C,D) were assayed in the presence and absence of Mg^{2+}, and the Mg^{2+}-dependent activity (cross-hatched area) calculated by difference. Results are from two separate determinations each done in triplicate.



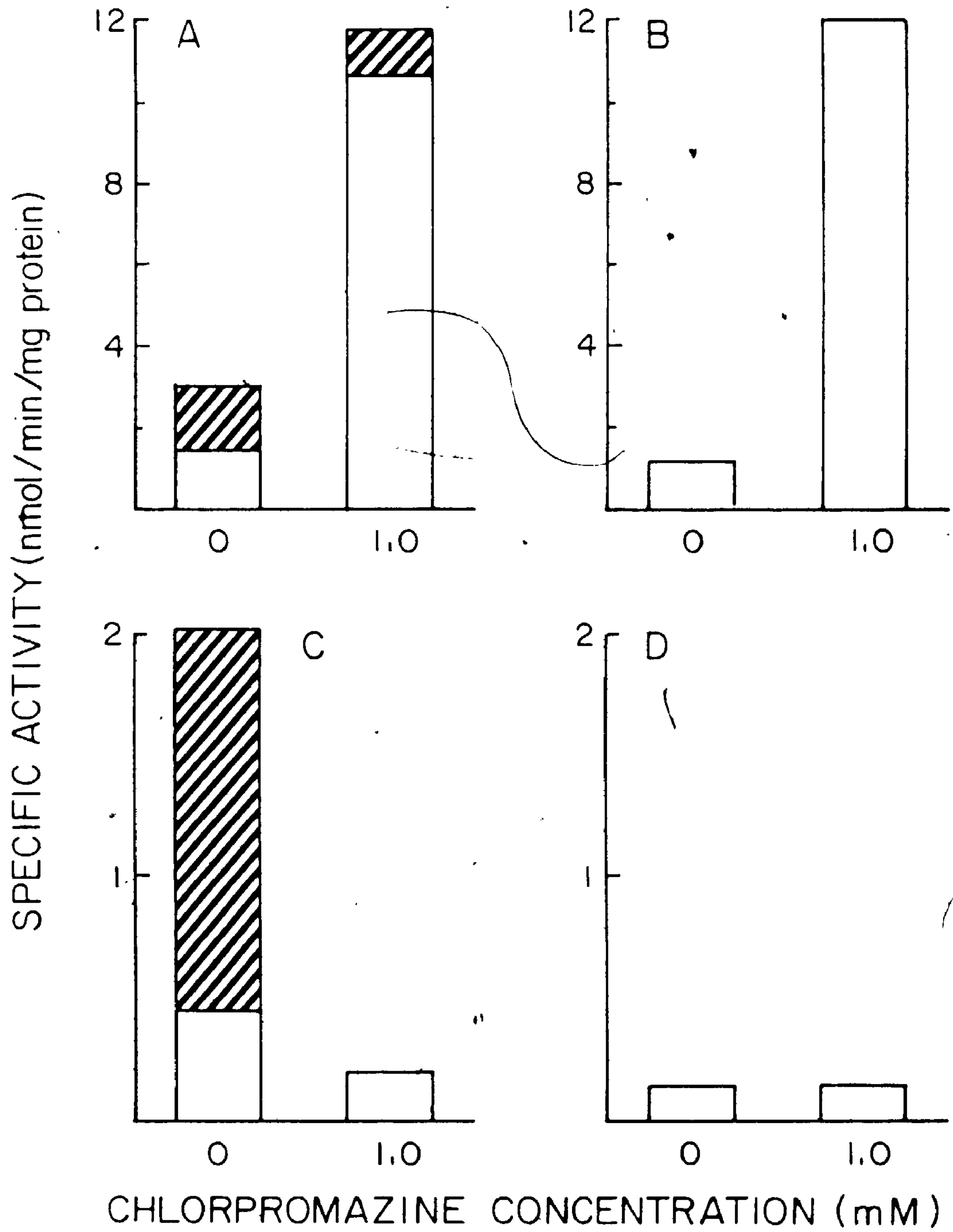
A
B
C
D
12
8
4
3
2
1
0
0.5
SPECIFIC ACTIVITY (nmol/min/mg protein)
TRITON X-100 CONCENTRATION (%, w/v)

5.3.3 Chlorpromazine

It has been suggested that cationic-amphiphilic drugs such as chlorpromazine can replace the requirement for Mg^{2+} in phosphatidate phosphohydrolase from liver (Bowley *et al.*, 1977; Sturton and Brindley, 1980). Although chlorpromazine is a competitive inhibitor of phosphatidate phosphohydrolase, it has been shown to be capable of stimulating phosphatidate phosphohydrolase activity in the absence of Mg^{2+} (Bowley *et al.*, 1977). This stimulation is masked by the addition of Mg^{2+}. These observations have prompted Brindley and coworkers to refer to the phosphatidate phosphohydrolase activity in liver as being "Mg^{2+}-stimulated" rather than Mg^{2+}-dependent (Brindley and Sturton, 1982). In order to determine whether the Mg^{2+} requirement of the Mg^{2+}-dependent phosphatidate phosphohydrolase in lung can be replaced by chlorpromazine, we measured the activity in the presence and absence of both Mg^{2+} and chlorpromazine. The results (Figure 24a) demonstrate that in microsomes 1.0 mM chlorpromazine does stimulate the phosphatidate phosphohydrolase in the presence or absence of Mg^{2+}, and therefore could be replacing the requirement for Mg^{2+}. The presence of 1.0 mM chlorpromazine inhibits the total cytosolic phosphatidate phosphohydrolase activity by 90% and abolishes the Mg^{2+}-dependent activity (Figure 24c). Heat inactivation experiments, such as those described above for Triton X-

Figure 24.

The effects of chlorpromazine on the phosphatidate phosphohydrolase activity of control or heat-inactivated microsomes and cytosols. Control (A,C) or heat-inactivated (B,D) microsomes (A,B) and cytosols (C,D) were assayed in the presence and absence of Mg^{2+}, and the Mg^{2+}-dependent activity (cross-hatched area) calculated by difference. Results are from two separate determinations each done in triplicate.


A
B
C
D
12
8
4
2
1
0
1.0
SPECIFIC ACTIVITY (nmol/min/mg protein)
CHLORPROMAZINE CONCENTRATION (mM)

100, reveal that heat-inactivated microsomes retain the stimulation in the presence of chlorpromazine. Using the same rational as described for Triton X-100, this stimulation could not be due to the Mg^{2+}-dependent phosphatidate phosphohydrolase, but must result from a stimulation of the Mg^{2+}-independent phosphatase. The apparent inhibitory effects of chlorpromazine upon phosphatidate phosphohydrolase, which lead to an accumulation of phosphatidate and acidic phospholipids as measured by in vivo experiments, can be confirmed by the inhibition of the Mg^{2+}-dependent phosphatidate phosphohydrolase activity in vitro. The stimulation of the Mg^{2+}-independent phosphatidate phosphohydrolase activity contradicts the in vivo observations and concurs with earlier findings in lung (Walton and Possmayer, 1984) that this activity is not responsible for glycerolipid biosynthesis.

These experiments illustrate an important point about the nature of the phosphatidate phosphohydrolase activity. In the absence of purified enzyme, there are many phosphatases in the cell that may be hydrolyzing phosphatidate in an opportunistic fashion. Without distinguishing between the Mg^{2+}-dependent and Mg^{2+}-independent activities, erroneous conclusions may be made regarding the stimulatory effects of a particular compound, if that compound stimulates the Mg^{2+}-independent phosphatases.

CHAPTER 6

EVIDENCE TO SUGGEST THAT THE MICROSOMAL AND CYTOSOLIC FORMS OF Mg^{2+}-DEPENDENT PHOSPHATIDATE PHOSPHOHYDROLASE ARE THE SAME ENZYME OF AN AMBIQUITOUS CHARACTER

6.1 INTRODUCTION

As has been previously demonstrated in Chapter 2, the Mg^{2+}-dependent phosphatidate phosphohydrolase is found in both the microsomal and cytosolic fractions from rat lung (Walton and Possmayer, 1985). Observation of this dual location has been reported previously for yeast (Hosaka and Yamashita, 1984), liver (Sturton and Brindley, 1978; Sturton and Brindley, 1980; Goldberg *et al*, 1981), adipose tissue (Jamdar and Fallan, 1973; Moller *et al*, 1981; Moller and Hough, 1982; Jamdar and Osborne, 1983; Jamdar *et al.*, 1984), lung (Casola *et al.*, 1979; Yeung *et al.*, 1979; Casola and Possmayer, 1981a,b; Walton and Possmayer, 1985), and muscle (Kunze *et al.*, 1985). Subcellular distribution studies demonstrate that the bulk of the activity resides in the cytoplasmic fraction after homogenization and differential centrifugation (Walton and Possmayer, 1985).

This distribution is puzzling, as the substrate for this enzyme, and indeed all other enzymes of phosphatidylcholine biosynthesis involving lipid intermediates, are located within the endoplasmic reticulum (Bell and Coleman, 1980). The cytosolic form of the Mg^{2+}-

dependent phosphatidate phosphohydrolase could have arisen from two sources: it could have been removed from the surface of the endoplasmic reticulum during homogenization and centrifugation, or there could be a pool of metabolically non-active enzymes in the cytosol under *in vivo* conditions.

In order to accept either of the previous two conclusions, it is necessary to demonstrate that the enzyme from microsomal and cytosolic fractions is the same activity and the same protein. Evidence for a single protein by direct purification has not yielded results to date. Partial purification of phosphatidate phosphohydrolase from liver (Sedgewick and Hubscher, 1967; Hosaka *et al.*, 1975; Sturton *et al.*, 1981) and lung (Casola, 1981a,b) have increased the specific activity by approximately 16-50 fold. Recently, purifications with a 400-600 fold increase in specific activity have been reported for rat liver (Butterwith, 1984) and Saccharomyces (Hosaka, 1984). However, both of these methods were for soluble phosphatidate phosphohydrolase and as yet, no comparable purifications for microsomal phosphatidate phosphohydrolase have been published. In addition, no purification to homogeneity, a requirement for amino acid analysis and sequence determination, has been reported. It has not been possible to obtain specific antibodies to either of these enzymes (Brindley, D.N. and Saggerson, E.D., personal communications).

Given the instability of phosphatidate

phosphohydrolase and its tendency to remain associated with other contaminating proteins during purification, homogeneous enzyme from both microsomes and cytosol remains the subject for later investigation. However, in the absence of pure protein and thus definitive proof, inference from circumstantial evidence may be made regarding the similarity of the enzyme activities from microsomal and cytosolic fractions.

6.2 MATERIALS AND METHODS

6.2.1 Enzyme Assays

Mg^{2+}-dependent phosphatidate phosphohydrolase assays of microsomes and cytosol were performed as described in Chapter 2.

6.2.2 Molecular Weight Estimations

Samples of cytosol or wash supernatant on ice were brought to 55% saturation with ammonium sulfate. The precipitated proteins were harvested by centrifugation at 12 000 x g for 20 minutes, and resuspended in 1.5 ml 50 mM Tricine (pH 7.4), 100 mM KCl, and 2 mM 2-mercaptoethanol. They were applied to a previously calibrated 53 x 1.5 cm column of Bio-Gel A5m and eluted at a flow rate of 9 ml/h. Fractions of 1.45 ml were collected and the Mg^{2+}-dependent phosphatidate phosphohydrolase activity assayed. The peaks in activity were compared to the position of known proteins for estimations of molecular weight.

6.2.3 Reassociation of Mg^{2+}-dependent phosphatidate phosphohydrolase to previously-stripped microsomes

Rat lung microsomes were treated by washing with 0.5 M NaCl for 10 minutes at 37°C and reisolated by centrifugation at 100 000 xg for 60 minutes. These microsomes were mixed with cytosol and incubated at various salt concentrations for 10 minutes at 37°C. After this incubation, the microsomes were reisolated by centrifugation as above. Microsomes and cytosols, before and after reassociation, were assayed for Mg^{2+}-dependent phosphatidate phosphohydrolase activity.

6.3 RESULTS

6.3.1 Removal of microsomal Mg^{2+}-dependent phosphatidate phosphohydrolase by washing with high salt buffers

Washing microsomes in buffers with NaCl concentrations of up to 1.0 M resulted in the progressive loss in activity of Mg^{2+}-dependent phosphatidate phosphohydrolase (Chapter 3). This activity could be quantitatively recovered in the wash supernatant (Chapter 3).

6.3.2 Reassociation of cytosolic Mg^{2+}-dependent phosphatidate phosphohydrolase with previously-stripped microsomes

Results in Table IV illustrate that cytosolic Mg^{2+}-dependent phosphatidate phosphohydrolase could reassociate with previously-stripped microsomes. The dependence on salt concentration of this reassociation (Figure 25) shows that low salt concentration favours binding between Mg^{2+}-

TABLE IV

Transfer of Mg^{2+}-dependent phosphatidate phosphohydrolase activity from cytosol to microsomes. Microsomes, previously-stripped with saline, were incubated with cytosol and reisolated as described in the Materials and Methods. The initial activity of microsomes was 0.8 nmol/min/fraction.

	TOTAL ACTIVITY nmol/min. per fraction		
Addition	Cytosol	Microsomes	Total
none	12.72 ± 1.26	--	12.72 ± 1.26
Ms-A	7.91 ± 0.51	6.35 ± 0.07	14.26 ± 0.58
Ms-B	8.83 ± 0.21	5.96 ± 0.19	14.80 ± 0.40

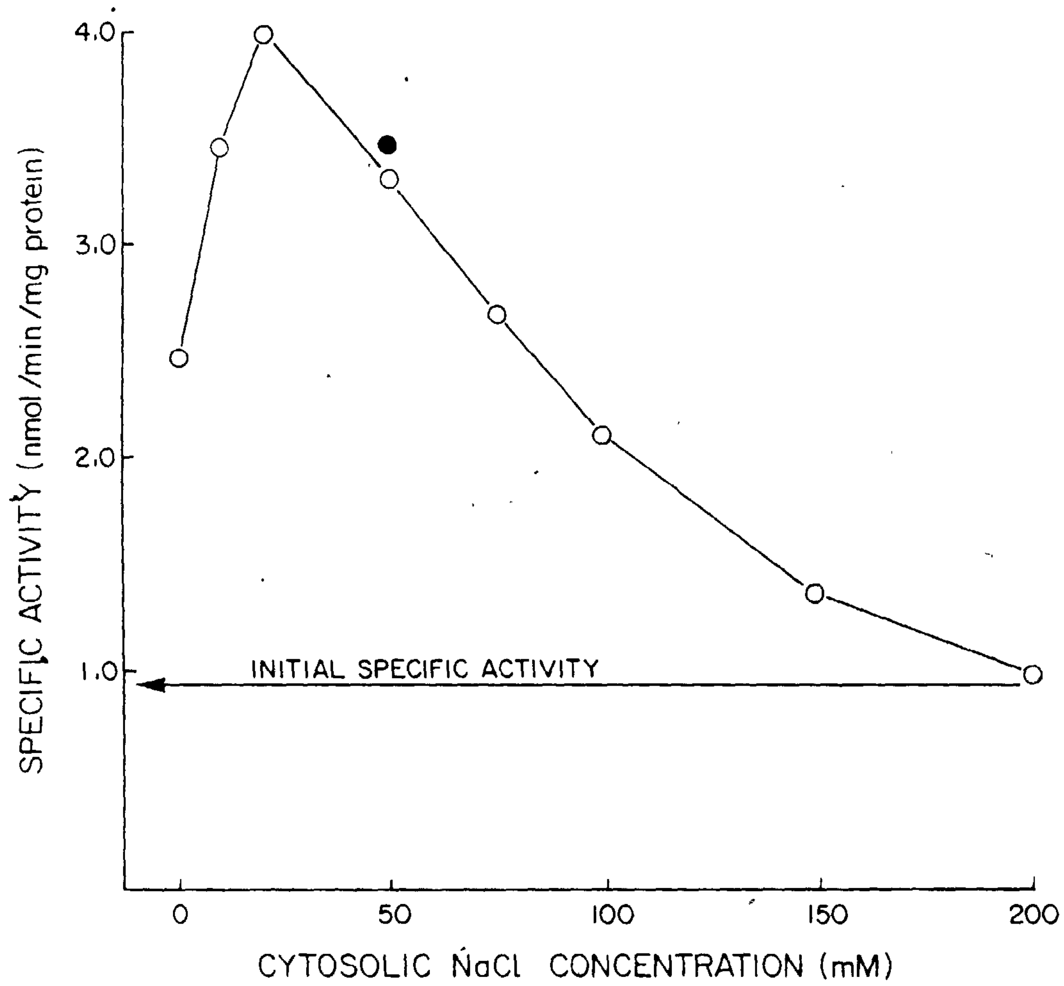
TABLE V

Reassociation of Mg^{2+}-dependent phosphatidate phosphohydrolase activity with heat-treated microsomes. Saline-stripped microsomes were placed in a boiling water bath for 5 minutes prior to incubation with cytosol and reisolation as described in Materials and Methods. Initial activity of the microsomes was 0.0 nmol/minute per mg protein. Specific activity reported in nmol/min. per mg protein.

	Protein Recovered	Specific Activity	Total Activity Recovered	Treated / Control
Ms (Control)	8.24 mg	0.40 ± 0.018	3.30 ± 0.15	
Ms (Heat Treated)	8.66 mg	0.54 ± 0.013	4.68 ± 0.11	1.42

Figure 25.

Effect of cytosolic NaCl concentration on the reassociation of Mg^{2+}-dependent phosphatidate phosphohydrolase with previously-stripped microsomes. Microsomes were incubated with cytosol at indicated NaCl concentrations and reisolated as described in Materials and Methods. Mg^{2+}-dependent phosphatidate phosphohydrolase activity was assayed in reisolated microsomes (open circles). Specific activity of microsomes freshly isolated from post-mitochondrial supernatant in 50 mM NaCl containing isolation buffer (closed circle) included for reference.


SPECIFIC ACTIVITY (nmol/min/mg protein)
4.0
3.0
2.0
1.0
INITIAL SPECIFIC ACTIVITY
0
50
100
150
200
CYTOSOLIC NaCl CONCENTRATION (mM)

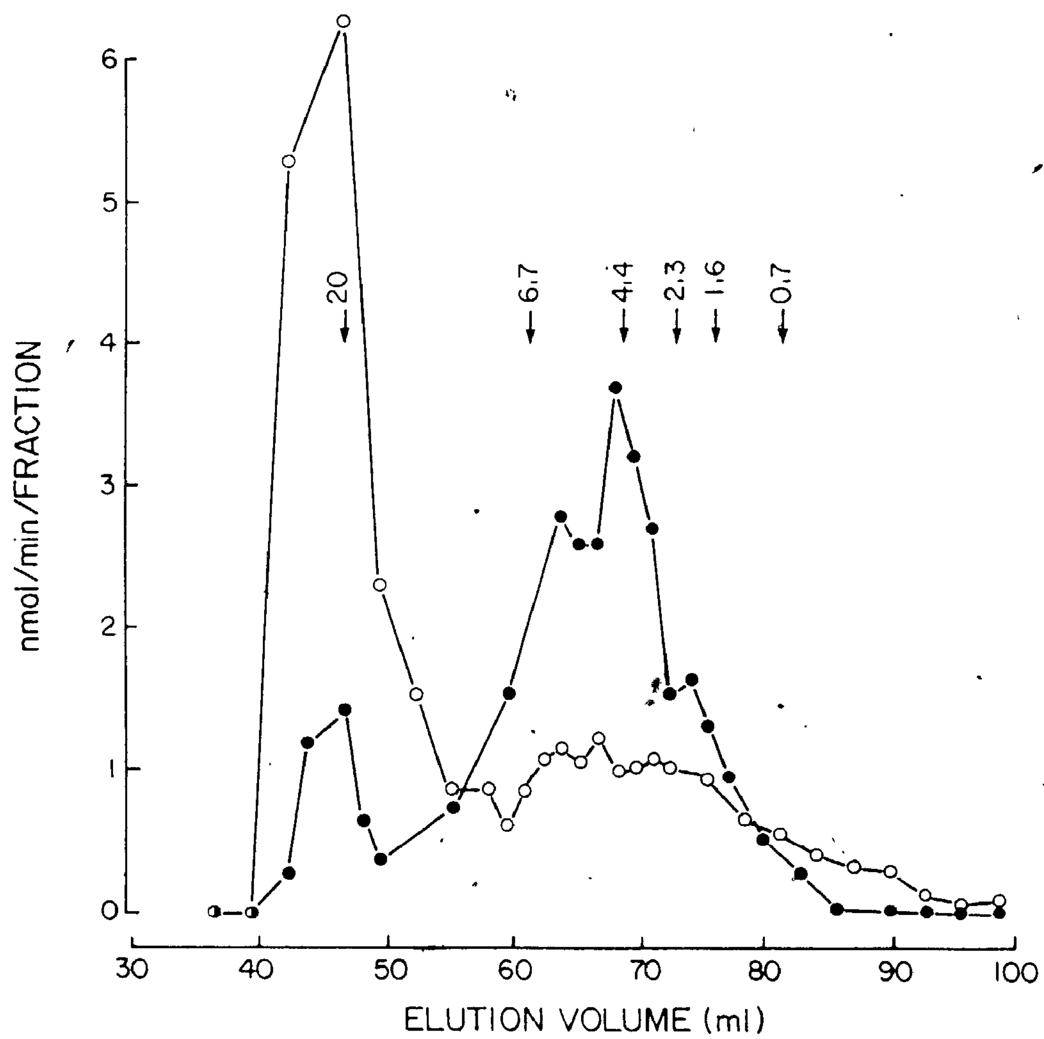
dependent phosphatidate phosphohydrolase and microsomes. Maximum association was observed at 20 mM NaCl. Reassociation at 50 mM NaCl gave specific activities equal to those for microsomes homogenized and isolated in homogenization buffers containing 50 mM NaCl. At 200 mM NaCl, no net change in the specific activity was observed. In order to determine if an intrinsic protein component on the microsomes (i.e. a docking protein) was necessary to facilitate reassociation or if the incubation with cytosol was adding a cofactor to a pre-existing, though inactive, phosphatidate phosphohydrolase activity, microsomes that had been treated in a boiling water bath for 10 minutes were also used for reassociation assays. The results (Table V) show that the boiled, stripped microsomes recovered 40% more activity than the control, stripped microsomes. These results suggest that a specific docking protein is unlikely and that the reassociation is possibly mediated by net membrane charge.

6.3.3 Molecular Weight Estimates

Estimates of molecular weight from the Bio-Gel A5m column (Figure 26) place the molecular weight of both cytosolic and wash supernatant activities at approximately 4×10^5 daltons. The large peak of activity seen at the void volume is particulate material with a molecular weight of greater than 2×10^6. It is assumed that this activity, which represents approximately 70% of the total activity,

Figure 26.

Molecular weight estimations of Mg^{2+}-dependent phosphatidate phosphohydrolase activity from cytosol (closed circles), and wash supernatant (open circles). Procedure of gel filtration as described in Materials and Methods. Arrows indicate elution volume of markers of known molecular weight (x 10^5).


nmol/min/FRACTION
6
5
4
3
2
1
0
20
6.7
4.4
2.3
1.6
0.7
30
40
50
60
70
80
90
100
ELUTION VOLUME (ml)

is aggregated proteins which did not solubilize after ammonium sulfate precipitation.

6.3.4 Other Parameters

Results presented in Chapter 2 demonstrated that the cytosolic and microsomal Mg^{2+}-dependent phosphatidate phosphohydrolase activities are equivalent for such parameters as: a) effects of PC/PA ratio in substrate liposomes, b) effects of Mg^{2+}, c) thermal stability, and d) inhibition by detergents. In addition, chlorpromazine inhibits the Mg^{2+}-dependent phosphatidate phosphohydrolase from both microsomes and cytosol (Chapter 5).

6.4 DISCUSSION

The term *ambiquitous enzyme* was first used by Wilson (1978) to denote an activity in two distinct subcellular locations. The control of enzyme location could then be used as a method of regulating enzyme activity. Hexokinase (Rose and Warms, 1967) is one of the most thoroughly studied of the ambiquitous enzymes. The mitochondrial and soluble forms of this enzyme are in a state of equilibrium with such factors as glucose-6-phosphate, ATP, and inorganic phosphate affecting the subcellular distribution. In times of glycolytic stress, the equilibrium shifts to increase the amount of mitochondrial enzyme, which, based on kinetic measurements, is thought to be the more active form. Other glycolytic enzymes, such as aldolase, LDH,

glutamate dehydrogenase and tyrosine hydroxylase (reviewed by Wilson, 1978) have also been demonstrated to exhibit ambiquitous characteristics. In the synthesis of phosphatidylcholine, the enzyme CPCT also behaves as an ambiquitous enzyme (reviewed by Pelech and Vance, 1984; Vance, 1984). Interconversion between inactive, phosphorylated CPCT and the active, dephosphorylated form is controlled by a protein phosphatase and cAMP-dependent protein kinase (Pelech et al., 1981). However, this method of control can be overridden by the presence of fatty acids (Pelech and Vance, 1984) which cause the CPCT to associate with the membrane of the endoplasmic reticulum. If phosphatidate phosphohydrolase, which also shows a distribution in both the microsomes and cytosol, is controlled by a similar method, this first step is to demonstrate that the cytosolic and microsomal activities are the same enzyme.

Previous experiments in lung have characterized four distinct phosphatidate phosphohydrolase activities (Casola and Possmayer, 1981a). These activities can be subdivided into two activities in two locations, the microsomes and cytosol. Recent results demonstrate that the Mg^{2+}-dependent phosphatidate phosphohydrolase activity, which appears to be equivalent to the activity measured using membrane-bound substrate, exists in both the microsomes and cytosol (Walton and Possmayer, 1985). These results indicated that the microsomal and cytosolic enzymes

preferred the same substrate liposomes, had the same Mg^{2+} requirements, and were both inactivated by detergents. Perhaps the most striking similarity between the two activities was that they were inactivated in a similar manner by high temperature with no regard for subcellular location. The microsomal activity appeared to gain no protection from its membrane association. This would be expected for a peripheral protein.

The demonstration that the microsomal activity could be washed from the membrane surface and quantitatively recovered in the wash supernatant means that the cytosolic enzyme could have come from the microsomes initially. Estimates of molecular weight confirm that the activity removed from the microsomes has the same molecular weight as that activity isolated in cytosol. The large peak of activity seen at the void volume apparently represents aggregated proteins which did not fully solubilize after the ammonium sulfate precipitation. Such aggregates have been reported previously for ammonium sulfate precipitates from cytosol (Casola and Possmayer, 1981b).

The observation that the cytosolic activity could reassociate with previously-stripped microsomes (Tables IV, V, Figure 25) indicates that under different salt conditions, the dissociation-reassociation phenomenon is reversible. This association has been demonstrated in adipose tissue (Moller and Hough, 1982) in which postmitochondrial supernatants with low salt (≈ 50 mM), when centrifuged, yielded microsomes of greater Mg^{2+}-

dependent phosphatidate phosphohydrolase specific activity than supernatant with high salt. An interesting observation of these dissociation and reassociation experiments is that the salt concentration at which half the activity was removed in dissociation experiments and no net change was observed in reassociation experiments was 200 mM. In salt-washing experiments 200 mM NaCl was the concentration at which 50% of the microsomal activity was removed. This value corresponds to the internal cationic concentration of the cell (Guyton, 1981) and indicates that under *in vivo* conditions, the subcellular location of Mg^{2+}-dependent phosphatidate phosphohydrolase is in equilibrium between the endoplasmic reticulum and cytosol. This equilibrium could then be modified by various cellular control mechanisms, such as availability of substrate or free fatty acids.

Although this chapter points out the equivalence of the micrasomal and cytosolic forms of Mg^{2+}-dependent phosphatidate phosphohydrolase in lung, and demonstrates that translocation from one subcellular location to another is possible under various salt conditions, no evidence of such a method of metabolic regulation in lung is presented. Indeed, in light of these observations, the subcellular distribution of Mg^{2+}-dependent phosphatidate phosphohydrolase in homogenized lung tissue must be questioned. Whether this interesting translocation is of biological consequence requires further study.

CHAPTER 7

TRANSLOCATION OF Mg^{2+}-DEPENDENT PHOSPHATIDATE PHOSPHOHYDROLASE BETWEEN CYTOSOL AND ENDOPLASMIC RETICULUM

7.1 INTRODUCTION

Mg^{2+}-dependent phosphatidate phosphohydrolase has been demonstrated to have an ambiquitous distribution as defined by Wilson (1978). This activity has been detected in microsomes and cytosol in liver (Brindley and Sturton, 1982), adipose tissue (Jamdar and Fallan, 1973; Moller _et al._, 1981; Moller and Hough, 1982; Jamdar and Osborne, 1983; Jamdar _et al._, 1984), and lung (Casola and Possmayer, 1979; Walton and Possmayer, 1985). In Chapter 6, evidence was provided to suggest that the activity in these two domains was the same enzyme. Other reports in adipose tissue (Jamdar _et al._, 1984) have drawn this conclusion, although the enzyme from both locations has yet to be purified. The dual location of this enzyme is unanticipated as the substrate is synthesized on the endoplasmic reticulum and does not exist as free monomers. Indeed, all other enzymes with lipid substrates in the biosynthetic pathway of phosphotidylcholine are integral proteins of the endoplasmic reticulum.

The presence of Mg^{2+}-dependent phosphatidate phosphohydrolase activity in microsomes and cytosol could arise in two different ways. First, this enzyme could be a loosely-associated peripheral protein that was removed from

the microsomal surface by the process of homogenization, or secondly, it may exist in two locations *in vivo* and its activity controlled by the intracellular location.

Investigations in adipose tissue demonstrated that treatment with lipolytic hormones or agents such as epinephrine, cAMP, theophylline, or dibutyryl cAMP increased the microsomal and decreased the cytosolic Mg^{2+}-dependent phosphatidate phosphohydrolase activities (Moller *et al.*, 1981). Adrenocorticotropin increased both microsomal and cytosolic activities. The effects of epinephrine could be inhibited by the β-adrenergic inhibitor propranolol, and enhanced by the α-adrenergic inhibitor phentolamine (Moller *et al.*, 1981). These results suggested that the activity of the Mg^{2+}-dependent phosphatidate phosphohydrolase in adipose tissue was being controlled by a β-adrenergic receptor and the activity of adenyl cyclase. The mechanism by which this control was imposed was through the translocation of enzyme from cytosol to the endoplasmic reticulum. Further investigations by Moller and coworkers (Moller and Hough, 1982) demonstrated that the amount of Mg^{2+}-dependent phosphatidate phosphohydrolase activity isolated with the microsomal fraction could be increased by adding KCl, in the range of 40-100 mM, to the homogenization buffer. This effect was not limited to KCl and NaCl, for Mg^{2+}, Ca^{2+}, and spermine could also enhance the adsorption to, or prevent the desorption of, Mg^{2+}-dependent phosphatidate phosphohydrolase from microsomes.

[^{14}C]-Glycerol-3-phosphate incorporation experiments with microsomes demonstrated that the greater the Mg^{2+}-dependent phosphatidate phosphohydrolase bound to the microsomes, the greater the label accumulated in diacylglycerol and triacylglycerol. These authors concluded that the subcellular location of Mg^{2+}-dependent phosphatidate phosphohydrolase could be acting as a control mechanism in glyceride biosynthesis. Additional experiments in adipose tissue (Jamdar and Osborne, 1983) demonstrated that spermine caused an increase in the amount of Mg^{2+}-dependent phosphatidate phosphohydrolase that was associated with the microsomal fraction and that this increase could not be accounted for by cytosolic contamination.

Experiments by Brindley and coworkers in liver have demonstrated that a translocation from cytosol to microsomes occurs *in vivo* as a response to a number of different stimuli (reviewed by Brindley, 1985). Using the digitonin method of Mackell *et al.* (1979) to produce leaky hepatocytes, these researchers demonstrated that oleic acid promoted the translocation of phosphatidate phosphohydrolase from cytosol to particulate fractions (Cascales *et al.*, 1984). Under control conditions, approximately 70% of the phosphatidate phosphohydrolase was cytosolic. When hepatocytes were incubated in 4 mM oleate for 60 minutes, this value fell to 3%.

Further experiments demonstrated that, in cell-free

extracts, acyl-CoA esters caused increased translocation to microsomes to a greater extent than fatty acids alone (Martin-Sanz et al., 1984). The addition of cAMP analogs inhibited the translocation effect in digitonin-treatment assays, but this inhibition could be reversed with oleate (Butterwith et al., 1984b). Insulin and glucagon had opposite effects on the translocation of phosphatidate phosphohydrolase. At low concentrations of oleate, insulin increased and glucagon decreased the proportion of phosphatidate phosphohydrolase associated with membranes (Pittner et al., 1985b).

A recent publication (Hopewell et al., 1985) demonstrated that the oleic acid-stimulated translocation of phosphatidate phosphohydrolase could be inhibited by albumin, which also removed the oleate from the membranes. In addition, the ability of spermine to cause translocation (Martin-Sanz et al., 1984) could be inhibited by ATP, GTP, CTP, or AMP. ATP had no inhibiting effects by itself. Chlorpromazine inhibited the translocation to the membranes. Based on these results, Brindley and coworkers have concluded that the negative charge of fatty acids, their acyl-CoA esters and possibly phosphatidate itself acts to cause the translocation of phosphatidate phosphohydrolase to membranes in liver cells (Hopewell et al., 1985).

To determine whether the previously observed ambiquitous character of the Mg^{2+}-dependent phosphatidate phosphohydrolase in lung could be regulated by oleate

exposure, the present studies in A549 cells were undertaken.

7.2 MATERIALS AND METHODS

7.2.1 Materials

A549 cells were a gift from Dr. Jacob Finkelstein, Department of Pediatrics, University of Rochester. [^{3}H]-oleate was obtained from NEN (Dorval). Digitonin was purchased from Sigma (St. Louis). Cell culture media and materials were purchased from Gibco (Burlington, Ontario).

7.2.2 Methods

7.2.2.1 Cell Culture

A549 cells were grown to confluence on 75 cm^2 flasks (Corning) in Dulbecco's MEM media with 10% (v/v) fetal calf serum. Cells were trypsinized and replated on 50 mm diameter culture dishes. The cells were incubated a further 24 hours with fresh serum-free medium and then used for [^{3}H]-oleate incorporation or digitonin experiments.

7.2.2.2 Lysis of Cells with Digitonin

Digitonin lysis was performed as described by Cascales et al. (1984). Control cells or oleate-pretreated cells were washed twice with Hanks balanced salt solution and culture dishes placed on a glass plate in an ice bath. 1.5 ml of an ice-cold solution of 0.25 M sucrose, 0.5 mM DTT, 10 mM Hepes (pH 7.4), and 4.07 mM digitonin (dispersed by

bath sonication) was added to each culture dish. The dishes were regularly rocked back and forth to wash released cytosol from the cells. Three samples of 0.4 ml each were removed at 1 minute intervals and the remainder removed after 4 minutes. The cell ghosts were scraped from the plate in 1.0 ml of the buffer described above, without the digitonin. Cell ghosts were homogenized by 5-10 passes through a 2.0 ml syringe with a 27-gauge needle. Digitonin was removed by centrifugation in a Beckman bench-top centrifuge at 12 000 x g for 2 minutes. All fractions were assayed immediately.

7.2.2.3 <u>Incorporation of [^{3}H]-oleate</u>

A549 cells in culture dishes were incubated with 1.0 mM oleate for up to 60 minutes. At 15 minute intervals, the cells were washed 3 times with Hanks balanced salt solution and twice with Hanks/ methanol (4:1) to remove oleate from the exterior of the cells. Cells were then scraped from the culture dishes in 2.0 ml methanol. To these homogenates was added 2.0 ml chloroform and the mixtures were vortexed thoroughly. Phases were broken by adding 1.8 ml 0.1 N HCl and the lower chloroform phase was divided into two aliquots. One aliquot was chromatographed on Whatman LK5D TLC plates in a solvent of petroleum ether/ether/acetic acid (80:20:1) to separate neutral lipids. The other aliquot was chromatographed on Whatman LK5D TLC plates in a solvent of chloroform/pyridine/formic acid (75:30:10.5) to separate the phospholipids. TLC

plates were lightly sprayed with Rhodamine 6G (0.1%, in methanol) and glycerolipid spots compared to pure standards. Lipid bands were scraped from the TLC plates into vials for scintilation counting.

7.2.2.4 Enzyme Assays

The activities of Mg^{2+}-dependent phosphatidate phosphohydrolase (Walton and Possmayer, 1984; Walton and Possmayer, 1985), Mg^{2+}-independent phosphatidic acid phosphatase (Yeung *et al.*, 1979) and lactate dehydrogenase (Harwood and Hawthorne, 1969) were assayed as described.

7.3 RESULTS

7.3.1 Release of Cytosolic Enzymes by Digitonin Treatment

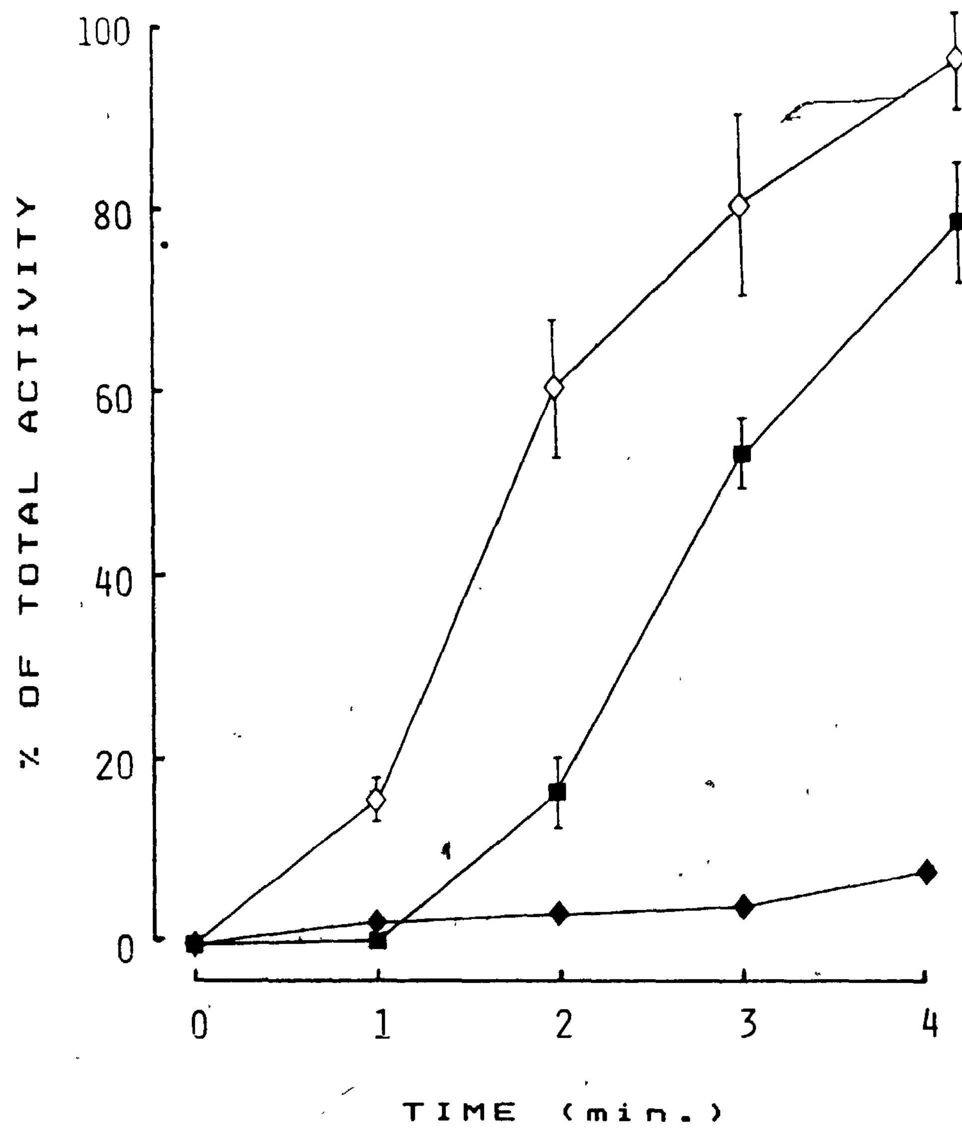
Addition of digitonin resulted in a rapid release in LDH activity from A549 cells (Figure 27). After 4 minutes, approximately 90% of the LDH activity was released from the cells. In contrast, only 7% of the Mg^{2+}-independent phosphatidate phosphohydrolase was released over the 4 minutes incubation. The Mg^{2+}-dependent phosphatidate phosphohydrolase was released more slowly than the LDH, although 78% of the activity was assayed in the soluble fraction after 4 minutes of incubation.

7.3.2 Effects of Oleate on the Release of Mg^{2+}-dependent Phosphatidate Phosphohydrolase Activity by Digitonin Treatment

Incubations with oleate up to 4.0 mM had no effect on

Figure 27.

Release of lactate dehydrogenase (◇), Mg^{2+}-dependent phosphatidate phosphohydrolase (■), and Mg^{2+}-independent phosphatase (◆) from A549 cell cultures, permeated with digitonin. Procedures as described in Materials and Methods. Results from four separate determinations each done in duplicate.


100
80
60
40
20
0
% OF TOTAL ACTIVITY
0
1
2
3
4
TIME (min.)

the distribution of LDH or the Mg^{2+}-independent phosphatidate phosphohydrolase (Figure 28). The Mg^{2+}-dependent phosphatidate phosphohydrolase activity released decreased from 66% in control incubations to 5% in incubations containing 4.0 mM oleate. Whether the decrease in control incubations seen between Figure 27 and Figure 28 is significant is not known.

7.3.3 Incorporation of [^{3}H]-oleate into Glycerolipids

In order to determine if the translocation prevented the increase in phosphatidate in A549 cells, cultures were incubated with 1.0 mM [^{3}H]-Oleate and lipids extracted over the 60 minutes time course. The results (Figure 29) demonstrate that, despite the synthesis of considerable quantities of glycerolipids, labelled phosphatidate did not accumulate at any time during the incubation. Other incorporation experiments with up to 2.0 mM oleate demonstrated no accumulation of phosphatidate (results not shown).

There occurred a considerable accumulation of monoacylglycerol over the 60 minute time course. Lung appears to have potent lipase activity and accumulations of monoacylglycerol have also been observed in glycerol-3-phosphate incorporation experiments with microsomes (Appendix I, Figure 32).

7.4 DISCUSSION

The use of A549 cells in this report must be prefaced

Figure 28.

Effects of oleate on the release of lactate dehydrogenase (◇), Mg^{2+}-dependent phosphatidate phosphohydrolase (■), and Mg^{2+}-independent phosphatase (◆) from A549 cells permeated with digitonin. Procedures as described in Materials and Methods. Results from three separate determinations each done in duplicate.

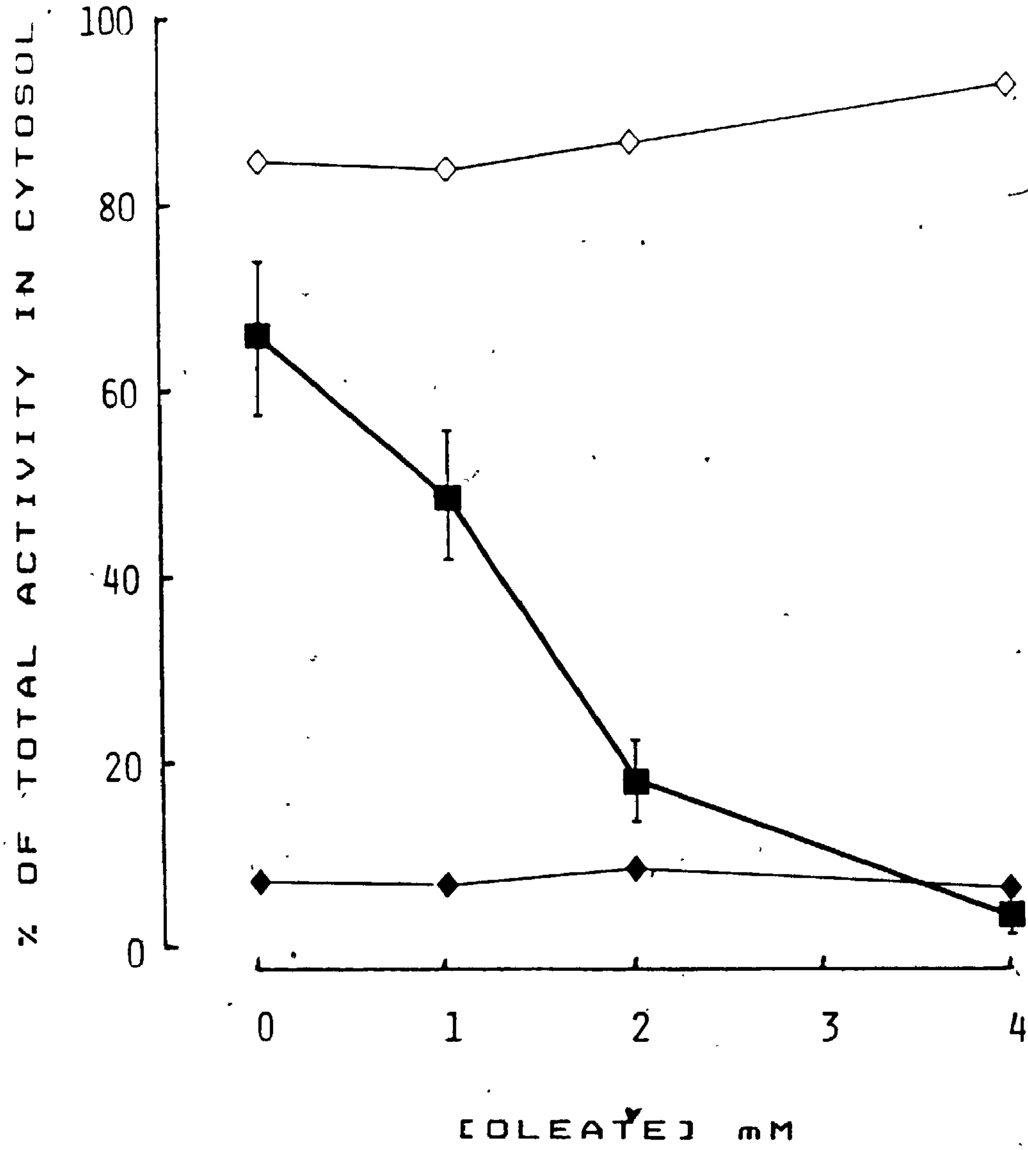
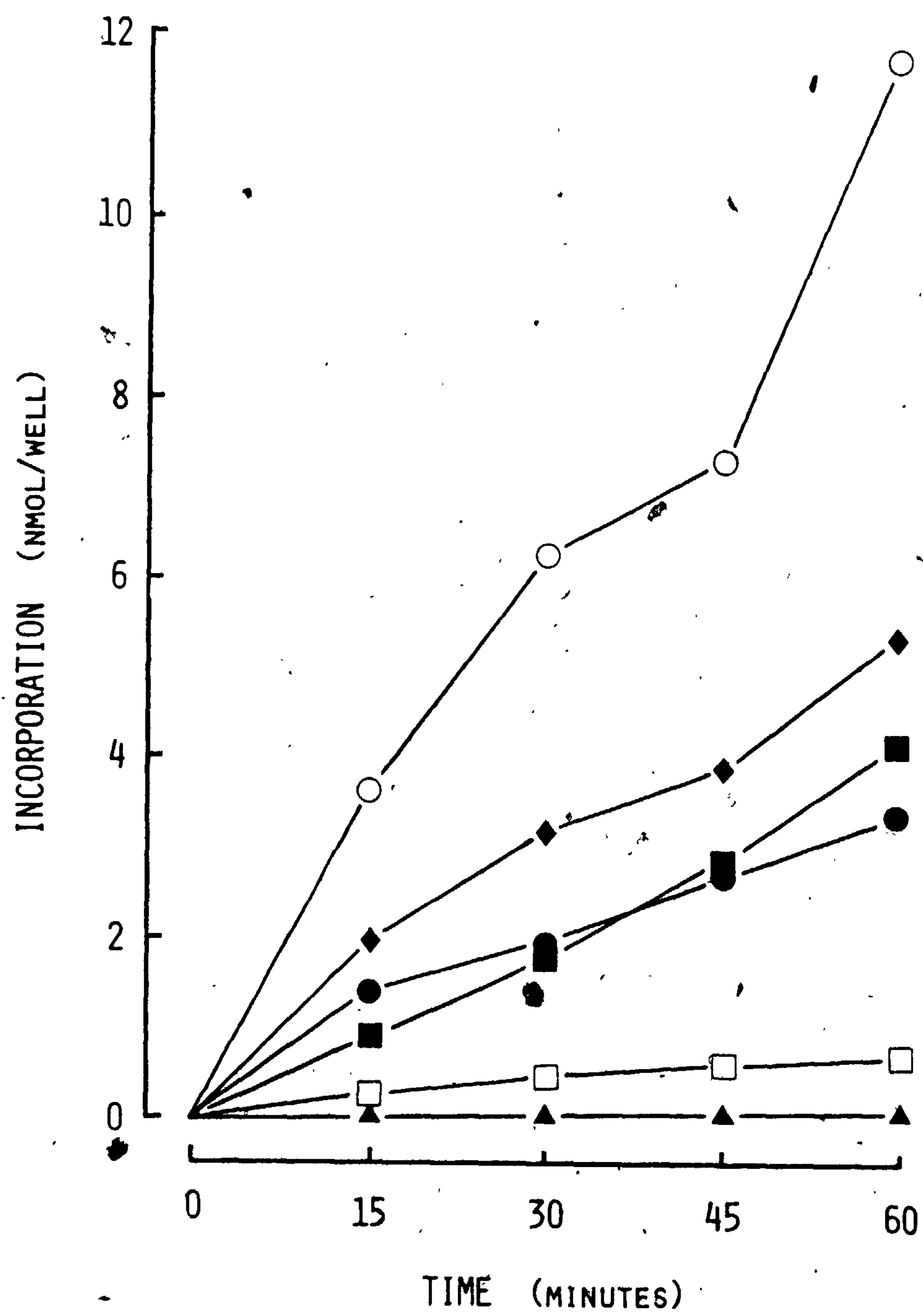

100
80
60
40
20
0
% OF TOTAL ACTIVITY IN CYTOSOL
0
1
2
3
4
[OLEATE] mM

Figure 29.

Time course of incorporation of [^{3}H]-oleate into glycerolipids in A549 cells. Confluent cells were incubated with 1.0 mM oleate (6.12 $\mu C_i/\mu mol$) for time indicated. Isolation and quantification of PA (▲), MG (○), DG (●), TG (◆), PC (■), and PE (□), as described in Materials and Methods. Results presented are of a typical incorporation of four.



INCORPORATION (NMOL/WELL)
12
10
8
6
4
2
0
0
15
30
45
60
TIME (MINUTES)

with a cautionary note. Originally described by Smith and coworkers (Lieber *et al.*, 1976; Smith, 1977), this malignant cell line had morphological and biochemical features of the pulmonary Type II cell. Its principle advantage was the number of cells available. However, recent experiments (Mason and Williams, 1980) have suggested that the phospholipid profile of the A549 cell is different from cultured Type II cells. Whether this difference in composition is due to the malignant cells or the isolated Type II cells is not well understood. However, in the absence of further studies comparing these two model systems, care chould be used in the interpretation of results from A549 cells.

The Mg^{2+}-dependent phosphatidate phosphohydrolase of A549 cells appeared to be capable of translocation from a principally soluble distribution to a location predominantly on cellular membranes. This distribution means that in times of biosynthetic flux through the pathway of phosphatidycholine biosynthesis, the reserve capacity of phosphatidate phosphohydrolase in cytosol could be employed to prepare diacylglycerol.

Whether this translocation acts as a control mechanism for phosphatidate phosphohydrolase activity is not known. Recent reports (Brindley, 1985; Hopewell *et al.*, 1985) suggest that this translocation is controlled by increasing negative charge on the membrane. Fatty acids and phosphatidate could donate this negative charge. The

translocation could then be acting to achieve greater glycerolipid synthesis when requirements rise and, as a protective mechanism within the cell to prevent the accumulation of phosphatidate under conditions of increased glycerolipid synthesis.

Membranes rich in phosphatidate are potentially unstable (for discussion, see Chapter 4.4), especially in the presence of divalent cations. Near the sites of phosphatidate synthesis, be it mitochondrial, peroxysomal or microsomal (reviewed by Bell and Coleman, 1980; Brindley and Sturton, 1982), phosphatidate would accumulate unless it was quickly metabolized. A cytosolic reservoir of phosphatidate phosphohydrolase would enable the cell to control phosphatidate levels at several sites of synthesis. However, the diacylglycerol produced by phosphatidate phosphohydrolase action also perturbs membrane stability (Cullis, P.R., personal communication) and must be rapidly metabolized. Results in liver, reviewed by Pelech and Vance (1984), indicate that CPCT translocation is also controlled by fatty acids. Hence, as the appearance of fatty acids signals the increase in glycerolipid synthesis, the translocation of phosphatidate phosphohydrolase and CPCT place these key enzymes in an active position to quickly pass the potentially perturbing intermediates through the pathway to phosphatidylcholine.

Future experiments to characterize the nature of this translocation should make use of homogeneous Type II cells (Douglas and Teel, 1976; Engle *et al.*, 1980; Simpson *et*

al., 1985). In addition, the development of fetal pre-Type II cells (Scott et al., 1983; Quirie and Possmayer, unpublished results) should facilitate investigations into the intracellular localization of the Mg^{2+}-dependent phosphatidate phosphohydrolase as the process of differentiation and surfactant production begins.

CHAPTER 8

SUMMARY

The results presented in this thesis serve to characterize the nature of the phosphatidate phosphohydrolase activity in rat lung. The initial aim of these investigations was to develop an assay which measured the phosphatidate phosphohydrolase activity upon membrane-bound phosphatidate. This activity had been considered responsible for glycerolipid synthesis in many tissues studied, although conclusive evidence in support of this conclusion was lacking.

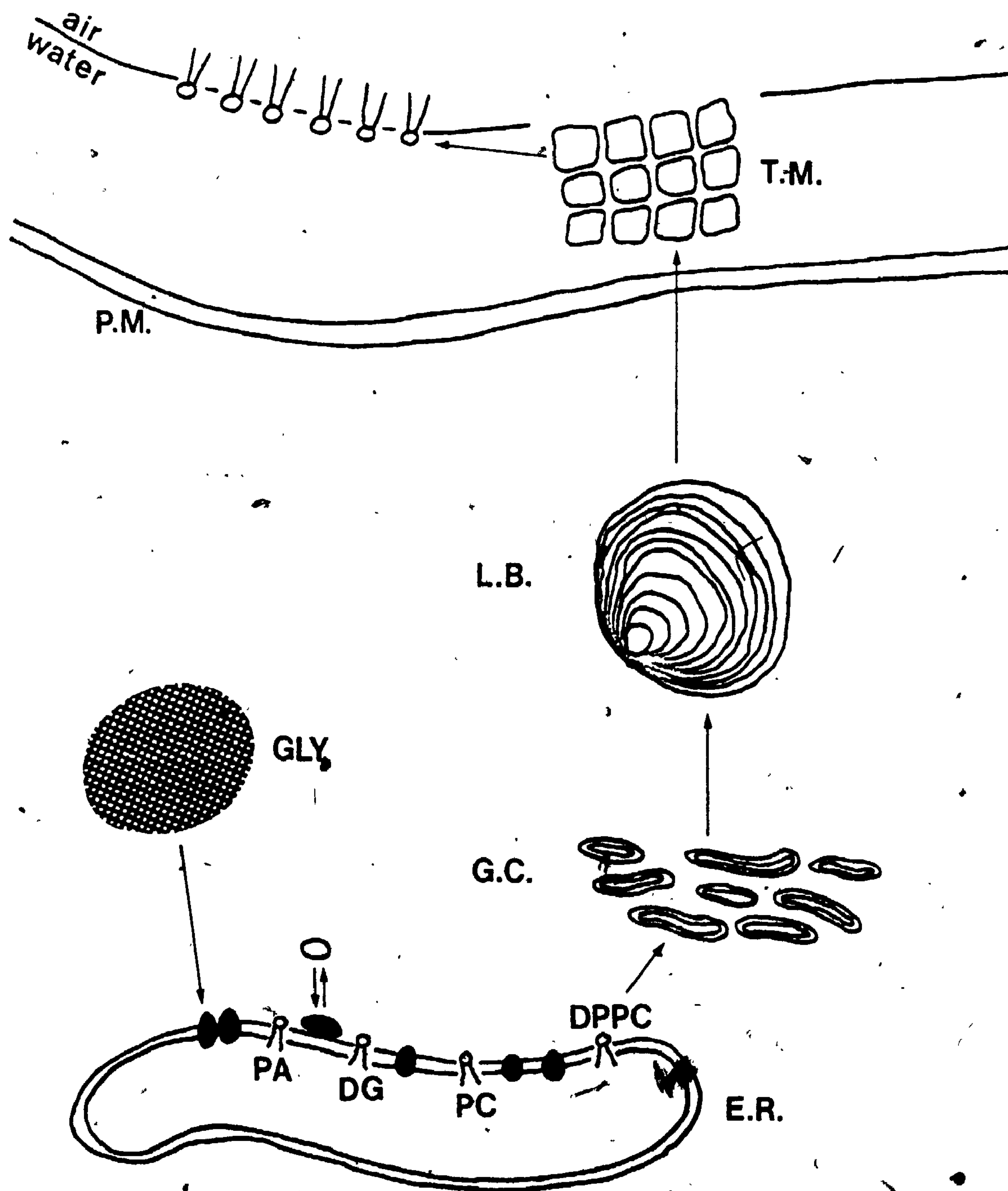
Having an assay of Mg^{2+}-dependent phosphatidate phosphohydrolase that measured the activity upon membrane-bound substrates, it was possible to distinguish which of the two previously described activities was important in the biosynthesis of glycerolipids. The chemically-defined substrate system for the assay of the Mg^{2+}-dependent phosphatidate phosphohydrolase also allowed for the investigation of a number of agents, including divalent cations, detergents, and amphiphilic cations and their effect on phosphatidate phosphohydrolase.

The subcellular distribution of the Mg^{2+}-dependent phosphatidate phosphohydrolase and its ability to dissociate and reassociate with the microsomes led to the conclusion that this activity may exist as an ambiquitous enzyme. Investigations with A549 cells as a model of the Type II pneumocyte provided information on the subcellular

Figure 30.

Schematic representation of the place of Mg^{2+}-dependent phosphatidate phosphohydrolase in the biosynthetic pathway of DPPC for pulmonary surfactant.

The Type II alveolar cell is considered to be the site of synthesis of the pulmonary surfactant. As the biosynthesis of pulmonary surfactant is initiated late in gestation, deposits of glycogen (GLY) provide the precursors for surfactant synthesis. The lipid intermediates in the *de novo* pathway-phosphatidate (PA), diacylglycerol (DG), phosphatidylcholine (PC), and dipalmitoylphosphatidylcholine (DPPC) - are synthesized by enzymes on the cytoplasmic side of the endoplasmic reticulum (E.R.). Following synthesis of PC and DPPC, fragments of the E.R. are pinched off and pass through the golgi complex (G.C.). Vesicles fuse and are encompassed in a limiting membrane to form the lamellar bodies (L.B.) which are the intracellular storage form of the surfactant. The lamellar bodies pass through the plasma membrane (P.M.) into the fluid lining the alveoli. Tubular myelin appears to act as an intermediate form between the lamellar bodies and the surfactant monolayer. Data from Batenburg (1984), Possmayer (1984), and Rooney (1985).


air
water
T.M.
P.M.
L.B.
GLY
G.C.
DPPC
PA
DG
PC
E.R.

distribution of the Mg^{2+}-dependent phosphatidate phosphohydrolase activity *in vivo*, and demonstrated that such agents as oleate could alter the subcellular distribution of the enzyme activity.

In conclusion, results of this thesis indicate that the Mg^{2+}-dependent phosphatidate phosphohydrolase activity of rat lung, as assayed by a mixed-lipid vesicle substrate system, is the important form of the activity *in vivo*. This activity requires Mg-phosphatidate as the salt form of the substrate, and the Mg^{2+}-requirement could not be replaced by chlorpromazine. Detergents, Ca^{2+}, F^{-}, and chlorpromazine inhibit the activity. Furthermore, the ability of the Mg^{2+}-dependent phosphatidate phosphohydrolase to translocate between the endoplasmic reticulum and cytoplasm of the Type II pneumocyte indicates that future studies on the metabolic regulation of this enzyme should concentrate on control of intracellular location.

APPENDIX I

I.1 INTRODUCTION

Phosphatidate phosphohydrolase activity can be measured by monitoring either the inorganic phosphate or diacylglycerol formed as products of the reaction. Theoretically, these two measures should be equivalent, but in practice other factors could lead to discrepancies between the two methods. The measurement of inorganic phosphorus released has been used in adipose tissue (Jamdar and Fallon, 1973; Moller *et al.*, 1977; Cheng and Saggerson, 1978a; Jamdar *et al.*, 1984), liver (Coleman and Hubscher, 1962; Caras and Shapiro, 1975; Van Heusden and Van den Bosch, 1978), brain (McCamon *et al.*, 1965), muscle (Kunze *et al.*, 1985), and lung (Schultz *et al.*, 1974; Jimenez *et al.*, 1974; Garcia *et al.*, 1976, Bleasdale *et al.*, 1978; Johnston *et al.*, 1978; Mavis *et al.*, 1978; Ravinutkala *et al.*, 1978; Casola and Possmayer, 1979;, Rooney *et al.*, 1979; Yeung *et al.*, 1979; Benson, 1980).

In liver, many investigations have also measured the production of diacylglycerol as a method of enzyme activity (Sedgewick and Hubscher, 1967; Smith *et al.*, 1967; Savolainen, 1977; Sturton and Brindley, 1978; Sturton *et al.*, 1978; Roncari *et al.*, 1979; Berglund *et al.*, 1982; Lamb *et al.*, 1982).

Results presented by Brindley and coworkers (Sturton and Brindley, 1978; Sturton and Brindley, 1980) indicate

that release of inorganic phosphate can overestimate the phosphatidate phosphohydrolase activity. As can be seen in Figure 31, inorganic phosphate can be released by any one of three reactions that phosphatidate or its subsequent products may enter into. In addition, the normal practice of measuring aqueous soluble radioactivity could further bias the results. Phospholipase A-type activities that catalyze the conversion of phosphatidate to lysophosphatidate and subsequently to glycerol-3-phosphate have been reported in hepatic microsomes and cytosol (Tzur and Shapiro, 1976; Sturton and Brindley, 1978; Sturton _et al._, 1978; Sturton and Brindley, 1980). In liver, a phosphatidate lipase activity that is stimulated by Mg^{2+} has been reported (Sturton and Brindley, 1980). It has been suggested by Brindley and coworkers (Sturton and Brindley, 1978) that measurement of phosphatidate phosphohydrolase activity by methods which quantify inorganic phosphate release may overestimate the real value. In order to verify that the methods developed for this thesis constitute an accurate evaluation of the Mg^{2+}-dependent phosphatidate phosphohydrolase activity, the present investigations were undertaken.

I.2 MATERIALS AND METHODS

I.2.1 Materials

These have been described previously in Chapters 2 and 3. Preparation of [^{14}C]-phosphatidate was as described by

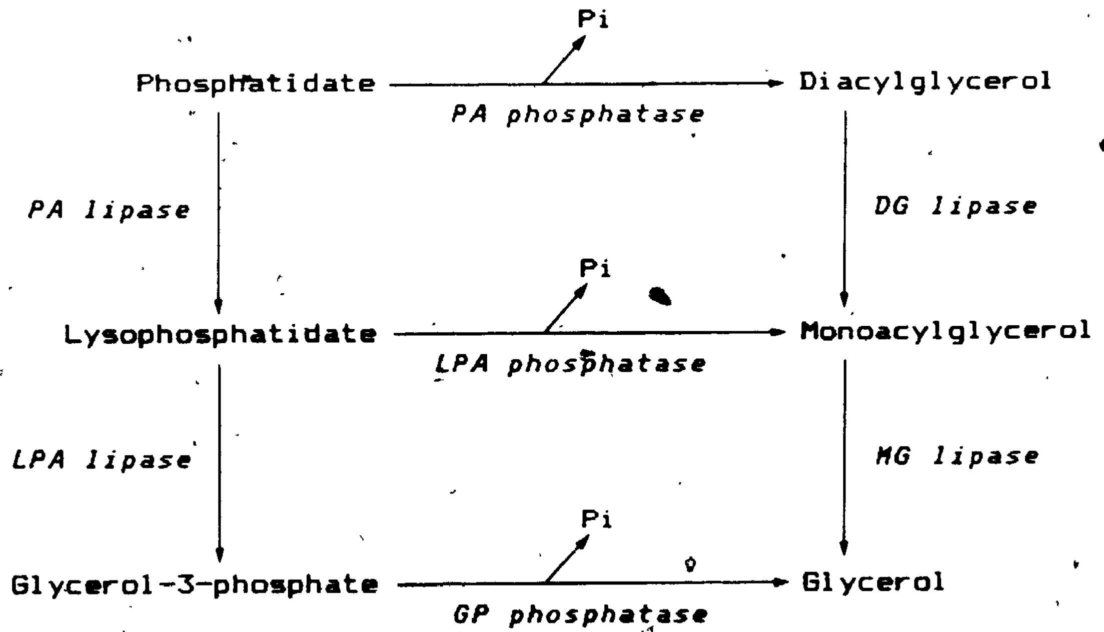


Figure 31.

Possible fates of phosphatidate under conditions of lipase and phosphatase action.

Germershausen et al. (1980), using salt-washed microsomes, and isolated using the same procedures as for [^{32}P]-phosphatidate (Chapter 3).

I.2.2 Methods

Assay of phosphatidate phosphohydrolase has been described in Chapters 2 and 3. Estimations of [^{32}P]-inorganic phosphate in the upper aqueous phase were made by taking a 0.5 ml sample of the aqueous phase, and adding 0.1 ml 10 N H_2SO_4 and 0.3 ml 5% ammonium molybdate to prepare the phosphomolybdate complex (Plaut, 1963). This complex was then extracted with 2.0 ml of isobutanol-benzene (1:1). A sample of the organic phase, which contained the phosphomolybdate complex, was placed in vials, dried, and its radioactivity determined.

Incorporation of [^{14}C]-glycerol-3-phosphate into lipid products was assayed as described in Chapter 3.

I.2.2.1 Assay of glycerol-3-phosphate phosphatase

The assay procedure was modified from that of Coleman and Haynes (1984) except that 100 µM glycerol-3-phosphate was used in assay conditions identical to those employed to measure the Mg^{2+}-dependent phosphatidate phosphohydrolase. The [^{14}C]-glycerol was separated from the precursor [^{14}C]-glycerol-3-phosphate by treating a 1.0 ml aliquot of the upper, aqueous phase of the Bligh and Dyer (1959) extraction with 0.2 ml slurry of an AG1 X-8 cation resin. Preliminary experiments demonstrated that the resin

quantitatively binds glycerol-3-phosphate but not glycerol. The resin was pelleted by centrifugation and an aliquot of the supernatent was taken for scintillation counting to measure the formation of glycerol.

I.3 RESULTS

I.3.1 Differences between aqueous [^{32}P]-phosphate and [^{32}P]-inorganic phosphate as measured by phosphomolybdate complex formation

Inorganic [^{32}P]-phosphate accounts for the majority of the total aqueous [^{32}P]-phosphate radioactivity in the Mg^{2+}-dependent phosphatidate phosphohydrolase assays of microsomes and cytosol (Table VI). Examination of the data revealed that with the microsomal fraction, 88% of the radioactivity released in the Mg^{2+}-dependent assays is also extractable as phosphomolybdate. The remaining radioactivity could be due to glycerol-3-phosphate but this was not examined further. It is also possible that the [^{32}P]-inorganic phosphate was not extracted quantitatively. Nevertheless, it is clear that under the experimental conditions used, the majority of the counts present in the aqueous layer of the Bligh and Dyer system can be attributed to inorganic phosphate released either directly or indirectly from the [^{32}P]-labelled phosphatidate.

I.3.2 Products of phosphatidate phosphohydrolase assays using [^{14}C]-phosphatidate

		^{32}PHOSPHATE RELEASED (nmol/min/mg protein)	
		TOTAL	MOLYBDATE EXTRACTABLE
Microsomes	+ Mg^{2+}	6.86 ± 0.14	6.02 ± 0.07
	− Mg^{2+}	4.85 ± 0.18	4.25 ± 0.20
	Mg^{2+}-dep.	2.01 ± 0.32	1.77 ± 0.27
Cytosol	+ Mg^{2+}	3.52 ± 0.14	2.82 ± 0.17
	− Mg^{2+}	1.03 ± 0.06	0.64 ± 0.02
	Mg^{2+}-dep.	2.49 ± 0.20	2.18 ± 0.19

Table VI.

Comparison between total aqueous ^{32}phosphate and molybdate extractable ^{32}phosphate products of the phosphatidate phosphohydrolase assay. Protocol as described in Materials and Methods. Results from three separate determinations each done in duplicate.

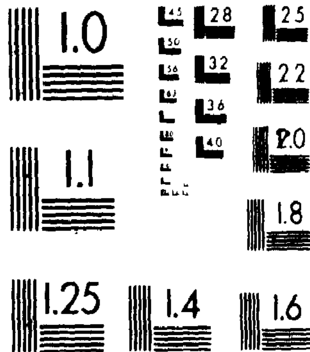
Results in Table VII reveal that diacylglycerol is the predominant product of enzyme activity upon [^{14}C]-phosphatidate. In microsomes, the measure of total [^{32}P]-phosphate and diacylglycerol formed are equal, and in cytosol, the amount of diacylglycerol formed is greater than the total [^{32}P]-phosphate. It is not known whether the 16% increase in diacylglycerol over total [^{32}P]-phosphate observed in cytosol is significant or represents an experimental error in the estimation of the neutral lipids. The discrepancy between the amounts of [^{32}P]-phosphate released (Table VI) and diacylglycerol produced (Table VII) is perplexing. This difference is especially large in the microsomal assays. The increase is limited to the Mg^{2+}-independent phosphatase activity in both subcellular fractions. The substrates used in these experiments may be the source of the discrepancies. The [^{14}C]-phosphatidate was made using equal mixtures of palmitate and oleate, the optimum ratio for incorporation into glycerolipids (results not shown). The [^{32}P]-phosphatidate was made by the phosphorylation of diolein by diglyceride kinase. Whether the dioleyl-phosphatidate is a more readily hydrolyzed substrate by the Mg^{2+}-independent phosphatase is not known. In addition, the nature of the contaminants in the two radioactive substrates may be different. The [^{32}P]-phosphatidate preparation was synthesized in the presence of 5% Triton X-100, and although the TLC methods should be expected to separate the phosphatidate and the Triton X-100, small amounts of

		PRODUCT FORMED (nmol/min/mg protein)				
		DG	MG	LPA	GP	GL
Microsomes	$+Mg^{2+}$	3.03 ± 0.23	0.52 ± 0.03	0.10 ± 0.03	0.07 ± 0.01	0.81 ± 0.03
	$-Mg^{2+}$	1.13 ± 0.03	0.38 ± 0.01	0.07 ± 0.02	0.01 ± 0.07	0.74 ± 0.02
	Mg^{2+}-dep.	1.90 ± 0.26	0.14 ± 0.04	0.03 ± 0.05	0.06 ± 0.08	0.07 ± 0.05
Cytosol	$+Mg^{2+}$	3.04 ± 0.09	0.35 ± 0.03	0.27 ± 0.05	0 ± 0.01	0.41 ± 0.02
	$-Mg^{2+}$	0.15 ± 0.01	0.15 ± 0.01	0.35 ± 0.03	0.01 ± 0.02	0.24 ± 0.02
	Mg^{2+}-dep.	2.89 ± 0.10	0.20 ± 0.04	0 ± 0.08	0 ± 0.03	0.17 ± 0.04

Table VII.

Products of phosphatidate phosphohydrolase assay employing [^{14}C]-phosphatidate. Protocol as described in Materials and Methods. Results are from three separate determinations each done in duplicate.

3 OF/DE 3



detergent may associate with the phosphatidate through the isolation. The presence of Triton X-100 may be acting to stimulate a microsomal, Mg^{2+}-independent phosphatase.

Significant accumulation of monoglyceride occurred in the presence and absence of Mg^{2+}, although the Mg^{2+}-dependent accumulation was less than 10% of the diacylglycerol formed.

In the microsomes and cytosol, the appearance of glycerol was observed in incubations with and without Mg^{2+}. Whether this glycerol is formed from monoacylglycerol or glycerol-3-phosphate cannot be determined with certainty. However, the low levels of glycerol-3-phosphate that accumulate and the lack of quantitative hydrolysis under these conditions (Figure 33) indicate that monoacylglycerol is the most likely source. Experiments containing 100 µM glycerol-3-phosphate, to block the hydrolysis of [^{32}P]-glycerol phosphate in a phosphatidate phosphohydrolase assay, demonstrated no change in the amount of inorganic phosphate released (results not shown) and as such, indicate that the formation of glycerol most likely occurs via the deacylation of monoacylglycerol. The Mg^{2+}-dependent accumulation of glycerol and glycerol-3-phosphate was small in relation to the other products.

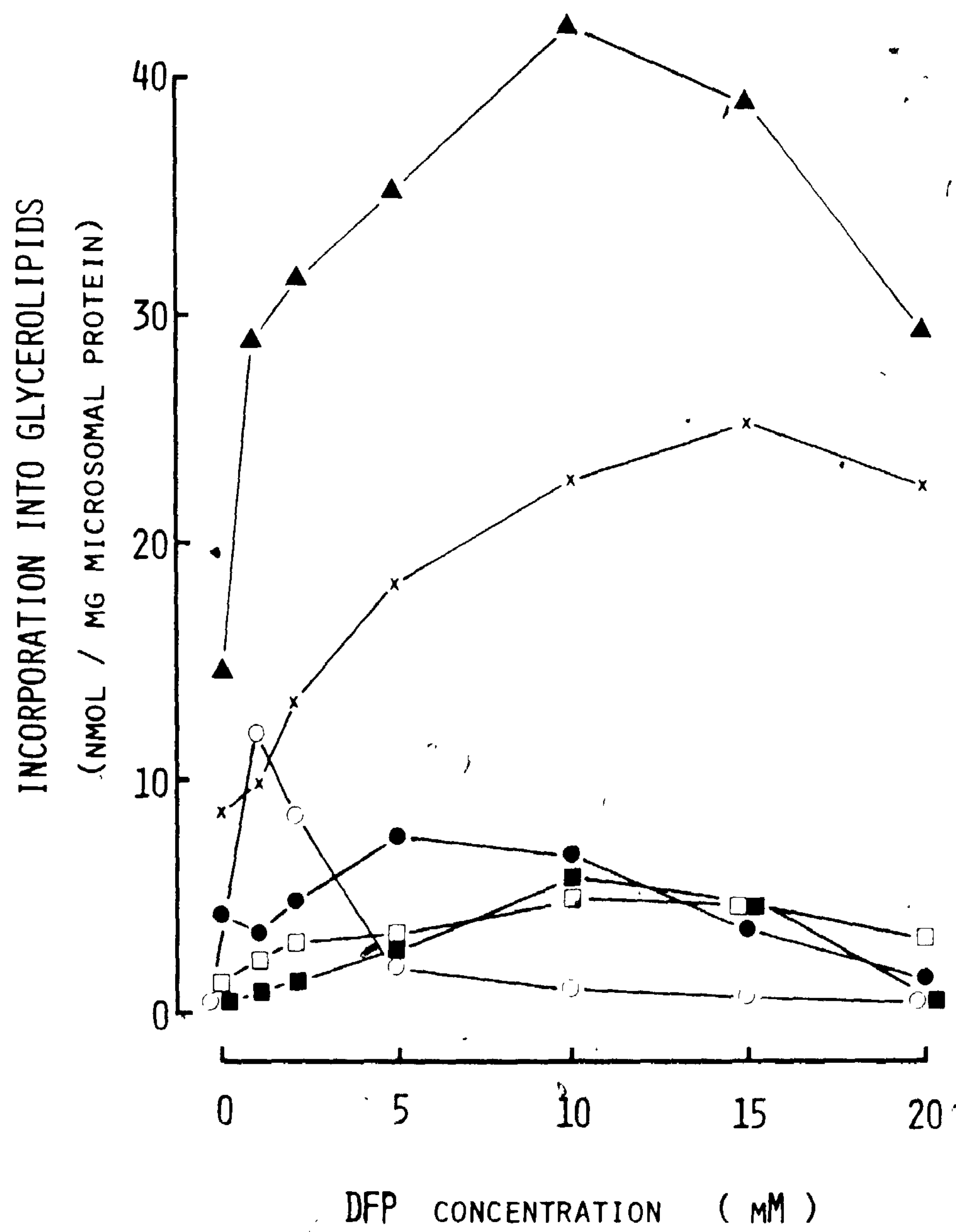
I.3.3 Inhibition of lipase activity with DFP

Results from Chapter 3 (Table III) indicate that 5 mM DFP was added to assays of [^{14}C]-glycerol phosphate

incorporation. DFP was required to inhibit lipase activity that caused a breakdown of endogenously-generated diacylglycerol and inhibited the formation of phosphatidylcholine. Figure 32 shows the accumulation of radiolabel from [^{14}C]-glycerol-3-phosphate into microsomal lipids at various DFP concentrations. At 1.0 mM DFP, the amount of label in monoacylglycerol had increased 28-fold, indicating that a potent monoacylglycerol lipase had been inhibited by this concentration of DFP. At higher levels (1-10 mM), the amount of label present in diacylglycerol, triacylglycerol, and phosphatidylcholine increased 5-fold, 12-fold, and 2-fold respectively. The decrease in label into monoacylglycerol over this range appears to be a result of diminished formation from diacylglycerol. Incorporation of label into phophatidate also increases 3-fold at 15 mM DFP. This increase could be explained by two mechanisms. The activity of phosphatidate phosphohydrolase could be inhibited by DFP or a phosphatidate lipase could have been inhibited. The evidence suggests that the inhibition of phosphatidate phosphohydrolase is more likely under these conditions. At intermediate levels of DFP (10-15 mM), the incorporation of label into phosphatidate is increasing while the incorporation of label into diacylglycerol, triacylglycerol, and phosphatidylcholine is decreasing. If the phosphatidate phosphohydrolase activity was unaffected by these levels, the label should accumulate in diacylglycerol. At high levels (25 mM) of DFP, the glycerol-3-phosphate acyltransferases appear to be

Figure 32.

Effects of DFP on the incorporation of [^{14}C]-glycerol-3-phosphate into microsomal glycerolipids. Assay conditions as described in Materials and Methods (Chapter 3). Incorporation of label into phosphatidate (✕), monoacylglycerol (○), diacylglycerol (□), triacylglycerol (■), and phosphatidylcholine (○). Total incorporation (▲) at 25 mM DFP was zero.


INCORPORATION INTO GLYCEROLIPIDS
(NMOL / MG MICROSOMAL PROTEIN)
40
30
20
10
0
0
5
10
15
20
DFP CONCENTRATION (mM)

inhibited and no label appears associated with the microsomal lipids.

I.3.4 Glycerol-3-phosphate phosphatase activity

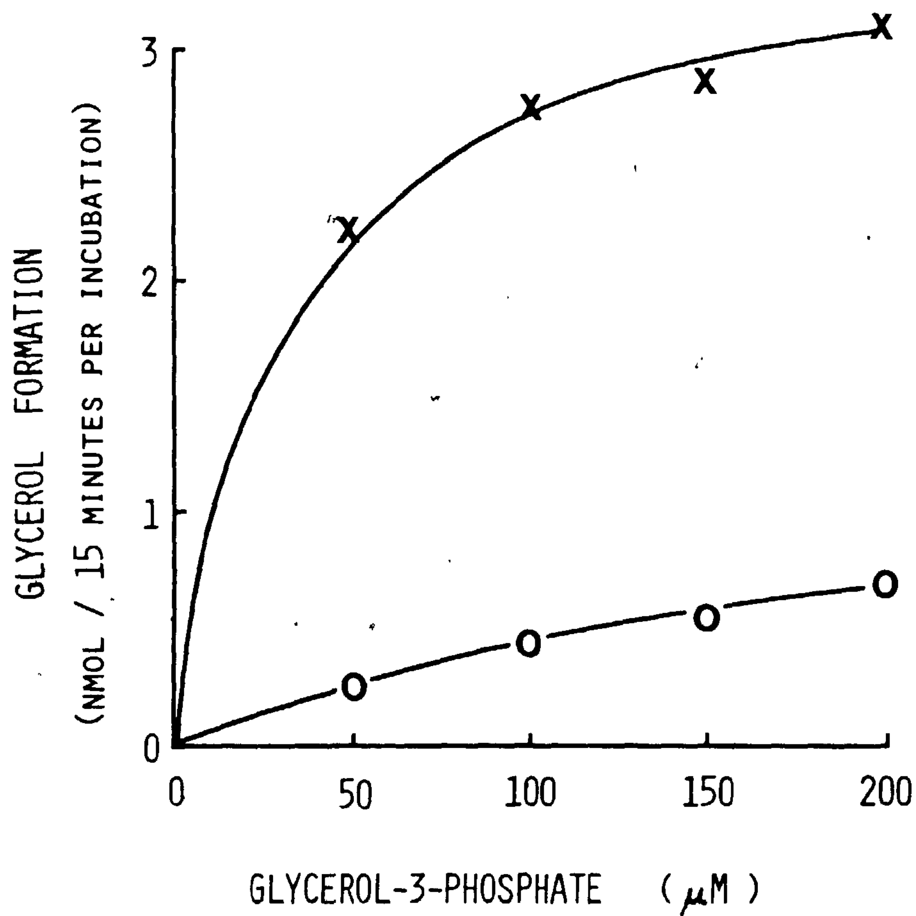
Results shown in Figure 33 indicate that there exists a Mg^{2+}-dependent glycerol-3-phosphate phosphatase activity in cytosol and microsomes under conditions employed to assay Mg^{2+}-dependent phosphatidate phosphohydrolase. Kinetic constants equalled: K_m = 26 µM, V_{max} = 3.44 nmol/15 minutes per incubation for microsomes, and K_m = 262 µM, V_{max} = 1.57 nmol/15 minutes per incubation for cytosol. These results demonstrate that in microsomes, quantitative dephosphorylation of glycerol phosphate could only occur under very low (less than 0.5 nmol formed per incubation) levels.

I.4 DISCUSSION

The studies by Brindley and coworkers (Sturton and Brindley, 1978; Sturton *et al.*, 1978; Sturton and Brindley, 1980) indicate that, in liver, a potent deacylase system is present, which is active upon phosphatidate. Consequently, assays based on [^{32}P]-inorganic phosphate release must be characterized to ensure that radioactivity measured in the aqueous phase of the extraction system is a result of the phosphatidate phosphohydrolase activity and not the result of deacylation to [^{32}P]-glycerol-3-phosphate. Furthermore, the hydrolysis of glycerol-3-phosphate to inorganic

Figure 33.

Conversion of glycerol-3-phosphate to glycerol under conditions used to assay phosphatidate phosphohydrolase. [^{14}C]-Glycerol-3-phosphate at concentrations indicated was added to standard phosphatidate phosphohydrolase assays for microsomes (X), and cytosols (O), and the formation of glycerol measured as described in Materials and Methods.


GLYCEROL FORMATION
(NMOL / 15 MINUTES PER INCUBATION)
0
1
2
3
0
50
100
150
200
GLYCEROL-3-PHOSPHATE (μM)

phosphate and glycerol may also contribute to falsely elevated [^{32}P]-inorganic phosphate levels.

In this report, we have characterized the Mg^{2+}-dependent phosphatidate phosphohydrolase assay by demonstrating that: 1) inorganic phosphate released in the aqueous layer is predominately molybdate extractable 2) amounts of Mg^{2+}-dependent diacylglycerol produced are comparable with aqueous [^{32}P]-phosphate measured 3) lung contains lipase activity which can degrade neutral lipids, particularly diacylglycerol, and lead to falsely decreased measures of phosphatidate phosphohydrolase activity if diacylglycerol is measured. These lipases are proportionately less active upon liposomal substrates than upon microsomal neutral lipids as the results of the DFP experiments demonstrate. Whether this is a result of the membrane-bound nature of the microsomal lipases and the inaccessibility of the liposomal lipids is not known 4) a glycerol-3-phosphate phosphatase activity in lung under conditions used to measure the Mg^{2+}-dependent phosphatidate phosphohydrolase has been observed. The activity could catalyze the quantitative conversion of glycerol-3-phosphate to glycerol and inorganic phosphate under low concentrations of glycerol-3-phosphate. However, inclusion of high levels of glycerol phosphate to block the glycerol phosphate phosphatase does not decrease the release of inorganic phosphate from [^{32}P]-phosphatidate.

In conclusion, the Mg^{2+}-dependent phosphatidate phosphohydrolase activity, as measured by phosphate

released in the presence and absence of Mg^{2+} provides a compatible measurement of phosphatidate phosphohydrolase activity with the assay of diacylglycerol formation in the lung. Furthermore, this assay is not subject to underestimation as a result of the neutral lipid lipase activities present in lung tissue.

APPENDIX II

SYNTHESIS OF [^{14}C-U]-DIHYDROXYACETONEPHOSPHATE

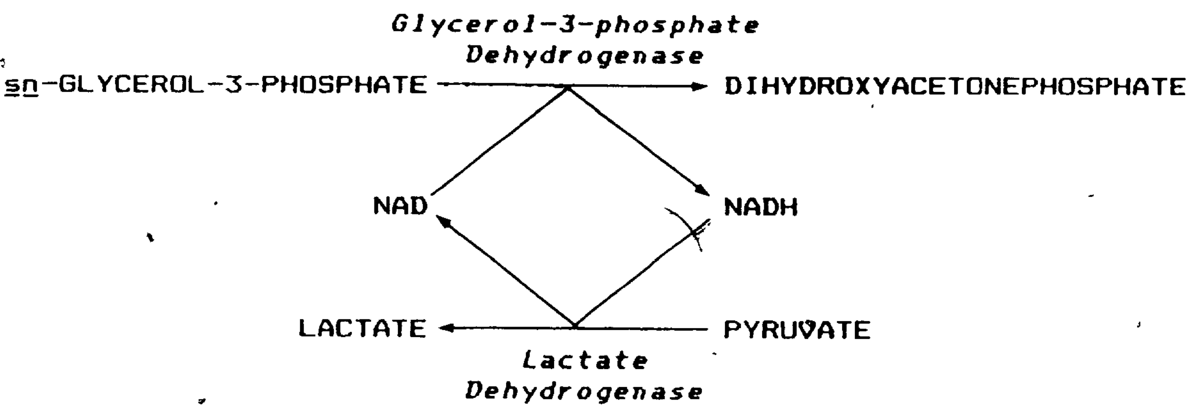
II.1 INTRODUCTION

Dihydroxyacetonephosphate (DHAP) can be prepared enzymatically from glycerol-3-phosphate (GP) via the action of glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) which catalyzes the conversion of these two phosphorylated trioses. The equilibrium constant of this conversion however is 1.0×10^{-12} (Telegdi, 1968), making the reaction direction strongly in favour of GP formation. In an attempt to decrease the ΔG for the reaction, conditions were sought to minimize the NADH/NAD ratio. Using high levels of NAD would slightly favour the production of DHAP, but relevant amounts of synthesis could only be achieved through the elimination of the NADH formed. With the NADH concentration approaching zero, the production of DHAP would be the predominant direction of synthesis, as expressed by the Principle of Le Chatelier.

A system of regenerating the NAD using the reduction of non-phosphorylated substrates was employed (Figure 34). Lactate dehydrogenase (EC 1.1.1.27) was chosen because its equilibrium constant strongly favours the production of lactate (Hakala et al., 1956). Under these conditions, NADH produced from the synthesis of DHAP would be reconverted to NAD via the action of LDH.

Figure 34.

Coupled reaction to synthesize dihydroxyacetonephosphate and to regenerate NAD. Method of synthesis as described in Appendix II.


Glycerol-3-phosphate
Dehydrogenase
sn-GLYCEROL-3-PHOSPHATE
DIHYDROXYACETONEPHOSPHATE
NAD
NADH
LACTATE
PYRUVATE
Lactate
Dehydrogenase

II.2 MATERIALS AND METHODS

GPDH, LDH, NAD, and pyruvate were purchased from Boehringer-Mannheim (Dorval). [^{14}C-U]-GP was purchased fro New England Nuclear (Dorval). BioRad AG-1 X-8 anion exchange resin was purchased from BioRad Laboratories (Mississauga). All other chemicals and reagents were obtained from Fisher Scientific (Toronto).

Reaction mixtures contained 130 mM pyruvate, 130 mM NAD, 25 units GPDH, 55 units LDH, 10 µCi [^{14}C-U]-GP (0.07µmol), 100 mM Tris buffer, pH 7.8 in a reaction volum of 2.3 ml. These mixtures were incubated for one hour at room temperature. After that time, the mixture was applie to an AG-1 X-8 anion exchange column, previously equilibrated in the formate cycle. Column volume was 16 m and the flow rate was 1.67 ml/minute. Reaction products were eluted from the column with a linear gradient of 1.0-4.5 M formic acid. 5 ml fractions were collected and samples taken for radioactivity and chemical determinations.

Fractions containing DHAP were pooled and the formic acid removed by four-fold extraction with two volumes of ether. Pooled fractions were then frozen in dry ice and lyophilized. [^{14}C-U]-DHAP was dissolved and and stored in 50 mM carbonate buffer at pH 4.5.

II.3 RESULTS

In order to determine if the LDH activity was stable

II.2 MATERIALS AND METHODS

GPDH, LDH, NAD, and pyruvate were purchased from Boehringer-Mannheim (Dorval). [^{14}C-U]-GP was purchased from New England Nuclear (Dorval). BioRad AG-1 X-8 anion exchange resin was purchased from BioRad Laboratories (Mississauga). All other chemicals and reagents were obtained from Fisher Scientific (Toronto).

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II.3 RESULTS

In order to determine if the LDH activity was stable

at pH 7.8, a pH curve was done (Figure 35). The results show that 80% of the maximum activity exists at pH 7.8. Time curves (results not shown) indicated that this activity was stable for greater than 60 minutes.

GP and DHAP could be separated using the AG-1 X-8 column (Figure 36). Both peaks tested positive for vicinal hydroxyl groups (periodate-Schiff) and peak 2 tested positive for keto groups (dinitrophenylhydrazine). Spectrophotometric measurements (Figure 37) indicate that the protein and cofactors come through the column in the first few fractions. Although not quantified in this study, it is assumed that the non-phosphorylated lactate and pyruvate also pass directly through the column.

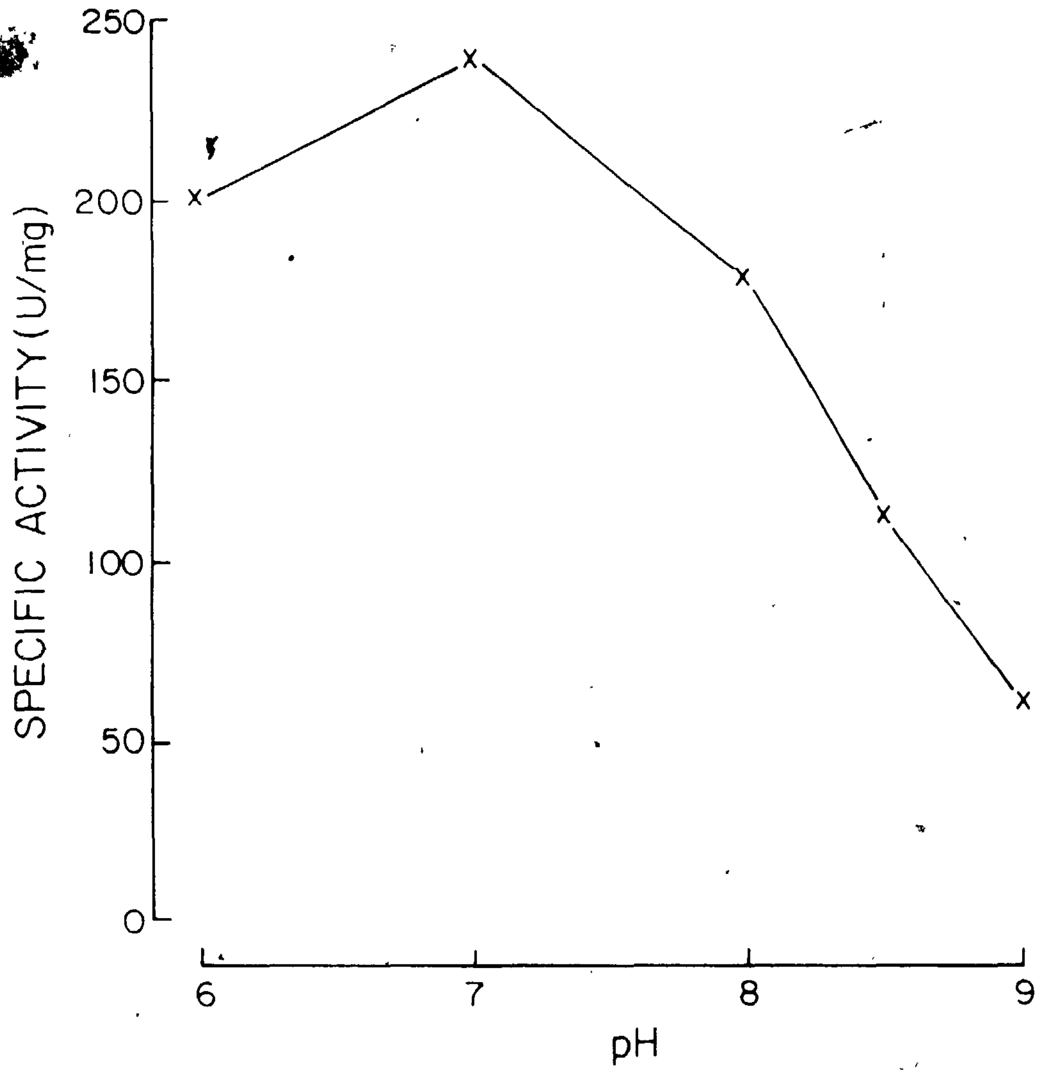
Under optimal conditions the conversion of GP to DHAP is virtually complete (Figure 38). Yields of 85-98% were recorded in five succesive syntheses.

Extraction of formic acid in pooled fractions with ether removed 95% of the titratable acidity (Figure 39) without removing any of the radioactivity (results not shown).

Lyophilized samples, made up in water and reapplied to the AG-1 X-8 column reappeared as the DHAP peak (results not shown), indicating that no breakdown occurred and recoveries of 80% could be achieved.

Figure 35.

The pH profile of lactate dehydrogenase.



SPECIFIC ACTIVITY (U/mg)
250
200
150
100
50
0
6
7
8
9
pH

Figure 36.

Separation of GP and DHAP by anion exchange chromatography.

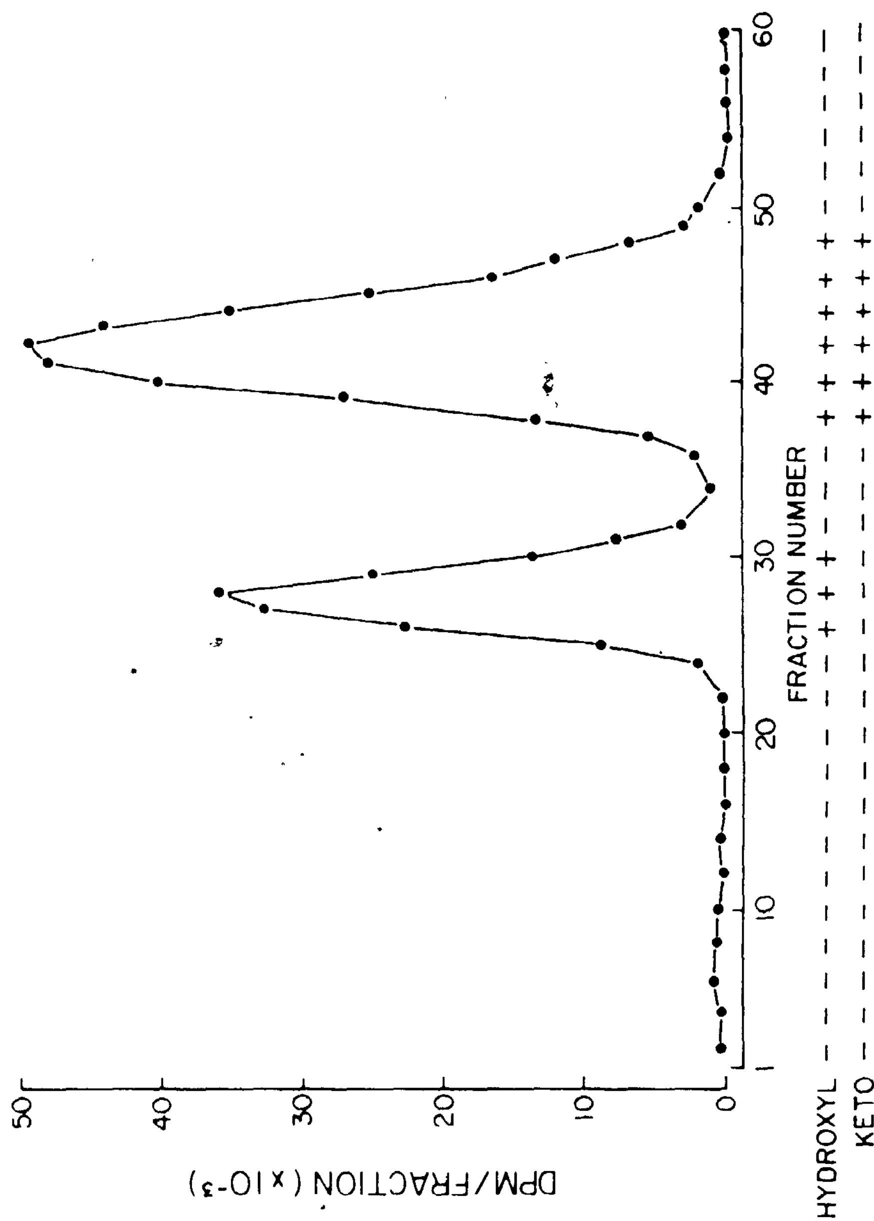
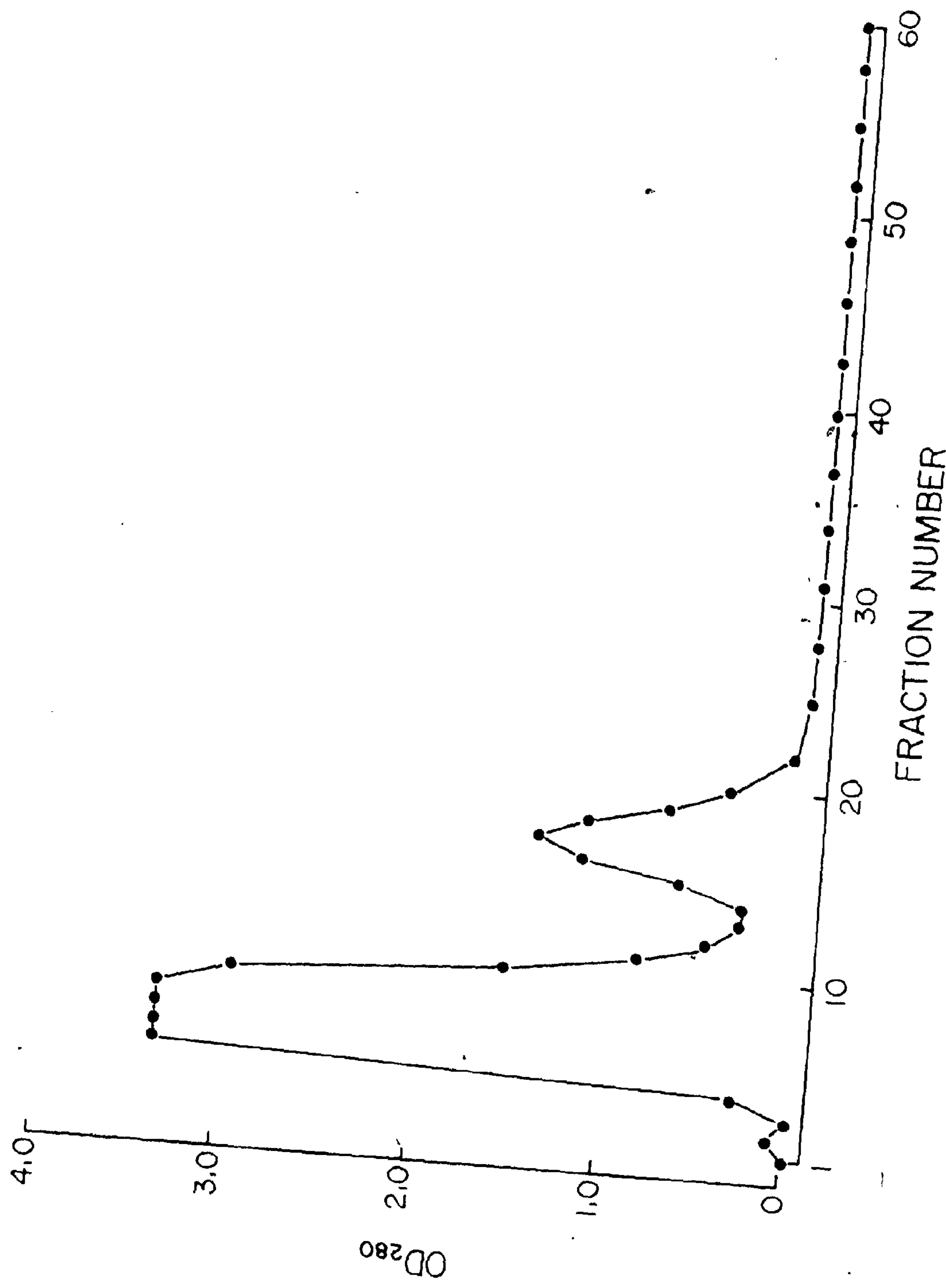

DPM/FRACTION (x 10-3)
50
40
30
20
10
0
1
10
20
30
40
50
60
FRACTION NUMBER
HYDROXYL
KETO

Figure 37.

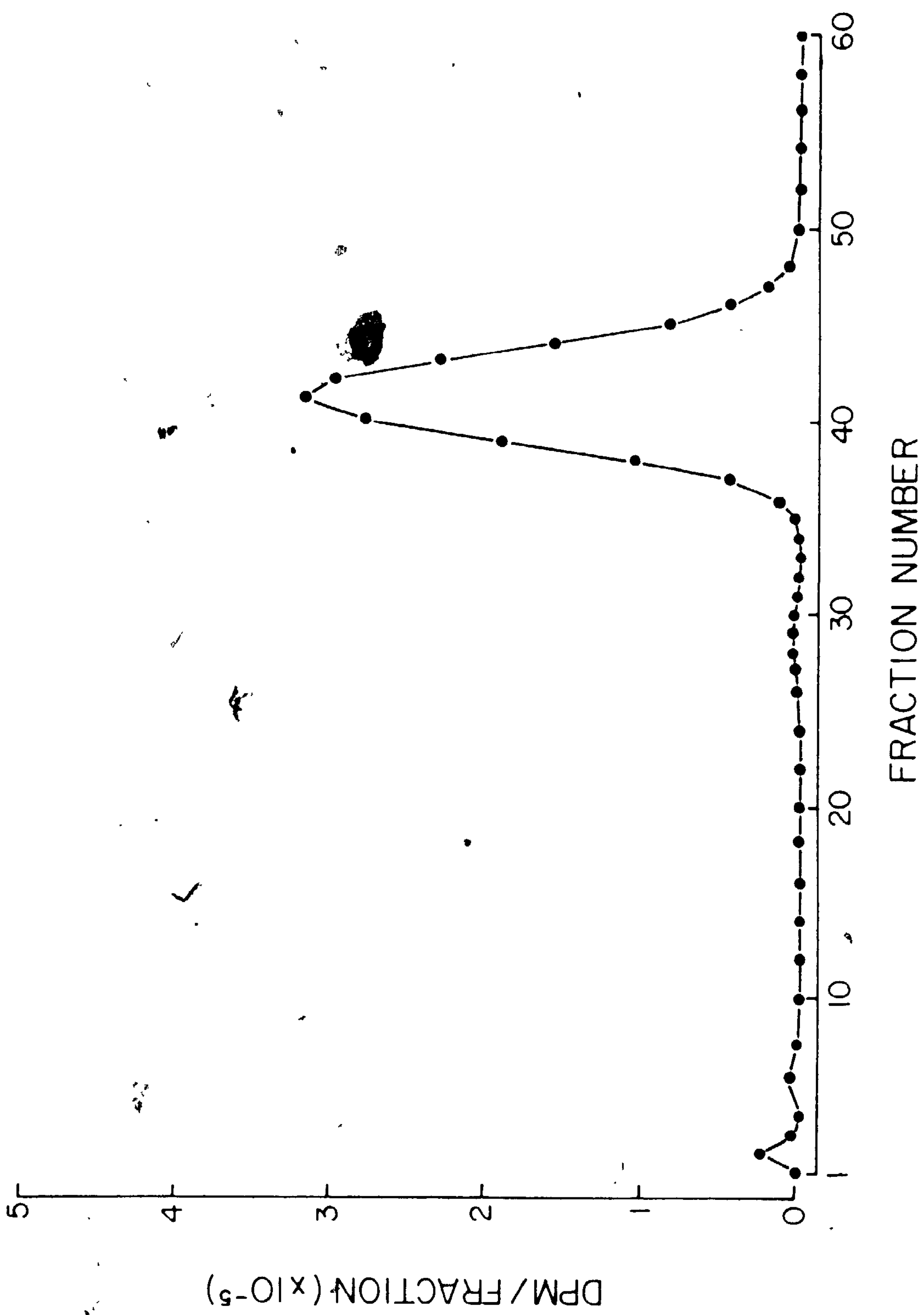
Position of protein and NAD elusion from AG1 X-8 column.



OD_{280}
4.0
3.0
2.0
1.0
0
1
10
20
30
40
50
60
FRACTION NUMBER

Figure 38.

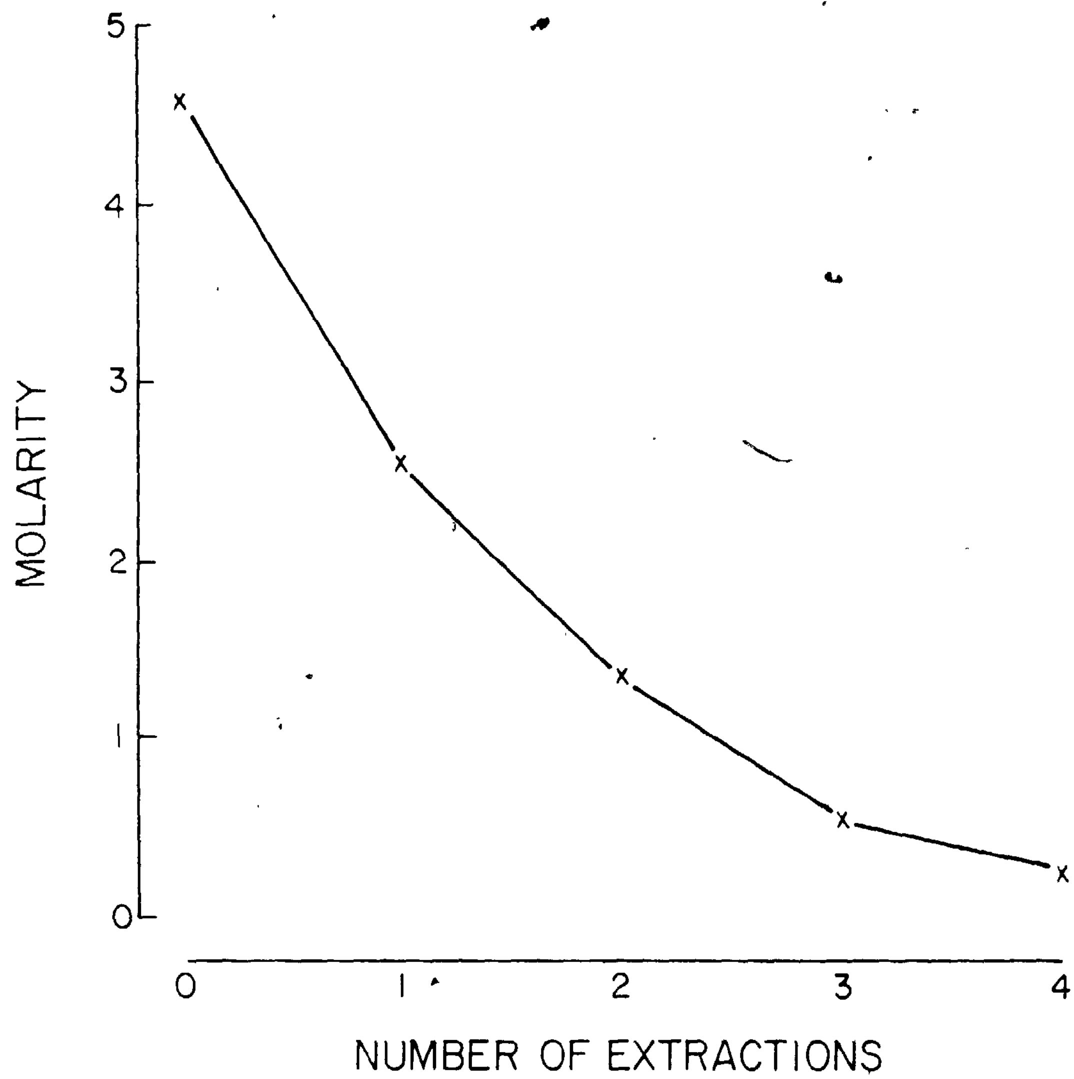
Elusion of products of DHAP synthesis from AG1 X-8 column.



DPM / FRACTION (x10^{-5})
0
1
2
3
4
5
FRACTION NUMBER
1
10
20
30
40
50
60

Figure 39.

Effects of ether extractions on the molarity of a formic acid solution.


MOLARITY
5
4
3
2
1
0
0
1
2
3
4
NUMBER OF EXTRACTIONS

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