STUDIES ON THE HORMONAL REGULATION OF HEPATIC PHOSPHOLIPID METABOLISM

by

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ABSTRACT

Investigations were carried out on the role of glucagon and calcium in the regulation of hepatic phospholipid biosynthesis. It was found that glucagon inhibits de novo phosphatidylcholine biosynthesis in cultured rat hepatocytes. This inhibition was associated with an inhibition of CTP:phosphocholine cytidylyltransferase activity, which is the regulatory enzyme for phosphatidylcholine biosynthesis. Calcium was shown to inhibit the uptake of choline in hepatocytes by decreasing the Vmax of the saturatable uptake system. It also slightly inhibited the rate of phosphatidylcholine biosynthesis by the de novo pathway, but not by the N-methylation of phosphatidylethanolamine. However, these experiments were difficult to interpret due to the use of ionophore A23187 to vary cytosolic calcium concentrations. This ionophore has many other effects on hepatocytes which could indirectly alter the synthesis of phosphatidylcholine. In vitro studies were carried out to determine the effect of calmodulin on CTP:phosphocholine cytidylyltransferase activity. Although calmodulin did not effect the activity under the conditions of the assay, an impurity of some calmodulin preparations was found which inhibited the cytidylyltransferase in a calcium independent fashion. The inhibitor had some peptide like properties. The effect of calcium on the incorporation of [3-^{3}H] serine into phospholipids was also investigated. Calcium was found to increase the amount of label recovered in phospholipid. It was also found that the label was rapidly transfered from phosphatidyserine to phosphatidylethanolamine. On the basis of these results, a model is presented for the relationships between calcium and phosphatidylserine metabolism.

LIST OF ABBREVIATIONS

ACS	aqueous counting scintillant
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphophate
CaM	calmodulin
cAMP	adenosine cyclic monophosphate
CDP	choline diphosphate
cGMP	cyclic guanosine monophosphate
Ci	Curie
CL	cardiolipin
CMP	cytidine monophosphate
CoA	coenzyme A
cpm	counts per minute
CTP	cytidine triphosphate
DG	diacylglycerol
dpm	disintegrations per minute
EDTA	ethylenediaminetetracetic acid
EGTA	ethyleneglycol bis(B-aminoethylether) N-N-tetracetic acid
g	gram
GTP	guanosine triphosphate
h	hour
Hepes	hydroxyethyl piperazineethanesulfonic acid
IPA	Ionophore A23187
Ka	association constant of an enzyme-activator complex
Km	Michaelis-Menten constant

l	liter
m-	milli-
M	molar
MEM^-	Dulbeccos modified Eagles Media formula 79-5141
min	minute
mol	moles
n-	nano-
NAD^+	oxidized nicotinamide-adenine dinucleotide
NADH	reduced nicotinamide-adenine dinucleotide
PA	phosphatidate
PBS	phosphate buffered saline, pH 7.4
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
Pi	inorganic phosphate
PI	phosphatidylinositol
PKA	cAMP dependent protein kinase
PKC	calcium phosphatidylserine dependent protein kinase
PKM	calmodulin dependent protein kinase
PS	phosphatidylserine
SAH	S-adenosyl homocysteine
SAM	S-adenossyl methionine
SD	standard deviation
SM	sphingomyelin
TG	triacylglycerol
TLC	thin layer chromatography
Tris	tris (hydroxymethyl) aminoethane
u-	micro-

UV	ultraviolet
Vmax	maximal velocity of an enzymatic reaction

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INTRODUCTION

Phospholipids are a major component of cell membranes, bile, lung sufactant, and lipoproteins. The pathways of phospholipid metabolism have been known for many years, largely due to the work of Kennedy and coworkers (1). Interest in the regulation of these pathways has intensified in recent years, as it is becoming increasingly evident that phospholipid metabolism is intimately related to other cellular processes. However, our knowledge of this regulation is still fragmentary and at a superficial level. The purpose of this thesis is to gain further insight into the processes by which lipid diversity, as well as total lipid synthesis, are maintained under different physiological conditions.

1.1. The Structure, Properties, and Function of Phospholipids

1.11 The Structure of Phospholipids

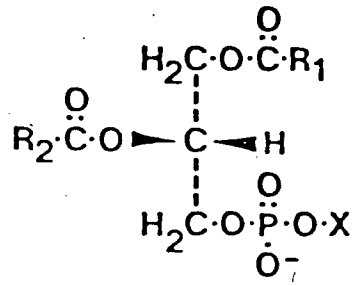
Glycerophospholipids can be considered to be derivatives of glycerol-3-phosphate. A wide range of compounds are possible by esterifying different fatty acids to the 1 and 2 positions of the glycerol, all giving rise to different types of PA. Stearate, palmitate, oleate, and linoleate are the predominant fatty acids found in animal membranes, with arachidonate being an important minor component. PA may be thought of as the parent structure of the other

glycerophospholipid classes, which can be generated by esterifying different organic bases or 'head groups' to the phosphate moiety (figure 1.1). This gives rise to the major phospholipids found in biological membranes, which generally contain predominantly PC, PE, PS, and PI. Glycerophospholipids which have one of their acyl groups removed are refered to as lysophospholipids (lyso-PE, lyso-PC, etc.). By varying the fatty acid and head group moieties, approximately 200 different phospholipid molecules can be generated, and most of them have been found in vivo. The term 'phospholipid' includes these compounds, as well as plasmalogens, sphingolipids, and phosphonolipids. However, with the exception of sphingomyelin, these compounds are present in only trace quantities in most animal systems.

1.12 The Properties of Glycerolipids

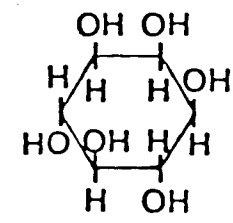
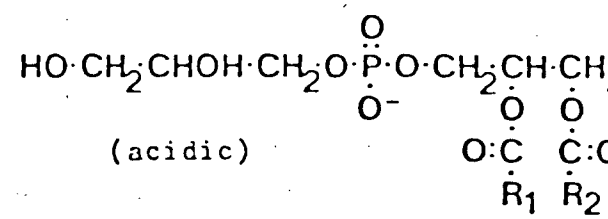
One can make certain generalizations which apply more or less to all lipid classes. Most lipids are amphipathic in that they contain long chain hydrocarbons on one end and a polar group on the other end. In aqueous solution, lipids counteract the hydrophobic effect of the carbon chains with the hydrophilic nature of the polar end by forming multimolecular aggragates, when the lipid concentration reachs high enough levels (the critical micellar concentration). One of the most prevalant structures observed in biological systems is the bilayer membrane, proposed by Gorter and Grendel (2) in 1925. Presently, the most widely accepted model of a biological membrane is the 'Fluid Mosaic Model' (see figure 1.2a), proposed by Singer and Nicolson in

Fig. 1.1 The structure of glycerophospholipids



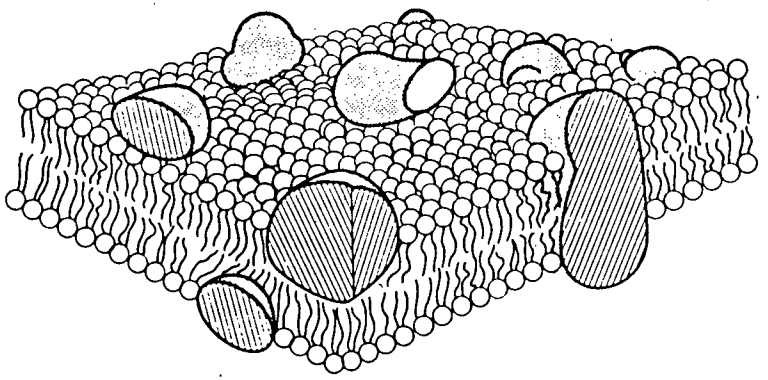
General Structure

The alcohols contributing the polar X groups in the major glycerophospholipids are shown below:

Glycerophospholipid	X
1,2-diacylglycerol 3-phosphate (phosphatidate)	H
Phosphatidyl choline (lecithin)	$HO{\cdot}CH_2CH_2\overset{+}{N}(CH_3)_3$ (basic)
Phosphatidyl ethanolamine (cephalin)	$HO{\cdot}CH_2CH_2\overset{+}{N}H_3$ (basic)
Phosphatidyl serine	$HO{\cdot}CH_2\underset{\overset{+}{N}H_3}{CH}{\cdot}COO^-$ (amphoteric)
Phosphatidyl inositol	 (neutral)
Diphosphatidyl glycerol (cardiolipin)	 (acidic)

1972 (3). This model basically accepts the idea of a bilayer, but suggests that the individual lipids and proteins in the structure move translationally and rotationally. Membrane proteins can be classified as to whether they are extrinsic (or electrostatically bound), or intrinsic (integrated into the hydrophobic region of the bilayer). This model has been able to assimilate the more recent concepts of: annular lipids on intrinsic proteins, phase transitions of bilayers, micro domains in bilayers differing in composition and structure, and membrane turnover, to name a few. More recent physical studies (4) show that lipids may also form non-bilayer structures such as hexagonal 2 phase and inverted micelle configurations (figure 1.2 b). It is highly possible that such structures may exist in vivo as a minor component, and it is likely that such stuctures are important in vivo as intermediates in membrane fusion events. There are many good reviews on these topics (4,5).

The exact structure of a lipid protein aggregate will depend to some degree on all the solutes present, but the structures of the individual lipid species is critically important. Differences in the net charge of the head group are important, and lipids can be classified according to whether they are acidic (PI,PS,PA,PG,CL), or neutral (PC,PE,DG,TG,cholesterol). Of course, the pH can change the charge of a lipid such as PE and many of its properties are pH dependent. Neutral lipids generally outnumber the acidic species in biological membranes. Acidic phospholipids have the property of forming complexes with divalent cations, particularly calcium. The addition of calcium to an acidic phospholipid mixture can cause isothermal phase transitions from bilayer to hexagonal 2 phase in some



A

Lipid	Phase	Molecular Shape
Lysophospholipids Detergents	Micellar	Inverted Cone
Phosphatidylcholine Sphingomyelin Phosphatidylserine Phosphatidylglycerol	Bilayer	Cylindrical
Phosphatidylethanol-amine (unsaturated) Cardiolipin - Ca^{2+} Phosphatidic acid - Ca^{2+}	Hexagonal (H_{II})	Cone

B

Fig. 1.2 Multimolecular lipid aggregates in aqueous solution (A) The fluid mosaic model. Globular proteins are inserted to varying degrees into a phospholipid bilayer. The proteins and lipids move rotationally and laterally with relative freedom. (B) The relationship between the average shape of a lipid species and its phase preference above the critical micellar concentration. (reproduced by permission (4)).

situations (4). The biological significance of these structures has not been fully elucidated but there are some systems where they are believed to be important. For example, the addition of PA to neuroblastoma cells has been shown to increase the permeability to media calcium dramatically (6). Lipids can be considered to be conical, cylindrical, or inverse conical (figure 1.2b), according to their average molecular shape. Cylindrical lipids, such as PC, are generally bilayer stabilizing lipids, as would be expected from their shape. The inverse conical and conical shapes form micelles and inverted micelles respectively, and are generally present as a minor species in membranes (4). The fatty acid composition can dramatically effect the shape of a molecule within a particular lipid class. Thus cis-enoic acids in PE can give it an inverted cone shape, relative to the cylindrical shape of a straight chain PE (4).

The fatty acid composition is also important in determining properties such as the transition temperature and the permeability of the membrane (4). Overall, it is evident that large variety of lipid species found in a cell allows for an incredible flexibility in the properties of its membranes.

1.13 The Function of Phospholipids

The most important function of phospholipids is their use in membranes, in which lipids and proteins are the major components. PC, PE, and SM are the major species found in animal membranes, with CL, PS, PI, and PG, being minor components. Traditionally, phospholipids have been thought of as basically inert structural components in

membranes, forming bilayers similar to those found in biological systems in vitro. The functional attributes of membranes have usually been assigned to proteins, with the exception of the lipids forming a relatively nonselective permeability barrier to ionic compounds and an annular sheath for the proteins. It was originally postulated that the phospholipid composion of a organism was species specific. However, with the development of more powerful analytical techniques it has been shown that lipid compositions depend more on the organelle than the species (table 1.1a). Moreover, the fatty acid composition of a particular phospholipid class may be very different from tissue to tissue and may change significantly in different biological situations. Phospholipids also turnover at markedly different rates (table 1.1b), and the fatty acid, glycerol, and head groups turnover independently as well (7). Thus it is becoming increasingly evident that the cellular membrane is a finely tuned, dynamic structure in which the properties of different lipids are exploited to give the optimal membrane composition to suit the membranes function and environment.

Additional functions of phospholipids in membranes are being uncovered, particularly in regard to the minor components. One function of phospholipid in a membrane is to provide a storage form of arachidonate, which may be released by the action of phospholipases A_2. In many systems (8), the concentration of arachidonate is rate limiting in the synthesis of the various prostaglandins. Indeed it appears that phospholipase A_2 play a key role in certain regulatory cascade systems.

Another function of some glycerolipids is their ability to

Table 1.1 Variations in lipid composition and turnover (A) The lipid composition hepatocyte membranes, expressed as a mole percent of total phospholipid. Collated by McMurray and McGee (149). (B) The half-life of phospholipid classes, as measured with various precursors. Redrawn from (150).

A

Lipid Class	Plasma membrane	Nuclear membranes	Endoplasmic recticulum	Golgi membranes	Mitochondrial membranes inner	Mitochondrial membranes outer	Lysosomal membranes
PC	34.9	61.4	60.9	45.3	45.4	49.7	33.5
PE	18.5	22.7	18.6	17.0	25.3	23.2	17.9
PI	7.3	8.6	8.9	8.7	5.9	12.6	8.9
PS	9.0	3.6	3.3	4.2	0.9	2.2	8.9
PG	4.8	*	*	*	2.1	2.5	*
CL	trace	0	*	*	17.4	3.4	6.8
l-PC	3.3	1.5	4.7	5.9	*	*	0
l-PE	*	0	0	6.3	*	*	*
SM	17.7	3.2	3.7	12.3	2.5	5.0	32.9

(* not determined)

B

Tissue	Phospholipid Class	Labeled Precursor	Half-Life (h)
Rat	PC	[^{32}P]phosphate	10.9
Liver	PC	[^{3}H]choline	10.0
	PC	[^{14}C]serine	1.2
	PE		2.1
	PS		7.0
Rabbit	PC	[^{3}H]choline	13.0
Lung	PC	[^{14}C]palmitate	10.0
	PC	[^{3}H]glycerol	8.0
	PC	[^{32}P]phosphate	88.0
Rat	PS	[^{14}C]serine	1.0
Intestine	PE		0.4

activate critical enzymes *in vivo*. For example, the concentration of plasma membrane PS and DG are crucial to the activity of protein kinase C (9), and PS is required for the activation of adenylate cyclase by glucagon (10). These and other examples of phospholipid coupling in regulation schemes will be discussed in section 1.5.

Lastly, changes in phospholipid concentrations in membranes have been implicated in basic cellular processes such as ion gating (6), membrane fusion phenomena (11), and nerve conduction (5). Once again membrane lipid metabolism responds in a highly specific manner to the particular physiologic situation.

The uses of phospholipids are not restricted to membrane phenomena. Phospholipid is also a major component of lipoproteins, bile, and lung surfactant. Again lipid metabolism is highly coordinated in order to provide optimal levels of lipids for each process.

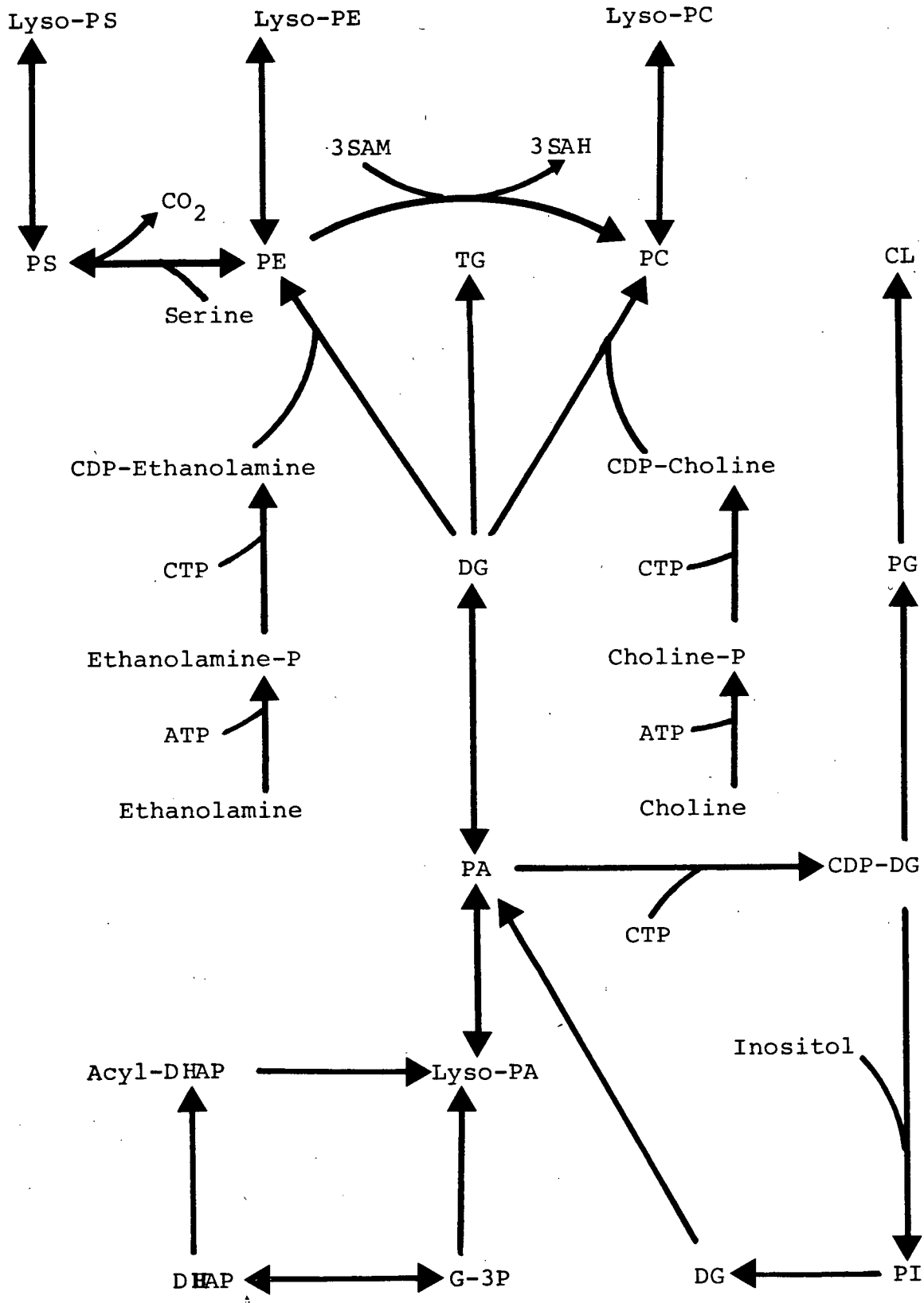
1.2. The Biosynthesis of Glycerophospholipids in Hepatocytes

The pathways of hepatic phospholipid metabolism are interrelated and complex. To facillitate discussion, the reactions have been divided into catagories according to whether they are synthetic, intermediate, or degradative. Throughout the following, the reader is refered to the pathway chart in figure 1.3.

1.21 Fatty Acid Synthesis and Activation

Fatty acids may either be taken up from the serum or synthesized

Figure 1.3: A summary chart showing pathways of lipid metabolism. Abbreviations as in text.



endogenously. All fatty acids are synthesized from ATP, NADPH, and Acetyl-CoA. Although this process occurs in all organisms, it is particularly prominent in the liver and mammary glands of higher animals.

The rate limiting step in the synthesis of fatty acids is the conversion of Acetyl-CoA and CO_2 to malonyl-CoA, which is catalyzed by acetyl-CoA carboxylase (EC 6.4.1.2.). The enzyme may exist as either a protomeric, inactive form, or as an active, aggregated form (12). The aggregation is stimulated by citrate. Acyl-CoA as well as malonyl-CoA esters inhibit aggregation and activation. Thus the enzyme can be coordinated with glycolysis through citrate levels and feedback inhibited by its own product. The aggregate is also depolymerized when phosphorylated by protein kinase A and is thus inhibited when the concentration of cAMP is elevated (14). It is also phosphorylated by a cAMP independent protein kinase and this inhibits activity as well (15). Acyl-CoA carboxylase may also be controlled transcriptionally (16). It is probable that the net activity is determined by a balance of all these factors.

The malonyl-CoA produced is then converted to fatty acid by a multi-enzyme system in the cytosol, termed the fatty acid synthetase complex . It is a dimer with a subunit molecular weight of 230K (17). The regulation of this complex has been extensively studied in recent years and there are several excellent reviews on this subject (18,64). Fatty acids which are taken up from serum are converted to the CoA derivative by the enzyme acyl-CoA synthetase (EC 6.2.1.1). Acyl-CoA is refered to as the 'activated form' of fatty acid because it is this derivative that is used in subsequent reactions. Acyl-CoA may be

acted on by a fatty acid elongation systems in the mitochondria or by specific monooxygenase systems in the endoplasmic recticulum; these introduce double bonds in the fatty acid. There is positional specificity in the placement of double bonds (19). Mammals can not synthesize linoleic or linolenic acids so these must be obtained from dietary sources. These essential fatty acids are required for arachidonate biosynthesis, the precursor of prostaglandins (20). The acyl-CoAs may be used for glycerolipid biosynthesis or utilized for B-oxidation.

1.22 The Synthesis of PA

The next step in the synthesis of glycerolipids is the synthesis of PA. PA may be synthesized by two pathways: one requiring glycerol phosphate, and the other requiring dihydroxyacetone phosphate. These compounds are in equilibrium with NADH and NAD^+ in the cell and so the relative amounts depend on the NADH:NAD^+ ratio. The total amount of these triose phosphates depends on the rate of glycolysis (the major source of dihydroxyacetone phosphate), the rate of lipolysis, and the rate of glycerol uptake from the serum. The glycerol obtained by these last two processes can be phosphorylated *in vivo* by glycerol kinase (EC 2.7.1.30) (21).

The synthesis of PA from glycerol phosphate and acyl-CoA was first observed in ginuea pig liver microsomes by Kornberg and Pricer in 1953 (22). Two acylations are required and the evidence suggests that two microsomal enzymes are required (23). PA generally has a saturated fatty acid at position one and oleic or linoleic at position

two *in vivo*. There is still controversy as to whether this difference is do to substrate specificity during synthesis, or whether it is introduced post-synthetically by a transacylase or by deacylase/reacylase activities. There is evidence that both methods could be used to some extent (23-25). Glycerol phosphate acyltransferase (EC 2.3.1.15) may also be controlled the level of calcium (26), and there is evidence that the enzyme can be regulated by cAMP in adipose tissue (27).

The acylation of dihidroxyacetone phosphate to acyl-dihydroxyacetone phosphate and its subsequent reduction to lyso-PA has been observed in rat liver microsomes by Hajra and Agranoff (28). The reduction requires NADPH as a cofactor. Despite numerous attempts to assess the significance of this pathway in the synthesis of PA (29), there is no general agreement on its contribution to PA synthesis. A third way PA may be synthesized is by the action of DG kinase on ATP and DG. This enzyme activity undergoes marked fluctuations *in vivo* and will be discussed in more detail in section 1.3.

1.23 The Biosynthesis of DG

PA occupies a unique position in the synthesis of glycerolipids; it may either react with CTP to give CDP-DG, and hence be directed toward acidic phospolipid synthesis, or it may be hydrolyzed to DG and used for the synthesis of PE, PC and TG. The hydrolysis of PA to DG is catalyzed by PA phosphatase (EC 3.1.3.4). This activity was first observed in animals by Weiss *et al*. in 1956 (30). PA phosphatase

activity has been demonstrated in mitochondria, lysosomes, microsomes, and cytosol (31). The cytosolic enzyme is moderately specific for PA containing one unsaturated fatty acid (32). Brindley et al. have demonstrated a positive correlation between PA phosphohydrolase activity and the rate of hepatic TG synthesis, and have also shown that the enzyme can be activated by glucocorticoids in vivo. Thus, it is likely that PA phosphatase can regulate TG synthesis in some situations (33).

1.24 The Synthesis of TG

DG acyltranferase activity (EC 2.3.1.20) was first observed in a particulate chicken liver fraction in 1956 by Weiss et al. The enzyme shows specificity for 1,2-DG, and unsaturated fatty acids are prefered over saturates (35). It has been shown that the enzyme is inhibited by glucagon (36). However, Groener et al. studied the effects of fasting and refeeding on the rate of rat liver TG synthesis in vivo (37), and found that the increased rate of TG synthesis after refeeding could not be totally explained by increased DG acyltranferase activity. He concluded that the increased DG levels stimulate TG synthesis directly. This agrees with a study by Sundler et al., who studied the effect of exogenous fatty acid on TG synthesis (38). Incorporation of labeled glycerol increased linearly into DG and TG with respect to fatty acid concentration. The results imply that TG synthesis may be regulated by the concentration of DG, as well as glucagon levels.

1.25 The de novo Synthesis of PC

PC is the most abundant phospholipid in animal membranes, and is also a major component of bile, lipoproteins, and lung surfactant. The major pathway for its biosynthesis was elucidated by Kennedy and coworkers in the 1950's (1). They found that the de novo synthesis of PC occurs in three steps, requiring three separate enzymatic activities. The first step is the phosphorylation of choline, catalyzed by the enzyme choline kinase (EC 2.7.1.32). ATP is the phosphate donor in this reaction. The second step in the synthesis is catalyzed by the enzyme CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15), which makes CDP-choline from phosphocholine and CTP. The final step is the transfer of phosphocholine from CDP-choline to DG, forming CMP and PC. This step is catalyzed by the enzyme cholinephosphotransferase (EC 2.7.8.2).

Although this pathway was demonstrated almost 30 years ago, very little advancement was made on the knowledge of its control for many years. Using the average concentrations of the PC related metabolites in rat liver, as well as the predicted intracellular equilibrium constants of the reactions involved, Infante made some theoretical calculations which implied that the first two steps in the synthesis are out of equilibrium (39). Thus either the choline kinase or cytidylyltransferase step could be involved in the regulation of PC biosynthesis. The third step is very close to equilibrium.

The phosphorylation of choline can be rate limiting in chicken liver. Studies by Vigo and Vance demonstrated that the rate of liver PC biosynthesis increased two fold after two days of diethylstilbesterol

treatment (40). There was a positive correlation beween this stimulation and the activity of choline kinase (41). Phosphocholine concentrations were also increased two fold. The concentration of phosphocholine in chicken liver was found to be about 0.15mM, and the Km of the cytidylyltransferase for phosphocholine is about 0.17mM (42). Thus the increase in choline kinase activity is directly translated into an increase of PC synthesis in this system. Subsequent studies by Paddon et al demonstrated that the diethylstilbesterol treatment increased the amount of immunotitratable choline kinase, suggesting that the stimulation was a result of increased choline kinase synthesis (43). This agrees with the widely accepted concept that steroids act at the level of gene expression.

The cytidylyltransferase step can also be regulatory in this system. In the same set of experiments, the rate of PC synthesis decreased relative to control values on the third day of diethylstilbesterol treatment (41). However, choline kinase activity as well as phosphocholine concentrations were still elevated. On the other hand the activity of the cytidylyltransferase was decreased by 2 fold. Thus both enzymes are involved in the regulation of PC biosynthesis in chicken liver.

The regulatory features of PC synthesis appear to be different in adult rat liver and Hela cells. In these cells the concentration of phosphocholine is about 1-2mM, which is well above the apparent Km of the cytidylyltransferase (0.17mM) (47). Thus, fluctuations in the concentration of this substrate would have little effect on the rate of PC synthesis. The concentration of choline in these cells in 5-10 fold lower than the the concentration of phosphocholine (45),

suggesting that choline is rapidly phosphorylated upon uptake, with the rate limiting step occuring subsequent to this step. Choline has two possible fates upon entering the cell: it may be phosphorylated and hence commited to PC synthesis, or it may be oxidized to betaine in the mitochondria. Studies by Pritchard and Vance on cultured rat hepatocytes showed that low concentrations of choline in the media decreased the amount of labeled choline that was oxidized, while the pool size of phosphocholine remained constant (45). Increasing media choline levels increased the amount of label oxidized to betaine produced. Very little radioactivity was recovered in CDP-choline This provides good evidence that the production of CDP-choline is rate limiting in this system.

The properties of CTP:phosphocholine cytidylyltransferase have received considerable attention in the past few years. The enzyme is found predominantly in cytosol when liver is homogenized in saline, but found in the microsomes when homogenized in distilled water (46). This implies that the enzyme is extrinsicly bound to the endoplasmic recticulum. Choy et al. partially purified the enzyme from rat liver cytosol (47). They found that the enzyme is markedly stimulated when rat liver phospholipid is added. They also found that the cytosolic enzyme, with a molecular weight of about 2.0×10^5 (L-Form), aggregates to a high molecular weight form with particle weights from 5.6×10^5 to about 1.3×10^7 (H-Form). H-Form had an increased basal activity but was also stimulated by rat liver phospholipid. Choy and Vance studied the lipid activation in more detail (48). They found that lyso-PE had the greatest activating properties, while PS and PI also stimulated the enzyme to a lesser extent. Some species of lyso-PC could

inhibit the enzyme by as much as 80%. They found a positive correlation between the activation of stored cytosol and the amount of lyso-PE present. The data implies that lyso-PE and acidic phospholipids could play a role in regulating the activity of the enzyme.

The aggregation of the enzyme was also studied in more detail by Choy et al. (49). They found that DG and PG stimulated the aggregation of the enzyme, although it was important that the DG be delivered in a proper form. Thus, phospholipase C treatment of cytosol increased the aggregation two fold. PG stimulates both the aggregation and activity of the enzyme (50). Although PG is present in only trace quantities in liver, it seems to be important in developing lung, where the production of PG and the activation of the cytidylyltransferase are closely correlated (50). Thus PG may serve to signal the production of lung surfactant in this system.

The implications of these in vitro observations for the regulation of liver PC synthesis are quite extensive. It could be postulated that the aggregation of the enzyme in vitro is a reflection of an in vivo form of regulation, functioning to translocate cytosolic enzyme to local DG domains in the endoplasmic recticulum. The enzyme would then produce its product, CDP-choline, in close proximity to the DG required in the final step of PC synthesis. This would significantly increase the efficiency of PC synthesis and allow the CDP-choline pool to remain very small, as it in fact is (44). A study by Pritchard et al showed that the rate of PC synthesis by the de novo pathway is stimulated by 3-deazaadenosine (66). They demonstrated a positive correlation between the increase in PC synthesis and the amount of cytidylyltransferase found in the

microsomes. The total amount of enzyme in the cytosol and microsomes remained constant. Another study showed that when embryonic chicken muscle cells were grown in the presence of phospholipase C, the cytidylyltranferase was tranlocated to the microsomal fraction and activated 3 fold (67). This was correlated ith a 3-5 fold increase in theincorporation of choline label. These experiments suggest that the aggregation and activation of the enzyme may be important regulatory features of the enzyme.

PC synthesis can conceivably be regulated by the concentration of CTP. Vance et al studied the mechanism by which polio virus stimulates the rate of PC synthesis in Hela cells (51). They found that that the increase in PC synthesis was due to a stimulation of the cytidylyltransferase step. A subsequent study by Choy et al. demonstrated that the increased rate of this reaction was due to an increase in the concentration of CTP in the Hela cells (52). The possibility that CTP regulates PC synthesis has not been verified in other systems to date. CTP is required for the synthesis of all phospholipid classes, although it is utilized in different ways. Thus it is tempting to speculate that CTP may be a universal signal for the synthesis of phospholipids. It would be interesting to do experiments withsynchronized mitotic cells and determine if CTP levels are associated with the increase in phospholipid synthesis that must occur in order to generate new membranes.

Recent studies by Pelech et al have shown that the rate of PC biosynthesis can be inhibited by cAMP analogs in cultured rat hepatocytes (53). This inhibition was correlated with an inhibition of the cytidylyltransferase. The inhibition of the enzyme activity could

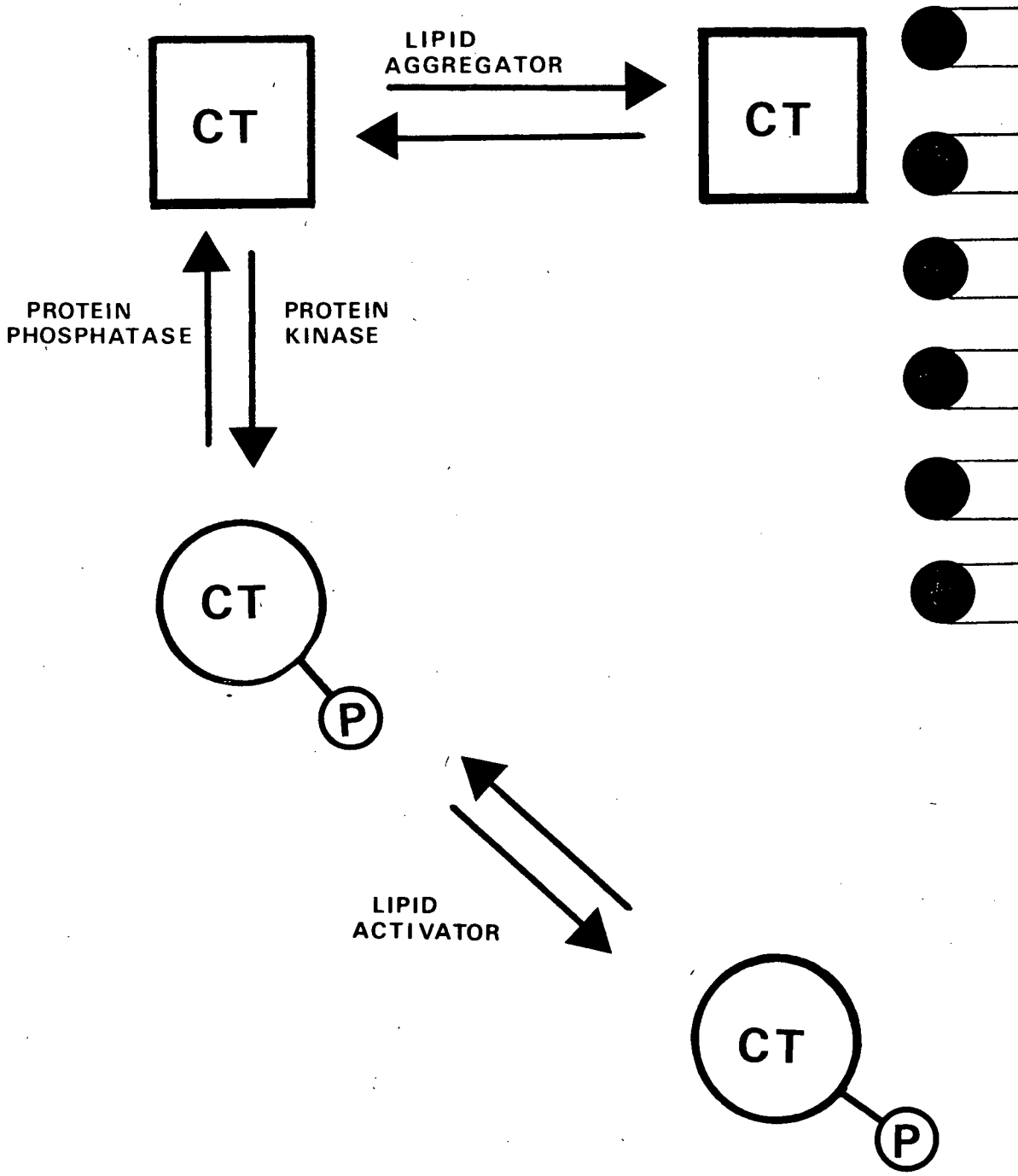
only be demonstrated when NaF and EDTA were added in the homogenizing buffer. NaF and EDTA inhibit protein phosphatases and protein kinases, respectively, and so it was postulated that the inactivation was due to a protein phosphorylation. Further support for this hypothesis was gained with a series of *in vitro* studies (54). It was found that NaF inhibits cytosolic cytidylyltransferase in a time dependent fashion. The inhibition could be mimicked with Mg-ATP or calcium, and supressed with protein kinase inhibitor protein isolated from rabbit skeletal muscle. However, the final proof that the enzyme is phosphorylated must await the purification of the enzyme so that direct phosphorylation can be demonstrated. The presumptive phosphorylation inhibited aggregation of cytosolic cytidylyltransferase and increased the Km for CTP. The inhibition could be reversed by addition of phospholipid.

However, a cAMP dependence of the *in vitro* could not be demonstrated. This raises the question of how the cAMP analogs work to inhibit the cytidylyltransferase in hepatocytes.

In figure 1.4 a model of cytidylyltransferase regulation is presented which combines the results discussed above into a single system. It should be emphasized that much more evidence must be obtained before this model can be accepted. Primarily, proof that the enzyme is phosphorylated must be obtained and the identity of the presumptive protein kinase must be discovered. Additionally, more experiments must be performed to demonstrate that activation and aggregation are important *in vivo*. Lastly, the importance of CTP in regulating the synthesis of PC must be established.

Choline phosphotransferase is an intrinsic microsomal enzyme, and

Figure 1.4 A model for the regulation of CTP:phosphocholine cytidylyltransferase: The model summarizes the known regulatory features of the enzyme, depicting the relationship between phosphorylation, aggregation, and activation. The unphosphorylated form (square form) may be translocated to the endoplasmic recticulum by interaction with aggregating lipids such as DG, increasing the particle weight, and allowing CTP-choline to be made in close proximity to DG. Alternatively, the enzyme may be inhibited by phosphorylation, (circle form). This inhibition may be reversed by acidic phospholipids, which decrease the Km for CTP.Thus the enzyme may be activated (thatched form) in either the cytosol or the microsomes.



catalyzes the final reaction in the de novo synthesis of PC. Its enzymatic activity is resticted to the cytoplasmic side of the endoplasmic recticulum (55). The reaction is reversible in vivo. Choline phosphotransferase has only a slight differential specificity for particular DG, implying that the acyl groups in PC are either regulated by availability or adjusted post synthetically (56). The enzyme activity fluctuates significantly in response to various stimulations but this is probably an adaptive response rather than a regulatory event.

1.26 The de novo Synthesis of PE

PE is formed from ethanolamine and diglyceride by a pathway equivalent to the synthesis of PC. Although the reactions are the same, it is clear that the enzymes involved in the two pathways are distinct (56,57). Less is known about the synthesis of PE than PC. It would seem that some of the regulatory features of the PE pathway must be different from those of the PC pathway, in order to maintain lipid diversity. When the media concentration of ethanolamine is low, it appears that the phosphorylation of ethanolamine can be rate limiting to synthesis of PE (58). Few experiments have been done with higher ethanolamine levels. It seems probable however that in this situation the synthesis of CDP-ethanolamine can be rate limiting (39). Ethanolamine phosphotransferase (EC 2.7.8.1) prefers hexenoic fatty acids at position 2 and this may help explain why 20% of the PE found in rat liver in vivo contains hexenoic acyl groups (56). PE may also

be made by the decarboxylation of PS (section 1.34) but it is not known how much of the cells PE is made by this pathway. However, this pathway does not result in net phospholipid synthesis.

1.27 The CDP-DG Pathway to Acidic Phospholipids

Instead of being converted to DG, PA may react with CTP to form CDP-DG. This reaction is catalyzed by the microsomal enzyme PA cytidylyltransferase (EC 2.7.7.41). Little is known about this enzyme; one study found a high percent of polyenoic CDP-DG *in vivo* relative to PA, but a preference for polyenoic fatty acids could not be demonstrated *in vitro* (59). It has not be determined then whether the high arachidonate content of the PI produced from CDP-DG is introduced at this step, or whether it is introduced post synthetically. The enzyme is stimulated by GTP (61). This stimulation is supressed by EDTA and NaF, and can not be seen with nonhydrolyzable GTP analogs. This suggests that there is a phosphorylation involved. There have been few studies investigating the relation of PA-cytidylyltransferase to PA-phosphohydrolase. One study showed that both enzymes have a high reserve capacity and normally operate well below maximal rates (62).

Once CDP-DG is formed, it is rapidly reacted with either inositol phosphate or glycerol phosphate to produce PI or PG phosphate, respectively. These reactions have been reported to occur in microsomes, Golgi apparatus, plasma membrane, and the inner mitochondrial membrane (63). The PG phosphate is hydrolyzed to PG and condenses with with another PG to make CL. This reaction occurs

predominantly on the inner mitochondrial membrane (64). Most of the CL formed remains in the mitochondria, and is enriched in linoleate (65). It is not clear how the selectivity for linoleate is achieved.

1.3. Intermediary PL Metabolism

In this section, reactions are dealt with that involve no net synthesis of phospholipids. Although these reactions obviously overlap with what are termed synthetic and degradative pathways, they are separated here to emphasize that this is where the 'retailoring' of phospholipids occurs, to maintain the proper fatty acid proportions in the phospholipid classes, and to provide another level of control over the head groups. It is becoming increasingly evident that the control of these reactions is involved in coupling phenomena and cascade pathways.

1.31 PE-N-Methylation

In addition to the Kennedy pathway, PC may be formed from PE by three sequential methylations. The reaction is catalyzed by PE methyltranferase (EC 2.1.1.17). The enzyme is intrinsically microsomal, uses SAM as a methyl donor, and is inhibited by SAH (65).The enzyme has been shown to prefer PE with polyenoic acyl groups, and this may contribute significantly to the amount of unsaturates found in PC *in vivo* (68). It has been reported that this pathway accounts for up to 20% of the PC in rat hepatocytes (69). The majority of PE made by the *de novo* pathway is methylated to PC in hepatocytes

(70). The pool sizes of the intermediates mono- and di-methyl-PE are extremely small (70). The enzyme has also been reported to occur in several other cell types, but the specific activity of the enzyme is very low in these tissues and thus the methylation pathway is probably of minor significance in these tissues (71).

There is evidence that the enzyme is regulated by cAMP. Castano et al. (72) have shown that the activity of PE methyltransferase increases upon addition of of glucagon to hepatocytes. Pritchard et al. (73) have shown the enzyme is activated by cAMP analogs, but the flux through the pathway was inhibited as measured by ethanolamine or methionine labeling experiments. It seems possible that the enzyme is activated as measured in vitro because there is more endogenous PE. However, further studies will be needed to accertain this.

PE-N-methylation has been reported to be involved in a number of exitation-response coupling systems in the plasma membranes of some cells (74,75); however this work has recently been criticized by Vance and Kruijff (71), who point out that the small number of PC molecules synthesized by PE-N-methylation could not account for the changes observed in membrane properties. Thus the role of PE methylation in these processes remains uncertain.

1.32 The Synthesis of PS

In 1961, it was shown that PS can be formed by a base exchange with serine and PE, forming PS and ethanolamine (76). The reaction occurs in the microsomes, is reversible in vitro, and stimulated by calcium (77). The enzyme, PS synthase (EC 2.7.8.8), is found in the

microsomes of some tissues, particularly brain and liver. While PS may also be made by a CDP-DG pathway in some bacteria and animals, no corresponding activity has been demonstrated in mammals (78). This is interesting in that it shifts the synthesis of PS from the mitochondria to the endoplasmic recticulum, and from the control mechanisms operating on the other acidic phospholipids, which are produced by the CDP-DG pathway. Although there is still some controversy about alternative pathways to PS (78-80), it appears at present that base exchange is the major, if not sole, source of PS (81).

1.33 Other Base Exchange Activities

Besides the synthesis of PS, base exchange activities have been reported for PC and PE in liver (81) and brain (82). The enzymes are found in microsomes and are calcium stimulated, similar to the PS synthase activity. However, they are clearly distinct enzymes, having been separated by gel filtration (83). One of these enzymesis probably the PS synthase discussed above. The other appears to be used for ethanolamine incorporation into phospholipids (81). Rate calulations by Bjerve indicate that the biosynthesis of PC by this pathway is probably negligible *in vivo* (81). His study also indicated that ethanolamine is preferentially incorporated into hexenoic containing phospholipids. Such was not the case for either serine or choline. *In vivo* studies by Orlando *et al*. indicate that the PE synthesized by this pathway turns over very rapidly. This provides some basis for other reports on PE-N-methylation (84) which

suggests that there are 2 pools of PE. The pool incorporated into PC from PE has more unsaturation and turns over faster. Thus the combination of base exchange and methylation provides another way in which arachadonate can be incorporated selectively into PE and PC.

1.34 The Decarboxylation of PS

Rat liver mitochondria contain an enzyme, PS decarboxylase (EC 4.1.1.65), which converts PS to PE and CO_2 (85). Very little is known about this enzyme, or about the contribution of this pathway to the cells ethanolamine, but it is interesting in that it provides a source of ethanolamine, and hence choline, from a nonessential amino acid. Moreover, it requires the intracellular migration of PS from the endoplasmic recticulum to the mitochondria to make PE, and back to the endoplasmic recticulum for PC synthesis. This cyclic migration occurs at rate faster than the average turnover time of either PE or PC, as labeled from their de novo precursors (see table 1.1b). This could also be a significant source of mitochondrial PE.

1.35 Phosphorylation of DG

In the section on glycerolipid synthesis, two ways were described by which the cell can make PA; one using glycerol phosphate, the other employing dihydroxyacetone phosphate. A third, and potentially very important, method is the phosphorylation of DG to PA by DG kinase (EC 2.7.1.). Unlike the enzymes in the other pathways, DG kinase activity undergoes marked fluctuations which have been correlated with

tissue specific stimulation (24,86). The enzyme operates at an functional branch point, and may give the cell increased flexibility in maintaining the ratio between PC&PE/acidic phospholipids. It is also important in maintaining the PI cycle, which is discussed below.

1.36 Exchange of Acyl Groups

Lysolipids generated by the action of phospholipases A_1 or A_2 (see section 1.5) are rapidly reacylated *in vivo*. Lands (24) studied the specificities of the enzymes involved in rat liver and found that the A_1 acyltransferases incorporate saturated fatty acid (primarily stearate). He also showed that the acyltransferases specific for the 2 position incorporate unsaturated fatty acids, and are responsible for introducing arachidonate into liver phospholipids produced from oleate or linoleate containing PA. Little else has been done toward elucidating the parameters determining these specificities. Thus it is difficult to say at this time whether the chief factor is enzyme specificity or substrate availability. It seems likely that both factors are important to some degree.

1.4. The Degradation of Phospholipids

The synthesis of lipid should obviously outweigh the breakdown of lipid in a growing tissue. However studies investigating lipid degradation have revealed that lipases are very active in many animal tissues. Considering what has already been said about the need to maintain a dynamic, flexible membrane lipid composition, it is

apparent that the lipids must turn over at a significant rate in order that they be replaced with lipids more suited to a changing environment. Also, in conjunction with some of the enzymes pathways mentioned above, partial degradation and resynthesis of lipid occurs to retailor fatty acid composition. Also, TG must be broken down when it is required for an energy source. Lastly, as was mentioned in section 1.2, phospholipases are responsible for the specific release of arachidonate.

1.41 Phospholipases

There are phospholipases for every ester linkage in a phospholipid; they are signified by A_1, A_2, C, and D, for the acyl linkages at positions 1 and 2, the glycerol-phosphate linkage, and the base-phosphate linkage, respectively. The A type phospholipases are ubiquitous, being distributed throughout the endoplasmic recticulum, plasma membrane, lysosomes, and Golgi apparatus of most tissues (24). Phospholipase A_2 activity is very important in the mitochondria as well.

The A types are generally stimulated by calcium (24). Unsaturated fatty acids are cleaved much more rapidly in the 1 position, while saturated fatty acids are cleaved faster at the two position (87). Combined with the reverse specificities for the acyltransferases, it is easy to understand how predominant fatty acid distributions are maintained. Phospholipase A_2 type activities attack bilayers much more readily if there are bilayerirregularities, including small radius curvature, dislocation, or any sort of defect which might be

found at aboundary between gel and liquid crystalline states (88). It seems possible that these conditions could occur under very specific physiological conditions. Phospholipase A_2 activities are responsible for releasing arachidonate from PC, PE and PS during calcium induced prostaglandin synthesis (89). Mitochondrial phospholipase A_2 activity also increases concommitantly with calcium release from mitochondria in hepatocytes (90). Thus, phospholipase A activity is very important for a variety of cellular processes.

Phospholipase C and D activities have been studied far less in animal systems. Besides PA-phosphatase, the only phospholipase C that has been investigated thoroughly is the PI specific activity found in the cytosol of some tissues (91). It is activated at concentrations of calcium that are far less than those required for phospholipase A activation, and has been strongly implicated in a variety of coupling schemes (92). The role of this enzyme in some of these events is discussed in section 1.5. There is almost nothing in the literature about animal phospholipase D activities *in vivo*.

1.5. The Hormonal Regulation of Hepatic Glycerolipid Metabolism

It is clear that the regulation of lipid metabolism is complex and operates on many levels. Considering the enzymes discussed in the previous sections, one can make some generalizations about enzymatic regulation. Regulatory enzymes are often found at the beginning of a biosynthetic pathway, or at a branch point. Such a reaction is said to 'commit' the substrate to the pathway. Regulatory enzymes must

catalyze reactions which are out of of thermodynamic equilibrium, so that the net rate of the reaction may be significantly changeable by a change in the enzymes activity. Also, the concentration of the reaction product involved must be less than the concentration of the substrate, and the product must turnover faster than the substrate; such a reaction is said to be rate limiting. In table 1.2, the enzymes involved in lipid metabolism that have regulatory functions are listed. These enzymes are the substratum upon which complex regulatory schemes are superimposed.

1.51 The Effect of Fasting on Hepatic Glycerolipid Metabolism

It has long been known that different nutritional states profoundly effect the hepatic TG levels, and yet the amount of phospholipid is much less effected. Fasting markedly reduces hepatic TG and DG levels, while refeeding rapidly increases the amount of these compounds (93). The rates of PC and PE synthesis are only slightly affected by nutritional states. This would then seem to be a case where the synthesis of various glyerolipids is coordinately regulated in response to serum conditions, and thus serves as a potential *in vivo* model to study hepatic lipid regulation.

In order to study this model in tissue culture it is necessary to determine what factors in the serum serve to coordinate hepatic lipid metabolism with the nutritional state of the organism. Of all the serum compounds which can show dramatic changes in concentration depending on the nutritional state, the concentration of glucose remains fairly constant at 4.5mM (94). This is because the functioning

Table 1.2 Regulatory enzymes in phospholipid metabolism:

See text for appropriate discussion and references.

ENZYME	INHIBITORS	ACTIVATORS
Acetyl-CoA Carboxylase	Acyl-CoA, cAMP	Insulin, Citrate
Glycerol phosphate Acyltransferase	cAMP, Calcium	
DG Acyltransferase	Glucagon	Insulin
PA-Phosphohydrolase	Glucocorticoids Noradrenalin	Insulin
CTP:Phosphocholine Cytidylyltransferase	cAMP, Calcium,	Insulin, Fatty acids Acidic phospholipids
CTP:phosphoethanolamine Cytidylyltransferase	cAMP (?)	
PS synthase		Calcium
PE-N-Methyltransferase	cAMP	
CTP:PA: cytidylyltransferase		GTP

of the CNS, and thus the sustanence of consciousness, critically depends on blood glucose. The liver is the tissue primarily responsible for maintaining blood glucose during nutritional stress, by converting glycogen, amino acids, and glycerol to glucose.

Two peptide hormones, insulin and glucagon, are widely accepted as the major signals which coordinate hepatic metabolism and with the nutritional state to sustain optimal serum glucose. Both hormones are secreted by the pancreas, from one of two cell types (95). Insulin secretion is stimulated when blood glucose levels are slightly elevated. Insulin causes glucose uptake in the periphery, glycogen synthesis in the liver and skeletal muscle, and TG synthesis in the liver and adipose (96). Through these processes, insulin decreases blood glucose and acts as a signal to store nutrients. On the other hand, glucagon is commonly refered to as the 'starvation signal', and is secreted when glucose levels are slightly decreased. Glucagon primarily operates on three types of cells; adipocytes, skeletal muscle, and hepatocytes. It causes lypolysis in adipocytes, and glycogenolysis in muscle. In hepatocytes glucagon stimulates glycogenolysis, gluconeogenesis, and ketogenesis. It also inhibits glycolysis, lipogenesis, and glycogen synthesis (95).

Glycerol and free fatty acid, which are released into the serum by glucagon treated adipocytes, are also believed to play important roles in coordinating hepatic lipid metabolism to the nutrional state. Glucocorticoids, secreted by the adrenal medula during long term starvation and ethanol stress, are important in regulating hepatic PA phosphatase (97), and may have other effects as well. The importance of these compounds in regulating hepatic lipid metabolism is discussed

below. Thus hepatic lipid metabolism is coordinated to the nutritional state of the organism by five major serum factors: glucagon, insulin, glucocorticoids, fatty acid, and glycerol.

Glucagon binds to specific receptors on the outside of hepatocyte plasma membranes and has a binding constant of about 4.5nm (98). It is widely accepted that the primary effect of glucagon on hepatocytes is to activate adenylate cyclase which produces cAMP from ATP (98). Many other hormones are known to activate adenylate cyclase, including adrenalin and vasopressin. This activation is a transmembrane event, in that the glucagon-receptor complex is on the serum side of the plasma membrane and adenylate cyclase is on the cytoplasmic side. Both the hormone receptor and adenylate cyclase are intrinsic membrane proteins, and the receptor-adenylate cyclase complex has been the topic of many studies. Levey (100) has solubilised the adenylate cyclase complex with a nonionic detergent and found it to be a complex with a molecular weight between 100-200K. Phospholipid added back to the system was required for restoration of the basal activity as well as the hormone responsiveness. Rethy et al. (101) studied adenylate cyclase in plasma membrane after treatment with phospholipase C or ether:butanol extraction, a process which selectively removes phospholipids. They found both the basal and hormonally stimulated rates reduced. Recovery of the basal level could be achieved by addition of PI but this had no effect on the hormonally activated rate. However addition of PS could restore adrenalin activation. These results imply that acidic phospholipids can be important couplers in adenylate cyclase activation, but it is too early to judge the physiological significance of this. The adenlyate cyclase receptor has

regulatory subunits which give it GTP sensitivity (102). x-Adrenergic stimulation and glucagon appear to involve separate GTP subunits and this may be important in determining the differential effects of these hormones. Some prostaglandins also activate adenylate cyclase by apparently by binding directly to the complex (103).

Once glucagon activates adenylate cyclase, intracellular cAMP levels can be elevated by upto 10 fold (104). cAMP activates protein kinase A, which then phosphorylates a number of proteins and so initiates a cascade system (99). The consequences of this cascade system on heptocyte metabolism are dramatic; lipid biosynthetic pathways which are known to be affected by cAMP levels are shown in figure 1.5b.

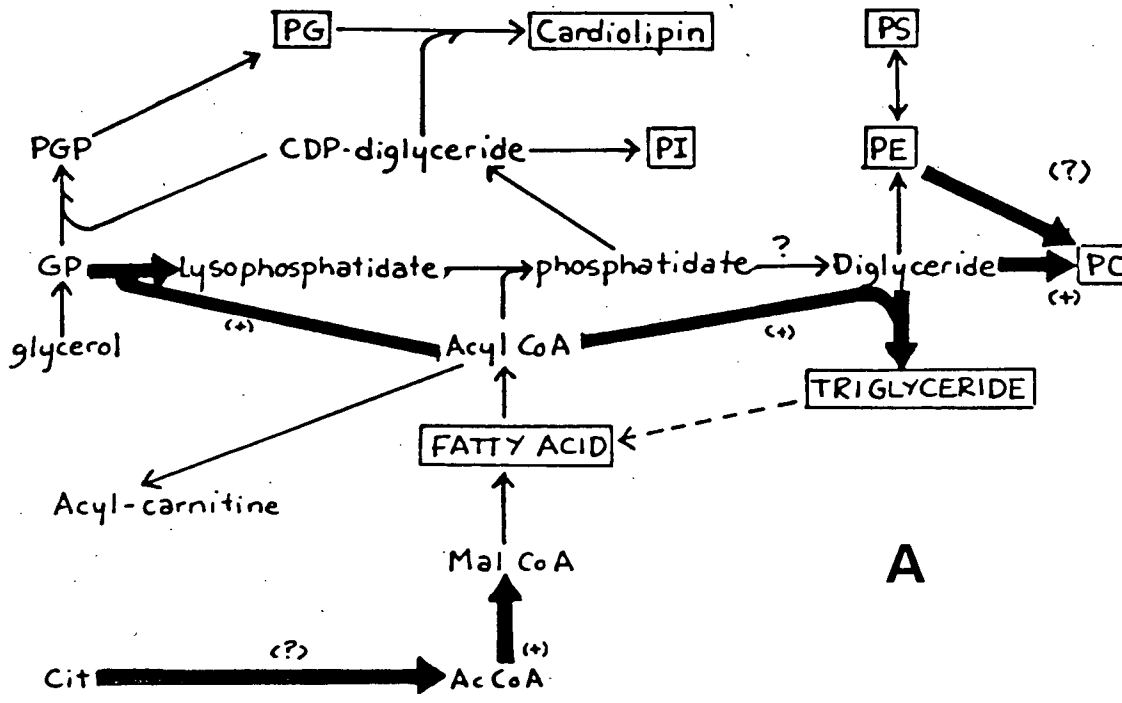
The effect of cAMP elevation is reversible in hepatocytes. When adenylate cyclase activating stimuli are removed, cAMP levels are rapidly returned to normal by the action of cAMP-phosphodiesterase, which converts cAMP to AMP (99). The proteins phosphorylated by the cAMP activated cascade system may be dephosphorylated by specific phosphoprotein phosphatases. It appears that the phosphatases are regulated as strictly as protein kinase A, but much less is known about these. It is possible that the protein phosphatases can activated by insulin. In the past few years some direct evidence has been obtained for this hypothesis, with the discovery of a second messenger of insulin, isolated from skeletal muscle (106) and adipocytes (107). The insulin mediator appears to be a peptide with a molecular weight of about 2000 daltons, and is released from plasma membranes after addition of insulin. It has been shown to activate the protein phosphatase responsible for the dephosphorylation of pyruvate

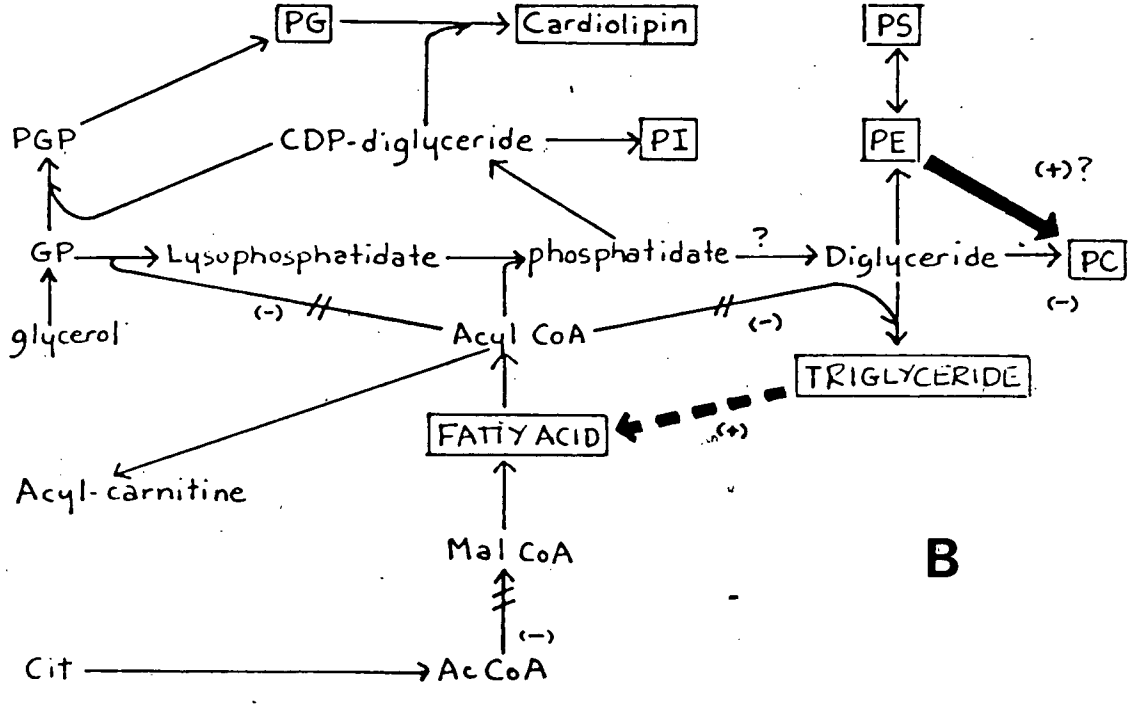
dehydrogenase (107). The generality of this insulin effect remains to be seen, but the hypothesis that insulin operates by a general scheme exactly opposing glucagon on many levels is attractive. Figure 1.5 a&b shows the opposing effects of insulin and glucagon on lipid biosynthesis.

The glucagon/insulin phosphorylation system adequately explains many of the *in vivo* effects of starvation on hepatic lipid metabolism. But there are still important effects that remain to be resolved. For one, the inhibition of PC synthesis observed in hepatocytes with cAMP analogs is notas pronounced during starvation *in vivo* (93). This could be because the increased serum and glycerol and fatty acid coming from adipocytes counteracts cAMP inhibition. This is supported by recent evidence that suggests that cAMP analog inhibition of PC biosynthesis can be counteracted by media glycerol and fatty acid in hepatocytes (54). Thus fatty acid and glucagon act together to channel DG away from TG in order to maintain PC synthesis. On the basis of this hypothesis, one would expect PC biosynthesis to be inhibited under extreme starvation conditions, when serum fatty acid and glycerol levels drop. No studies have been done to determine this.

Even less is known about the effect these serum factors on PE synthesis. Studies by Geelen *et al*. on hepatocytes indicate that PE synthesis could be enhanced with the addition of glucagon (108). However, the study was inconclusive as there was not sufficient data presented to determine if the rate of PE synthesis was enhanced, or whether the results were due to isotope dilution effects. The observations could also be explained by the inhibition of the methyltranferase pathway to PC, resulting in an increased accumulation

Figure 1.5 The effects of insulin and glucagon on glycerolipid biosynthesis (A) The glucogenic, glycolytic, lipogenic liver, (high insulin). (B) The glycogenolytic, gluconeogenic, ketogenic liver (high glucagon).





of label in PE. Such inhibition of the methyltransferase pathway has been shown to occur with cAMP analogs (73).

The effect of glucocorticoids on glycerolipid synthesis has not been carefully examined. One exception to this is a series of studies by Brindley and coworkers (33). They provided convincing evidence that glucocorticoids increase the activity of PA phosphatase, which has the effect of increasing the rate of DG synthesis. The steady state DG levels are relatively constant however (37), and the final effect of glucocorticoids is to increase the production of TG and cause fatty liver (33). This reaction prevents the fatty acid concentrations in the liver from rising to a toxic level during long term starvation. It would be interesting to see if glucocorticoids influence the synthesis of lipid synthesizing enzymes.

1.52 The Effect of Calcium on Glycerolipid Metabolism

The discovery of the cAMP cascade system by Sutherland and coworkers was truly a breakthrough in our understanding of hormonal action (109). And while it is true that glucagon, adrenalin, vasopressin, and many other hormones activate adenylate cyclase, it would be naive to postulate that these hormones all affect a cell in exactly the same way. But it has only been within the past 5 years or so that differences in the response to different hormones have been detected. The major discovery which has served a key role in understanding these different responses was the realization that calcium can serve as a messenger in a manner equivalent to cAMP. Thus calcium dependent kinases, calcium dependent enzymes, and calcium

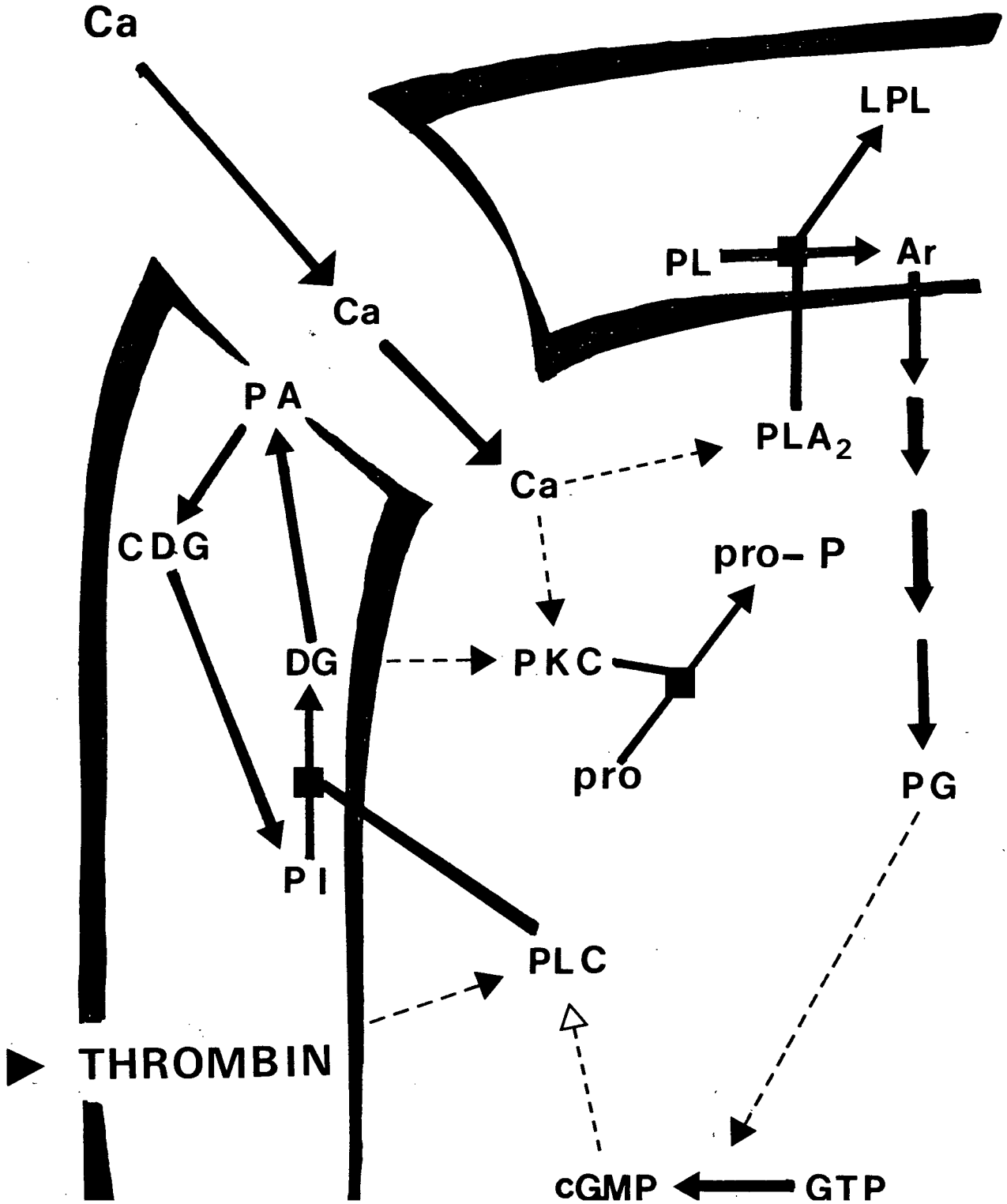
binding proteins with regulatory functions (CaM and Troponin C) have been purified, and calcium activated cascade systems have been elucidated (110).

Before considering specific calcium dependent processes operating in vivo, several general points should be made regarding the ways in which calcium is employed in the cell. First, cytoplasmic calcium levels are generally very low in comparison to serum calcium concentration (10uM compared to 1mM) (111,112). This gradient is maintained by Mg-ATPases operating at the plasma membrane, the endoplasmic recticulum, and the inner mitochondrial membrane, which transport calcium out of the cytoplasmic compartment (113). Mitochondria contain about 70% of the total cellular calcium while the endoplasmic recticulum contains about 20% (114), and these pools are hormonally responsive (115). Thus there are several ways in which to increase cytosolic calcium concentrations, depending on the pool or pools which are gated. Concerning the relation of hormone activation to calcium mediated responses, calcium can be considered a second, third, or forth messenger, in contrast to cAMP, which has always been observed as a secondary messenger. Moreover, situations have been described (see sec 3.2) where calcium can be considered a purely intracellular messenger, in that cytoplasmic concentrations can rise in response to the oxidation state of the mitochondria, irrespective of the hormonal state. It is perhaps more useful to consider calcium as a 'coupling factor' to provide a common conceptual framework for these various possibilities.

There are currently many model systems in which calcium mediated responses are being actively studied. One of the most revealing systems

that has been studied concerns thrombin induced platelet aggregation (116-122), shown schematically in Fig. 1.6. One of the first noticable events following thrombin is the activation of a phospholipase C. (116). The phospholipase C is activated by basal levels of calcium and is specific for PI (92). The mode in which its activation is coupled to thrombin binding is a matter of controversy, but one intriguing possibility is that the change in the cells shape, caused presumably by the cytoskeleton in response to thrombin binding at the plama membrane, makes the PI in the plasma membrane more available to the cytosolic phospholipase C (92). In any case, once phospholipase C is 'activated', DG is rapidly formed in the plasma membrane which can activate protein kinase C (117). The mechanism of protein kinase C is interesting in itself: protein kinase C requires acidic phospholipid and calcium for activity; however DG reduces the Ka for calcium to basal calcium levels (118). Thus protein kinase C can be activated by PI hydrolysis or increased calcium, given that acidic phospholipids are available. The DG formed is quickly acted on by diglyceride kinase *in vivo* to form PA (119). PA has ionophore properties and physiological levels of PA can cause calcium influx (120). Thus it is plausible that the production of PA is responsible for the calcium influx seen *in vivo*. The PA so produced is acted on by diglyceride cytidylyltransferase and inositol phosphotransferase to complete what is known as the PI cycle. Thus the net effect of these reactions is to increase the turnover of PI, with the consequent increases in steady state DG and PA concentrations. Once cytoplasmic calcium levels increase, phospholipase A_2 activities are stimulated and cause the release of arachadonate from PE, PC, and PS, and subsequently increase

Figure 1.6 A model for thrombin induced platelet aggregation
See text for discussion. PLC = phospholipase C, CDG = CDP-DG, Ca = calcium, PKC = protein kinase C pro = protein, pro-P = phosphoprotein, PLA_2 = phospholipase A_2, PL = phospholipid, LPL = lyso-phospholipid, Ar = arachidonate, PG = prostaglandin. (---▶) denotes enzyme activation, (——■) denotes cataylsis, (——▶) denotes an enzymatic reaction, and (---▷) denotes enzyme inhibition.



the production of prostaglandins (92). Protein kinase C phosphorylates membrane proteins which are closely associated with secretion of platelet granules (121). Lastly, the prostaglandins formed activate guanylate cyclase, and the product, cGMP, feedback inhibits phospholipase C (122).

Thus, in this system, calcium and phospholipid are important couplers in the stimulus-response sequence. The generality of this cascade system has not been firmly established but stimulation of PI turnover by different effectors has been noted in many tissues.

Although adrenergic or vasopressin activation stimulates the PI cycle in hepatocytes, the situation is different in that it does not involve calcium influx from the serum (123). Rather, it seems that intracellular calcium pools are gated to provide transient increases in cytoplasmic calcium. Thus, adrenergic activation causes calcium release from the mitochondria, vasopressin causes release from the mitochondria and endoplasmic recticulum, and glucagon does not significantly change cytoplasmic calcium at all (124).

As was discussed above, all of these hormones activate adenylate cyclase by similar mechanisms. However, it seems nessecary to postulate separate messengers in addition to cAMP for adrenalin and vassopressin, to produce the different effects on calcium gating. Discovery of such messengers would be an important breakthrough in understanding hormone action. One possible way in which adrenalin could cause calcium release could be through the GTP subunit. Adrenalin activation of adenylate cyclase is known to be associated with GTP hydrolysis (102). As was discussed in section 1.2, mitochondrial diglyceride cytidylyltranferase is stimulated by GTP. If

the adrenalin induced hydrolysis is sufficiently active to decrease mitochondrial GTP levels, it could be that this enzyme is inhibited. If this were the case, then PA levels would increase in the inner mitochondrial membrane. This could allow calcium efflux from the mitochondria. It would be very interesting to do experiments which could test this hypothesis.

Regardless of how the adrenalin message reaches the mitochondria, it is fast; maximal calcium release occurs within 5 minutes after adrenalin addition (124). Concommitantly with calcium release from the mitochondria, phospholipase A_2 activities are activated on mitochondrial membranes (90). It is not clear what is causal at this step, and there is much controversy regarding the sequence of events. Changes in the oxidation state of the mitochondria alone can cause lipase activation and calcium release (125), irrespective of the hormonal state.

As a consequence of the mitochondrial response, cytosolic calcium levels rise transiently, where the calcium can bind to CaM and various enzymes, most notably phosphorylase a (124). However, cytosolic calcium levels decrease to basal levels within 15 minutes, due to the rapid uptake of the calcium by the endoplasmic recticulum. As was mentioned in section 1.3, PS synthase is activated by calcium, and the calcium-PS can then activate protein kinase C activity at the endoplasmic recticulum. It would also be interesting to see if any of the lipid synthesizing enzymes can be phosphorylated by protein kinase C, particularly phosphocholine cytidylyltransferase. There is also a mystery regarding the calcium; one would intuitively expect the calcium to make it back to the mitochondria somehow, yet no mechanism

has been proposed. A possible mechanism for the return of calcium to the mitochondria is presented in the results section.

1.6. Experimental Approach and Rationale

Clearly, many important questions remain to be answered before the regulation of hepatic lipid metabolism can be understood as a coordinated system. The mammalian liver is an ideal tissue to study lipid metabolism for several reasons. It is nearly homogenous in composition, being composed of over 80% hepatocytes. Tissue culture techniques have been developed that allow culture of virtually 100% hepatocytes. Thus one can easily interpret experiments in terms of a single cell type. In addition, hepatocytes have many additional functions for lipids that are not found in most other tissues in that they secrete bile and lipoproteins, and are active in fatty acid synthesis. Hepatocytes are also very responsive to hormones and different nutritional states and these physiological responses give excellent foundations for biochemical studies on regulatory phenomena. The response of liver lipid metabolism to physiological stimuli is generally coordinated with the responses of other tissues such as adipose, heart, and kidney, and so learning about the regulation of the liver will undoubtedly shed light on the regulation of other tissues as well. Lastly, the results obtained in such studies are useful for far more than purely academic purposes. With stress, starvation, and environmental toxins increasingly afflicting the human populace, understanding how the liver deals with these and other diseases will certainly be of use in the medical community in their

search for more effective clinical treatments.

Of particular interest in this thesis was the descrepancy between the response of hepatocyte phosphocholine cytidylltransferase and the cytosolic enzyme to cAMP analogs. The observation that the rate of PC biosynthesis in hepatocytes was inhibited by cAMP analogs prompted further studies with glucagon and calcium on this pathway. Also, *in vitro* studies on phosphocholine cytidylyltransferase were carried out in an attempt to demonstrate CaM dependent inhibition.

1.61 The Effect of Glucagon on PC Biosynthesis

Two approaches were employed to study the effect of glucagon on PC biosynthesis. Both involved the use of cultured rat hepatocytes. The first approach used was a series of pulse and pulse chase studies with radiolabeled choline, glycerol and palmitate radiolabels. These are the primary precursors for PC biosynthesis. The effect of glucagon on the incorporation pattern of these labels as a function of time and concentration was studied. The second approach was a study on the effect of glucagon on the choline kinase, phosphocholine cytidylyltransferase, and cholinephosphotransferase activities at glucagon concentrations shown to have an effect on the labeling pattern.

1.62 Studies on the Effect of Calcium on Phospholipid Metabolism

The effect of calcium on select phospholipid pathways was

examined in hepatocytes using radiolabel techniques similar to above. In order to vary the cytosolic calcium levels, it was nessecary to use the ionophore A23187 (IPA). Some hormones also raise cytosolic calcium levels transiently, but they also elevate cAMP levels, and this would make the data very difficult to interpret. IPA is specific for calcium and is highly soluble in biomembranes. The major drawback in using IPA is that it releases calcium from the mitochondria and thus decouples oxidative phosphorylation. This can cause decreases in cellular ATP levels (126), with the possibility that many energy dependent pathways are inhibited. As a positive control, a serine label was used to see if the base exchange reaction was enhanced. These conditions were then used to study the effect of calcium and IPA on PC synthesis. The limitations inherent in this approach make all data obtained in this way inconclusive in regard to the *in vivo* situation but provides a good foundation for future studies nonetheless.

1.63 In Vitro Studies on CTP:Phosphocholine Cytidylyltransferase

In order to examine the possibility that CaM or protein kinase M could effect CTP:phosphocholine cytidyltransferase activity, studies were done using the standard CTP:phosphocholine cytidylyltransferse assay described below. An attempt was made to purify liver CaM, and at all steps the effect of the preparation on phospocholine cytidylyltransferase could be checked. This type of study provides a good counterpart to the hepatocyte studies.

MATERIALS AND METHODS

2.1. Chemicals and Isotopes

Chemicals and isotopes were purchased from the following companies.

Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri, 63178 U.S.A.

Disodium adenosine triphosphate, disodium cytidine triphosphate, phosphorylcholine chloride, betaine hydrochloride, insulin, glucagon, Hepes,Trisma base, collagenase type 1A, phenol red, and dithiothreitol, Dowex 1.

Grand Island Biological Company, 4534 Manilla Rd. S.E., Calgary Alberta.

Dulbeccos MEM formula #79-5141 (MEM^-), Hanks salts without bicarbonate, Hanks salts without calcium, magnesium or bicarbonate, Earles Salts without calcium, magnesium, or bicarbonate, Vitamin suppliment 100x, essential amino acid concentrate, 100x.

Amersham/Searle, 505 Iroquois Rd., Oakville, Ontario, L6H 2R3

[Me-^{3}H] choline (15Ci/mM), [1(3)-^{3}H] glycerol (2-5Ci/mM), [3-^{3}H] serine (19Ci/mM), [1-^{3}H] ethanolamine

(25Ci/mm), [1-^{14}C] palmitic acid (55mCi/mM), cytidine-5'-diphospho [Me-^{14}C] choline (50mCi/mM), Aqueous Counting Scintillant (ACS).

Serdary Research Laboratories, Cytidine diphosphocholine, phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylserine, di-oleoyl glycerol, sphingomyelin.

Bio-Rad Laboratories, 2580 Wharton Glen Ave., Mississauga, Ontario, L4X 2A9

Protein assay dye reagent concentrate.

British Drug House Chemicals, 15 West 6th Ave., Vancouver, B.C., V5Y 1K2.

Choline chloride.

J.T. Baker Chemical Company c/o Canadian Laboratory Supplies, 237-7080 River Rd., Richmond, B.C. V6T 1B7.

Tween 20

Brinkman Instruments, 50 Galaxy Blvd., Rexdale, Ontario, M9W 4Y5.

Silica Gel G-25 thin layer chromatography plates.

Merck, Sharpe, and Dohme, Montreal, Canada

Plastic Backed Silica Gel G-60 thin layer chromatography plates.

Calbiochem-Behring Corporation P.O. Box 12087 San Diego, California 92112. U.S.A.

Ionophore A23187, Calmodulin.

Flow Laboratories, 1625 Sismet Rd., Unit 10, Mississauga, Ontario, L4V 1V6.

Fetal calf serum, Vitrogen 100.

Pharmacia 2044 St. Regis Blvd., Dorval, Quebec., H8P 3C9.

Sepharose 4B.

2.2. The Isolation and Culture of Rat Hepatocytes

Hepatocytes were released from rat livers by collagenase perfusion, essentially by the method of Berry et al. (127). The perfusion was carried out in situ, as adapted from Davis et al. (128) by Pritchard and Vance (45). The cells were purified by differential centrifugation, and cultured on collagen coated petri plates in a medium supplemented with 20% fetal calf serum, 10ug/ml insulin, and 0.4mM ornithine, but without arginine. This media selects for hepatocytes as their urea cycle is active and synthesizes arginine from ornithine.

2.21 Isolation of Liver Cells

A 100-125g female Wistar rat was anesthetized with Nembutal.

The rat was placed under a heat lamp in a laminar flow hood and the abdomen was swabbed with ethanol. A midline incision was made through the skin from the thorax to the bladder, and the skin was torn back. The abdomen wall was cut back and the intestines were moved to the right hand side, exposing the portal vein and the inferior vena cave proximal to the renal vein. A loose ligature was placed around the vena cava anterior to the renal vein, and two loose ligatures were placed around the portal vein. The portal vein was clamped off, and a cut was made in it large enough to accept a cannula needle. A cannula was inserted into the portal vein. The initial perfusion media was Hanks salts without calcium or magnesium and contained 4.5g/l glucose, 10mg/l insulin,0.5mM EGTA, and 25mM Hepes. It was kept at 40°C and was pregassed with O_2. The vena cava was cut below the renal vein and the ligatures were tightened around the cannula. The perfusion rate was adjusted to 15 ml/min for 15 seconds to flush the blood out of the liver. The perfusion rate was maintained at 7.5ml/min for the rest of the perfusion. The thorax and diaphram were removed and a loose ligature was placed around the vena cava just posterior to the atria. The atria were cut with scissors and the ligatures around the vena cava were tightened. One or two small cuts were made in the tip of each liver lobe, and EGTA solution was run through the liver for another 4 minutes. At that time the perfusion was changed to collagenase solution, which was Hanks salts containing 4.5 g/l glucose, 10mg/ml insulin, 25mM Hepes, and 1g/l collagenase type 1A. The collagenase solution was pregassed with O_2 and kept at 40°C. The liver was perfused

for 10 minutes more, at which time the liver was essentially disrupted.

After the perfusion was completed, the liver was carefully removed and transferred to 10mls of fresh collagenase solution in a 60mm petri plate. The liver was diced by cutting it twelve times with scissors, and then it was carefully poured into a 50ml centrifuge tube. The centrifuge tube was agitated at $37^{\circ}C$ for five minutes to completely disrupt the tissue. The cells were then filtered through a coarse mesh sterile nylon filter into about 40mls of ice cold culture media and more media was added to make a suspension of 100mls. The culture media was MEM^{-} containing 20% fetal calf serum and 10mgs/l insulin. The suspension was transferred to two 50ml centrifuge tubes and spun at 90xg for 1 minute. The supernatant was discarded and the pellet washed two times with culture media. The cells were suspended in more culture media and filtered through a sterile 75uM mesh nylon filter and the volume was made up to 100mls. The cells were counted in a hemocytometer and the volume was adjusted to give a cell density of $1x10^{6}$ cells/ml. The yield was generally about $4x10^{8}$ cells/ 125g rat.

2.22 The Culture of Hepatocytes

The hepatocytes were plated out by pipetting 3mls of the suspension onto each 60mm petri dish. The petri plates had previously been coated with collagen, as follows: A sterile solution of collagen was made by adding 20mls of Vitrogen 100 (a

commercial preparation of beef skin collagen, 3mgs/ml) to 160mls of 0.01N HCl at 4°C. Two mls of this solution was pipetted into each 60mm Lux Contur petri plate under sterile conditions. The lids were left partially off for 12 hrs under a filtered air flow and UV light to allow the acid to evaporate. The plates were used the same day or stored at 4°C until use.

The viability of the cells was accessed by their ability to exclude 0.4% trypan blue and was generally about 90-95%. The cells were left undisturbed in a 37% incubator under an atmosphere of air:CO_2 (95:5) for 24 hours prior to use. After 24 hours the media was changed to serum free MEM$^-$ with 100nM insulin (unless otherwise mentioned) and preincubated for 3 hours more to diminish the hormonal effects of the fetal calf serum. All experiments were started directly after this preincubation.

2.3 Pulse-Chase Experiments

2.31 Choline Uptake Studies

In preliminary studies, control cells were incubated (pulsed) for one hour with MEM$^-$ containing 28uM [Me-^{3}H] choline (119mCi/mmol), 100nM insulin, and with or without $CaCl_2$, as indicated in the figures and tables. Other cells were treated with a similar media which also contained 1uM IPA. At the end of one hour, the cells were harvested as described in section 2.36. In other studies, cells were incubated in MEM$^-$ containing 100nM insulin, 1-100uM [Me-^{3}H] choline

(119mCi/mmol), and with or without 200mg/l $CaCl_2$ and 1uM IPA as indicated in the figures and tables. At the end of one hour, cells were rinsed once in MEM$^-$ and harvested as described in section 2.36.

2.32 Detection of a Change in the Rate of de novo PC Biosynthesis

Cells were pulsed for one half hour with 28uM [Me-^{3}H] labeled choline (178mCi/mmol) in MEM$^-$ containing 100nM insulin. After one half hour the media was aspirated off and the cell were rinsed once in MEM$^-$ containing 100nM insulin. Some cells were harvested at the end of the pulse period in each experiment. Labeled choline was efficently taken up and metabolized as previously observed (45). The rest of the cells were then incubated (chased) in unlabeled MEM$^-$ plus 100nM insulin, and with or without glucagon or IPA as indicated in the figures and tables. Cells were harvested at the times indicated in the figures and tables, as described in section 2.36.

2.33 Detection of a Change in the Rate of PC Synthesis by the Methylation Pathway

Cells were pulsed for 90 minutes with 15uCi <(1)-^{3}H] labeled ethanolamine (10Ci/mmol) in MEM$^-$ containing 100nM insulin. The cells were then rinsed once in MEM$^-$. Some cells were harvested at the end of the pulse. At that time the

majority of the label appeared in PE, with only about 15% in PC and only a trace in other intermediates. The rest of the cells were then chased with MEM^- with 100nM insulin, and with or without 1uM IPA as indicated in the figures and tables. Cells were harvested at one and three hours after the pulse as described in section 2.36. During the chase period, almost all of the label in PE was transfered to PC with a half time of about 1.5 hours. This is consistent with previous experiments (73). The PE pool was identically labeled in all experiments, and so it was assumed that any change in the rate of transfer of label from PE to PC induced by IPA during the chase period would result from a change in the rate of PC synthesis by PE N-methylation.

2.34 Incorporation of [(2)-^{3}H] Serine into Hepatic Phospholipids

Cells were pulsed with 15uCi [(2)-^{3}H] serine per dish in MEM without nonessential amino acids (to enhance serine uptake) with 100nM insulin. For pulse studies, cells were harvested at the times indicated in the tables and figures (section 2.36). Cells efficiently incorporated serine label, but the majority of the label was found in protein rather than lipid. For pulse-chase studies, the labeled media was aspirated off the cells after one hour and the cells were rinsed once with MEM^-. The cells were then incubated in MEM^- with 100nM insulin and with or without 1uM IPA. Cells were harvested as described in section 2.36 at the times indicated in the tables and figures.

Sufficient label was incorporated into lipid to observe the transfer of label from serine to PS through to PE and PC during the chase period. A minor fraction of the label was recovered in SM, but it was not sufficient to allow accurate quantitation.

2.35 Studies on the Incorporation of Other Lipid Precursors

Experiments were also done by pulsing each dish with 10uCi [(1)-^{3}H] glycerol in MEM^{-} with 100nM insulin and with or without 100nM glucagon. Cells were harvested after 20, 40 and 60 minutes as described in section 2.36. Label was incorporated linearly for at least one hour into TG, PC, and PE, while the DG and other intermediates approached steady state levels within 30 minutes.

In a similar manner, cells were pulsed with 200uM [(1)-^{14}C] palmitate (7.5mCi/mmol) per dish in MEM^{-} with 100nM insulin, and with or without 100nM glucagon. Cells were harvested after 20, 40 and 60 minutes as described in section 2.36. The cells incorporated labeled palmitate linearly into all lipids for at least one hour.

2.36 Harvesting of Cells for Lipid Extraction

In all of the pulse chase studies described above, cells were harvested in a manner similar to that described previously (45). At the time of harvesting, the media was removed, and the cells

were washed with 4mls ice cold PBS (two times for pulse plates, one time for chase plates). The cells were then scraped off into 3.6mls of ice cold methanol:H_2O (1:0.8,by volume) with a rubber policeman, and the suspension was pipetted into 4mls of ice cold chloroform. This is the equivalant of a Folch extraction (129).

2.37 Thin Layer Chromatography Analysis of Phospholipids

Aliquots of the upper phases (0.1-1.0ml) were put directly in scintillation vials for counting. For choline labeling experiments another aliquot was saved for separation of upper phase compounds. The lower phases were washed two times with chloroform:methanol:saline (3:48:47, by volume) to remove any contaminating non lipid radiolabel. One ml aliquots of lower phase were dried down in scintillation vials for counting of label. Another 1 ml aliquot was dried dowm with 25ul of total rat liver phospholipid (20mgs/ml, prepared as previously described (130)). and taken back up in 100ul chloroform:methanol (9:1,by volume) and applied to a TLC plate. When labeled choline was used, an aliquot of the media was kept for quantitation of labeled betaine.

Lipid and upper phase compounds were separated by TLC using the following systems:

System #1; Methanol:0.6% NaCl:NH_3 (10:10:1,by volume) (131). This system was to separate choline, phosphocholine, and betaine on Silica Gel G-60 TLC plates. 100uL of upper phase was spotted in each lane of a G-60 silica plate and run with carrier

compounds (0.6mgs choline; 1.5mgs phosphocholine:0.6mgs betaine per lane). The bands were clearly resolved as visualized by I_2 vapors, and were scraped into scintillation vials for counting. This system was also used for the separation of CDP-choline from phosphocholine required for CT assays (see below).

System #2; $CHCl_3$:Methanol:Acetone:H_2O, (50:30:8:3, by volume) (132). This system was used with Silica Gel G-25 TLC plates to separate SM, PC, PS, DPE, and MPE/PE. MPE and PE were not resolved but were scraped together as one band. It has been shown (73) that MPE has only 1% of the label found in PE and it was felt that this was not significant for these studies. This system was found sufficient to separate all compounds of interest when using ethanolamine label. However, PS ran too close to PC to use for serine labeling experiments, in which case it was used in conjunction with system #3 described below.

System #3; THF:Acetone:Methanol:H_2O (50:20:40:8, by volume) (133). This system was used two improve the separation of PS from PC when using a serine label. The plate was first run 15cm in system #3, and then the plate was dried and run 15cm in system #2. This gave a very clear resolution of SM from LPC, PC, PS, DPE, and PE, as visualized under I_2 vapors.

System #4; Diethyl Ether: Hexane: Acetic Acid (40:60:1,by volume) (134) This system was used in conjunction with System #2 to separate the neutral lipids in palmitate or glycerol labeling experiments. The plate was run 9cm in System #2 to separate PE, from PC, leaving the neutral lipids at the solvent front. The plate was dried and run for 20cm in System #4 to resolve the

neutral lipids into fatty acid, DG, and TG fractions.

2.38 Scintillation Counting

All samples were counted in 1ml of water with 9mls of ACS. When counting scrapings from TLC plates, the samples were left in a shaker for two days to ensure that the counts would completely elute into the cocktail. All samples were counted for 1 minute on two channels, one with a nearly open window, and the other covering only the first one third of the beta spectrum. The channels ratio was then calculated. Standards containing a known amount of the isotope of interest were counted simultaneously, and their dpm/cpm ratio (the counting efficiency) as well as their channels ratio was calculated. A quench curve was generated and the dpms in each sample was then calculated.

2.4 Enzyme Activity Studies

2.41 Incubation of Hepatocytes

24 hour hepatocytes were prepared as usual,and were preincubated for 3 hours in serum free MEM with no insulin. A pulse-chase study with [Me-^{3}H] choline was carried out prior to this to determine if there was any difference between the glucagon response with or without insulin in the preincubation media. No significant difference was observed. After the preincubation, cells were incubated for one hour with MEM with

100nm insulin and with or without 100nm glucagon. Hepatocytes were maintained at 37°C throughout the incubation.

2.42 Harvesting of Hepatocytes and Cell Fractionation

At the end of the one hour incubation in the presence or absence of glucagon, 5 sets of triplicate dishs of hepatocytes were harvested and fractionated, by a modification of a previously described technique (12). The medium was aspirated and the cells were rinsed once with ice cold PBS, and then scraped off into 2.5mls of homogenizing buffer ice cold (0.145M NaCl; 10mM Tris-HCl, pH 7.4; 7mM EDTA; 10mM NaF;) with a rubber policeman. The combined suspensions from each triplicate were homogenized with 20 cycles of a tight fitting glass dounce, and the homogenate was transferred to a polycarbonate centrifuge tube and spun for 15 minutes at 14,000 rpm in a Ti75 rotor to remove nuclei, unbroken cells, and mitochondria. The supernatant was spun for 60 minutes at 50,000 rpm to separate the microsomes from the cytosol. The microsomal pellet was resuspended in a buffer containing .25M sucrose; 10mM Tris-HCl, pH 7.4; 1mM EDTA; and 10mM NaF. The entire process was carried out at 4°C.

2.43 Enzyme Assays and Protein Estimations

Enzyme assays were performed on the microsomal and cytosolic fraction. The assays rely on previously established procedures which are summarized below.

a) Choline Kinase (135): Assays for choline kinase were carried out on the hepatocyte cytosols. The reaction mix contained (in 100ul) 0.1M Tris-HCl,pH 8.5, 0.010M MgCl2, 0.010M ATP, and 1.0mM [Me-^{3}H] labeled choline (20mCi/mM). Assays were initiated by the addition approximately 30-50ug of protein to the other components. The mixture was incubated for 20 minutes at 37°C. The reaction was terminated by boiling for two minutes. 50ul of this solution was spotted onto a Silica G-60 plate with 10uL of 150 mM phosphocholine carrier and run for 10cm in solvent system #1 described in section 2.37. The phosphocholine band was visualized on a light box and the band was counted for radioactivity. Control studies were carried out previously to assure that all assays were kineticly linear.

b) Phosphocholine cytidylyltransferase (53): Assays for cytidylyltransferase were performed on rat liver cytosol or microsomes. The reaction mixture contained (in 100ul), 1.5mM [Me-^{3}H] labeled phosphocholine (40mCi/mM), 2mM CTP, 8mM $MgCl_2$, and 0.08M Tris-HCl, pH 6.5. The reaction was started by the addition of cocktail to 40-150 ug of protein, and it was allowed to react for 15 minutes at 37°C. In some cases the assay was carried out in the presence of total rat liver phospholipid, which was dried down under nitrogen in the tube, and then resuspended in the enzyme preparation by vortexing. The reaction was terminated by boiling for two minutes, and centrifuged for 10 minutes at 7000 rpm to remove denatured protein lipid aggregates. 50ul of the supernatant was spotted on a plastic backed G-60 Silica TLC plate with 10ul of CDP-choline

carrier (50mgs/ml) and run for 10cm in solvent system #1. The CDP-choline band was visualized on a light boxand then cut out into a scintillation vial for counting of radioactivity. Previous control studies were carried out to assure that all assays were kineticly linear.

c)Choline phosphotransferase (136): This enzyme was assayed in hepatocyte microsomes prepared as described above. The reaction mix contained (in 250ul), 50mM Tris-HCl, pH 8.0, 0.20M MgCl2, 0.50mM DTT, 0.40mg/ml Tween 20, and 0.50mM [Me-^{14}C] CDP-choline (4uCi/uM). The reaction was started by the addition of cocktail to approximately 150-200ug of protein, and the reaction mixture was maintained at 37^{o}C for 20 minutes. The reaction was terminated by the addition of 1.6mls $CHCl_3$:MeOH (1:1,by volume). 0.8mls of chloroform and 0.35mls H_2O was added subsequently to give the equivalant of a Folch (129) extraction. 20ul of CDP-choline carrier (20mg/ml) and 20ul of total rat liver phospholipid (20mg/ml) were then added and the assay tube was spun at 7,000 rpm for 10 minutes. The upper phase was discarded and the lower phase was washed twice with theoretical upper phase. An aliquot of the lower phase was dried down and counted for radioactivity. Previous control studies were carried out to assure that all assays were kineticly linear.

d) Protein Determinations (137): Protein concentrations were estimated by a modification of the Bio-Rad protein assay system, as follows. Twenty mls of freshly filtered Bio-Rad Protein Assay Dye Reagent Concentrate was dilute to 85mls with H_2O. Samples containing 10 to 125ud protein were diluted to a final volume of

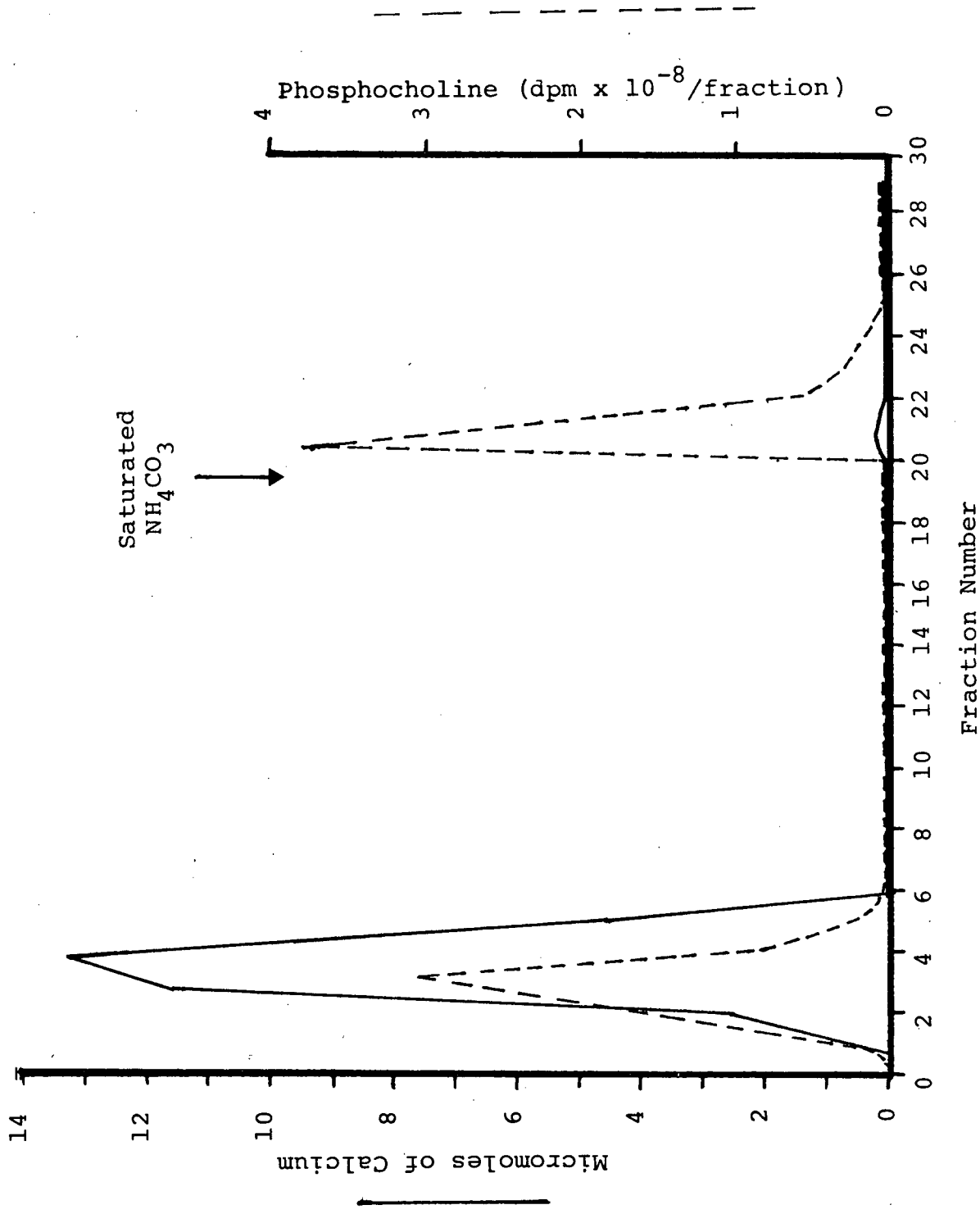
0.5ml. To the protein sample 2.5 mls of diluted dye reagent was added and vortexed gently. After sitting for 15 minutes the absorbance was read at 595nm. The solutions were stable for at least one hour. The results were compared to a standard curve generated with IgG protein which was linear from 0 to 125ug protein.

2.5 Studies on Phosphocholine Cytidylyltransferase in vitro

2.51 Preparation of Calcium-Free Labeled Phosphocholine

[Me-^{3}H] phosphocholine was prepared as previously described (138), and unlabeled phosphocholine was added to give a specific activity of 20uCi/uM. 0.72mCi of this preparation in a total of 9mls was applied to a Dowex 1 (H form) ion exchange column of bed volume 1ml and the calcium was washed through with 55 mls of distilled H_2O. The phosphocholine was eluted with 32mls of saturated ammonium carbonate. 3.2 ml fractions were collected and assayed for tritium. Calcium was determined by atomic absorption (see figure 2.1). The calcium-free phosphcholine fractions were pooled and concentrated by lyophilyzation. Distilled water was added back to give a 7.5mM solution of phosphocholine. The yield was 48%. The remainder of the radiolabel eluted with the calcium and was not characterized further. Atomic absorption for calcium was carried out on all components of the phosphocholine cytidylyltransferase assay and the total calcium in the assay was 17 micromolar.

Figure 2.1 Ion exchange chromotography of calcium phosphocholine chloride. (——) denotes calcium and (- - -) denotes phosphocholine. See text for details.


Phosphocholine (dpm x 10^{-8}/fraction)
Saturated NH_4CO_3
Micromoles of Calcium
Fraction Number

2.52 Partial Purification of CaM

CaM was partially purified from rat liver cytoplasm using the protocol of Cheung et al., for the isolation of CaM (105). 40 grams of rat liver were homogenized in 120 mls of cold Buffer A (20mM Tris-HCl, pH 7.5,with 20mM $MgSO_4$). The homogenate was spun for 30 minutes at 12,000xg. It was then boiled for 4.5 minutes, and spun again to remove denatured protein. The supernatant was dialyzed 3 times against 4 liters of the Buffer A. The dialysate was assayed for phosphocholine cytidylyltransferase inhibition, then applied to a 1.5 cm x 20 cm column of DEAE Sepharill which was previously equilibrated with buffer A containing 0.15M ammonium sulfate. The sample was eluted with 200mls Buffer A containing 0.15M ammonium sulfate, then a 150mls of a linear gradient from 0.15M to 0.3M ammonium sulfate in buffer A. Subsequent to this another 200mls 0.3M ammounium sulfate in Buffer A was added then 30mls 0.5M ammonium sulfate in buffer A. 100 fractions of 4.5mls were collected and assayed for phosphocholine cytidylyltransferase inhibition, protein, and conductance. Peak 2 (see results) was applied to an Amicon column eluate concentrator and ultrafiltrated. This destroyed the inhibitory activity.

RESULTS AND DISCUSSION

3.1. The Effect of Glucagon on Phosphatidylcholine Biosynthesis

3.11 The Effect of Glucagon on [^{3}H] Choline Incorporation into Hepatocyte Phospholipid

These experiments were designed to test the effect of glucagon on PC biosynthesis by the *de novo* pathway. Choline label has proved to be the best indicator of the rate of PC biosynthesis in past studies (41,45,53). The incorporation pattern of [Me-^{3}H] choline into hepatocytes after a one half hour pulse is shown in Table 3.1. These results agree with previous studies (45), and show that over half of the choline label was rapidly oxidized to betaine under these conditions, while most of the rest of the label was phosphorylated. Of the label that was phosphorylated, the vast majority was found in phosphocholine, while only 2% had appeared in PC. The amount of label in CDP-choline was exceedingly small, and was not quantitated in this study. This supports the accepted view that CTP:phosphocholine cytidylyltransferase is the rate limiting enzyme in the synthesis of PC.

The effect of adding 100nM glucagon to the chase media is shown in figure 3.1. Transfer of label from phosphocholine to PC was inhibited rapidly, amounting to 50% inhibition ($p<.001$) within 15 minutes. The inhibition was sustained to some extent throughout the 2 hour chase period (about 30% at the end of one hour). The total amount of label in

Table 3.1 The incorporation pattern of [Me-^{3}H] choline into cultured rat hepatocytes. Cultured rat hepatocytes were incubated for one half hour in MEM containing 100nM insulin and 28uM [Me-^{3}H] Choline (178mCi/mmol) at 37 C. The cells were harvested and analyzed as described in section 2.3.

	Incorporation of [Me-^{3}H] choline (10^{-5} dpm x 10 /dish)	Percent Incorporation
Choline	6.5±.05	12%
Phosphocholine	18.0±.1	33%
Phosphatidylcholine	1.1±.1	2%
Betaine	28.0±.2	52%
Total	53.6	100%

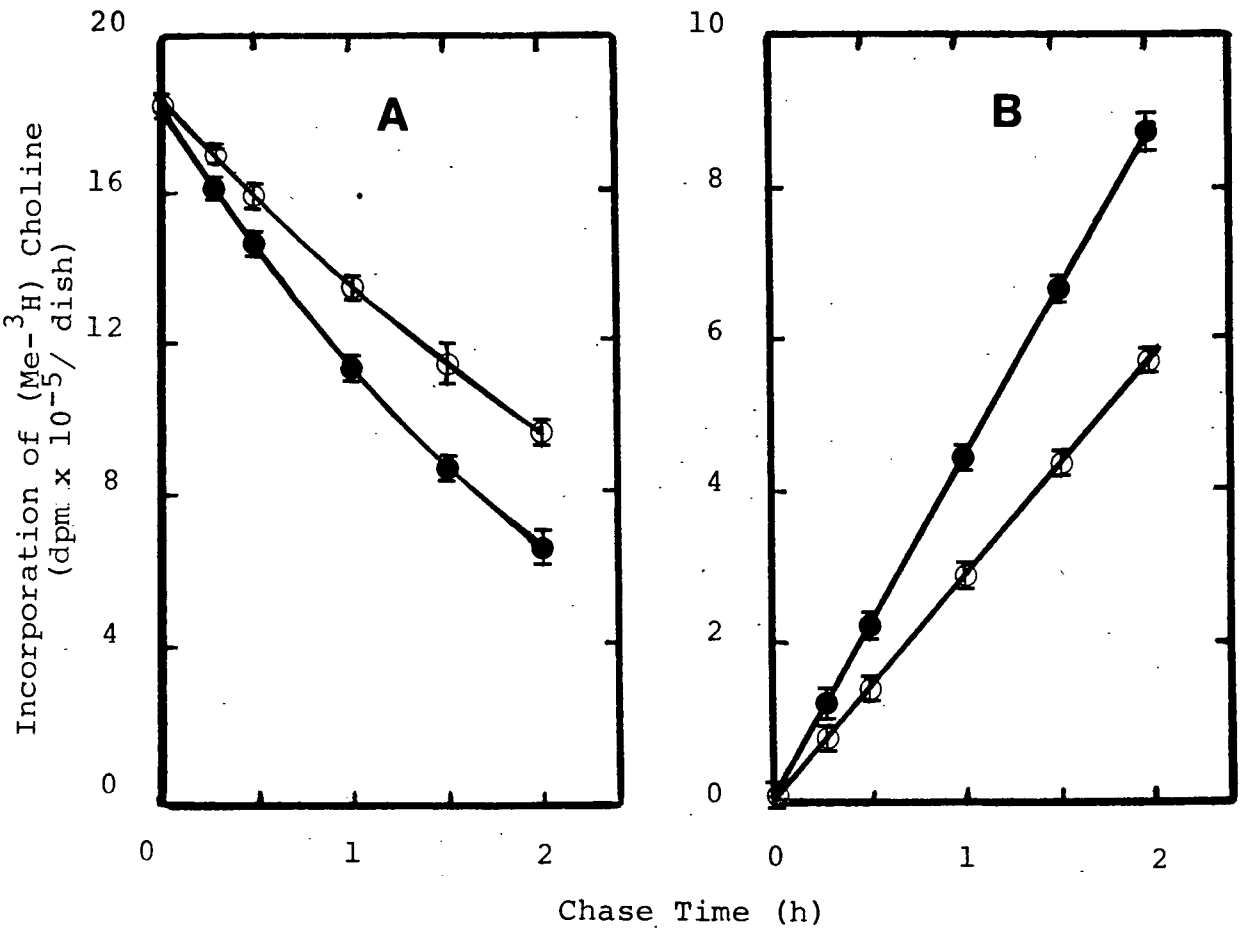


Figure 3.1 The effect of glucagon on the disappearance of [Me-^{3}H] choline from cellular phosphocholine and the accumulation into phosphatidylcholine. Cultured rat hepatocytes were pulse labeled for one half hour with 10uCi [Me-^{3}H] choline. The cells were washed and fresh medium with (O) or without (●) 100nM glucagon was added. At various times, the cells and media were collected, and the radioactivity was quantitated in (A) phosphocholine; and (B) phosphatidylcholine. Each point reprtesents the mean of three dishes, and SE is indicated by bars.

phosphocholine and PC was constant throughout the chase period, which supports the hypothesis that the phosphorylation of choline effectively commits it to the synthesis of PC (45). The halflife of phosphocholine was calculated from this experiment (1.5-2.5 h), and is similar to previous studies (53). The amount of label in choline was uneffected by this treatment. Also, there was no effect on the secretion of betaine into the media (figure 3.2). These results are similar to previous studies on the effect of cAMP analogs on the rate of PC biosynthesis (53), and are consistent with the hypothesis that glucagon acts on hepatocytes metabolism primarily by raising the intracellular cAMP levels (102).

In figure 3.3, a glucagon titration curve is presented with respect to the labeling pattern of phosphocholine and PC. The data was taken after a one hour chase period with various concentrations of glucagon. Half maximal inhibition of label transfer to PC occurs at 10nM glucagon, which is close to the Km reported for hepatic glucagon receptors (4.5nM) by Rodbell et al (98). The fact that the experimental Ki is slightly higher than the receptor binding constant can be rationalized by considering that insulin concentration in the media was 100nM, and it is well documented that the insulin:glucagon ratio may be more important to the cellular state than the absolute concentration of either hormone (95). Moreover, it has been shown that the liver degrades glucagon at a substantial rate (95), and thus the average glucagon concentration over the hour chase could be significantly lower than the concentration at the beginning of the chase. With these factors considered, it thus seems probable that this inhibition occurs in vivo.

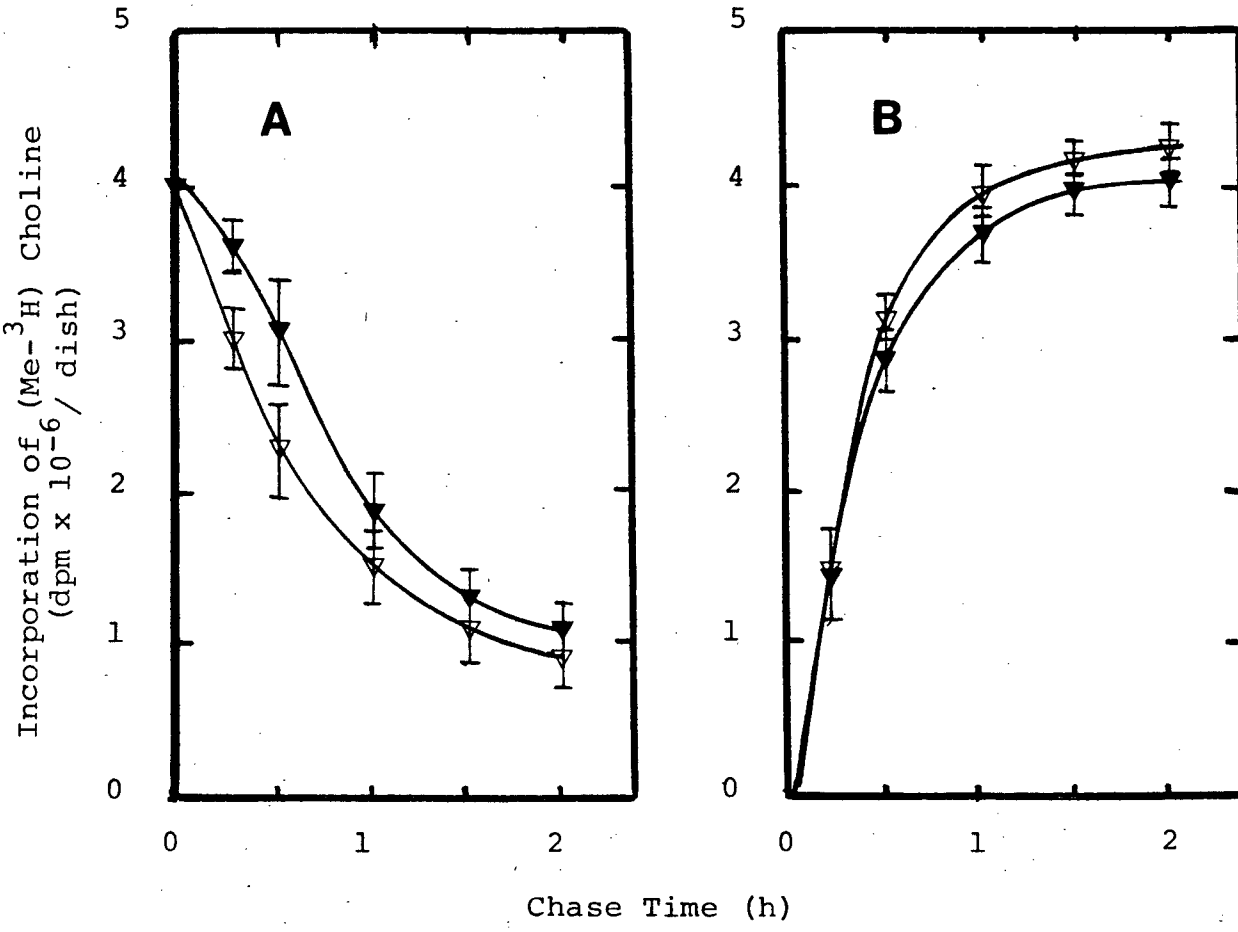


Figure 3.2 The effect of glucagon on the sectetion of [Me-^{3}H] betaine into the culture media. Cultured rat hepatocytes were pulse labeled for 30 minutes with 10uCi [Me-^{3}H] choline. The cells were washed and fresh medium with (▽) or without (▼) 100nM glucagon was added. At various times, the cells and media were collected, and the radioactivity was quantitiated in (A) cellular betaine; and (B) media betaine. Each point represents the mean of three dishes. and SE is indicated by the bars.

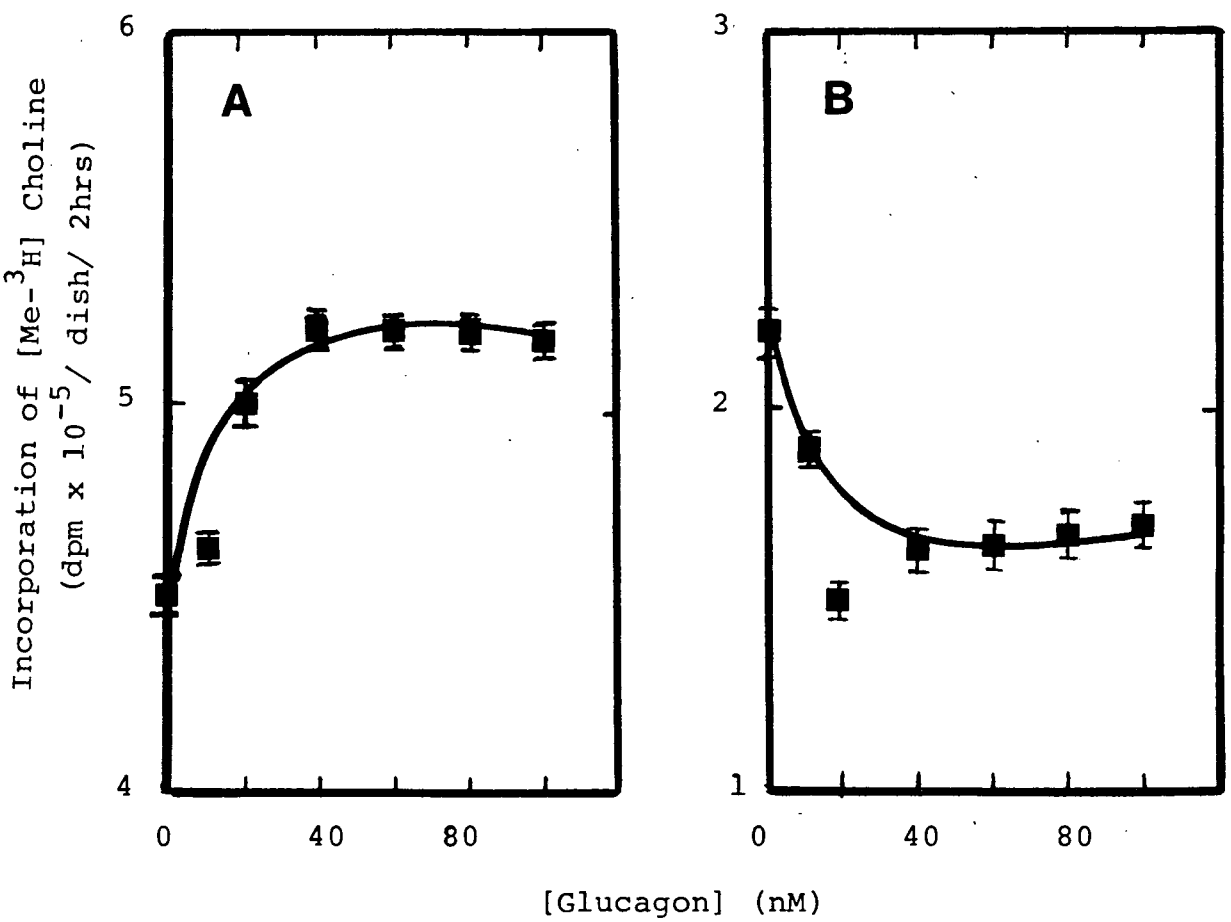


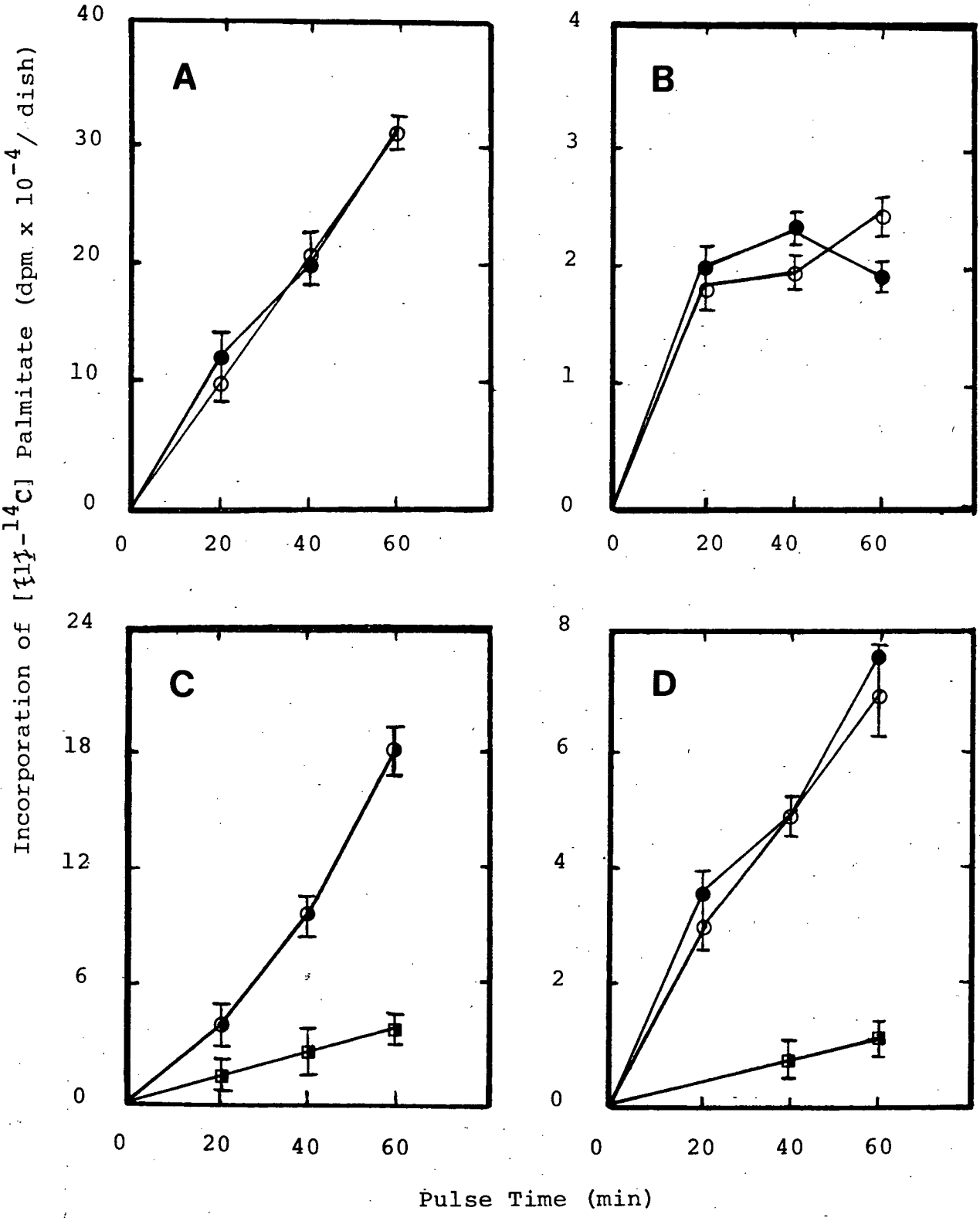
Figure 3.3 The effect of glucagon concentration on the incorporation of [Me-^{3}H] choline into cellular phosphocholine and phosphatidylcholine. Cultured rat hepatocytes were pulse labeled for one half hour with 10uCi [Me-^{3}H] choline. The cells were washed and fresh medium with various concentrations of glucagon was added. After one hour, the cells and media were collected, and the radioactivity was quantitated in (A) phosphocholine; and (B) phosphatidylcholine. Each point represents the mean of three dishes, and SE is indicated by bars.

It is known that cAMP analogs decrease choline uptake (53). This would act to increase the specific radioactivity of the phosphocholine pool, which means that the inhibition of PC synthesis by glucagon is greater than the labeling data indicates. Glucagon probably inhibits the rate of PC biosynthesis at a step subsequent to choline phosphorylation. This could be achieved by decreasing the availability of either DG or CDP-choline. It is unlikely that DG availability is decreased as the amount of hepatic PC has been shown to remain be relatively constant under conditions where DG concentrations have varied markedly (37). Definitive studies have not been done on hepatocytes, however. This could be tested if a sensitive assay for DG was available. It is more probable that the inhibition of PC synthesis is a reflection of an inhibition of the cytidylyltransferase step, caused by a decrease in CTP:phosphocholine cytidylyltransferase activity, or of CTP levels.

3.12 The Effect of Glucagon on the Incorporation of [^{14}C] Palmitate into Hepatocyte Glycerolipids

The effect of glucagon on the incorporation of palmitate into glycerolipid was studied to see if the inhibition of the rate of PC biosynthesis could be observed with palmitate label as well. A pulse experiment was performed as a preliminary study. The incorporation pattern of [1-^{14}C] palmitate into hepatocytes is shown in figures 3.4a-d, with and without 100nm glucagon. The total uptake, shown in 3.4a, was linear up to one hour, and was independent of glucagon under these conditions. The incorporation into the different classes of

Figure 3.4 The effect of glucagon on the incorporation and metabolism of [1-^{14}C] palmitiate by cultured rat hepatocytes. Cultured rat hepatocytes were pulse labeled for various times in culture media with 200uM [1-^{14}C] palmitate and without glucagon (open symbols), or with 100nM glucagon (closed symbols). The cells were harvested and the radioactivity was determined in (A) total lipid; (B) free fatty acid; (C) diglyceride (□,■) and triglyceride (○, ●); and (D) phosphatidylcholine (○, ●) and phosphatidyethanolamine (■, □). Each point represents the mean of three dishes, and SE is indicated by bars.


A
B
C
D
Incorporation of [1-^{14}C] Palmitate (dpm x 10^{-4} / dish)
Pulse Time (min)
0
20
40
60
0
10
20
30
40
1
2
3
4
6
12
18
24
8

lipids, is shown in figures 3.4b&c. The pattern of incorporation was also unaffected by the addition of glucagon.

The recovery of palmitate label as fatty acid plateaued at 20 minutes (figure 3.4b). This is expected as free fatty acid is an intermediate present only in trace quantities in hepatocytes (64). Thus, the specific radioactivity of the fatty acid pool rapidly increases to a maximum because the label is rapidly oxidized or esterified. Glucagon has no significant effect on fatty acid label incorporation because fatty acid is utilized as fast as it is taken up, irrespective of the hormonal state. DG is also an intermediate, although the pool size is much larger and the label does not reach a steady state concentration within the pulse period. Glucagon has no effect on the label recovered in DG. DG levels are known to decrease during fasting, however (37). It is possible that the effect of glucagon is masked by isotope dilution effects. Thus, the amount of label in DG is the same although the DG in the glucagon treated cells has a higher specific activity. This seems likely if it is assumed that the amount of acyl-CoA produced from endogeneous fatty acid is decreased while that produced from exogeneous fatty acid remains the same. The linear incorporation of label into phospholipids and TG (figure 3.4c&d) is expected because the pools are much larger than their precursors and hence the label would take much longer to equilibrate and turnover. The finding that glucagon had no effect on the incorporation of palmitate label into these lipids can be mimicked by cAMP analogs (54). This would suggest that the decrease in glycerolipid synthesis caused by glucagon was masked by isotope dilution effects. One way to rationalize this would be to postulate

that the diglyceride label was not diluted as much by endogenously synthesized fatty acid in the glucagon cells, and therefore the specific radioactivity a of the diglyceride pool is higher and masked the inhibition. This interpretation seems likely as it is well known that glucagon inhibits the rate of fatty acid biosynthesis in hepatocytes (141). Again it would be useful to measure the pool size of DG to test this possibility.

These findings are to be contrasted with the findings of Geelen et al (142), who reported an inhibition of palmitate incorporation into TG with 85nm glucagon, although the rate of phospholipid synthesis was unaffected. However, the conditions of their experiment were different than the one described here, in that the palmitate concentration was 540uM, as opposed to the concentration of 200uM used in this study. Thus it is plausible that with the higher concentration of exogenous fatty acid the label could be channelled towards B-oxidation under a glucagon load. This would cause a decrease in the radioactivity appearing in DG, and consequently in TG, PE, and PC. However, recent work has shown that palmitate can increase the rate of PC synthesis when it has previously been inhibited by cAMP analogs (54). Thus under the conditions of Geelens experiment it is possible that the rate of PC synthesis was maintained by the balance of two opposing effects. This interpretation is in keeping with the in vivo situation, where serum fatty acid concentrations increase concommitantly with glucagon levels under starvation conditions. Thus one could propose that glucagon inhibits the rate of synthesis of both PC and TG, but as exogenous fatty acid concentrations rise, PC synthesis and B-oxidation increase, the effect being a channelling of

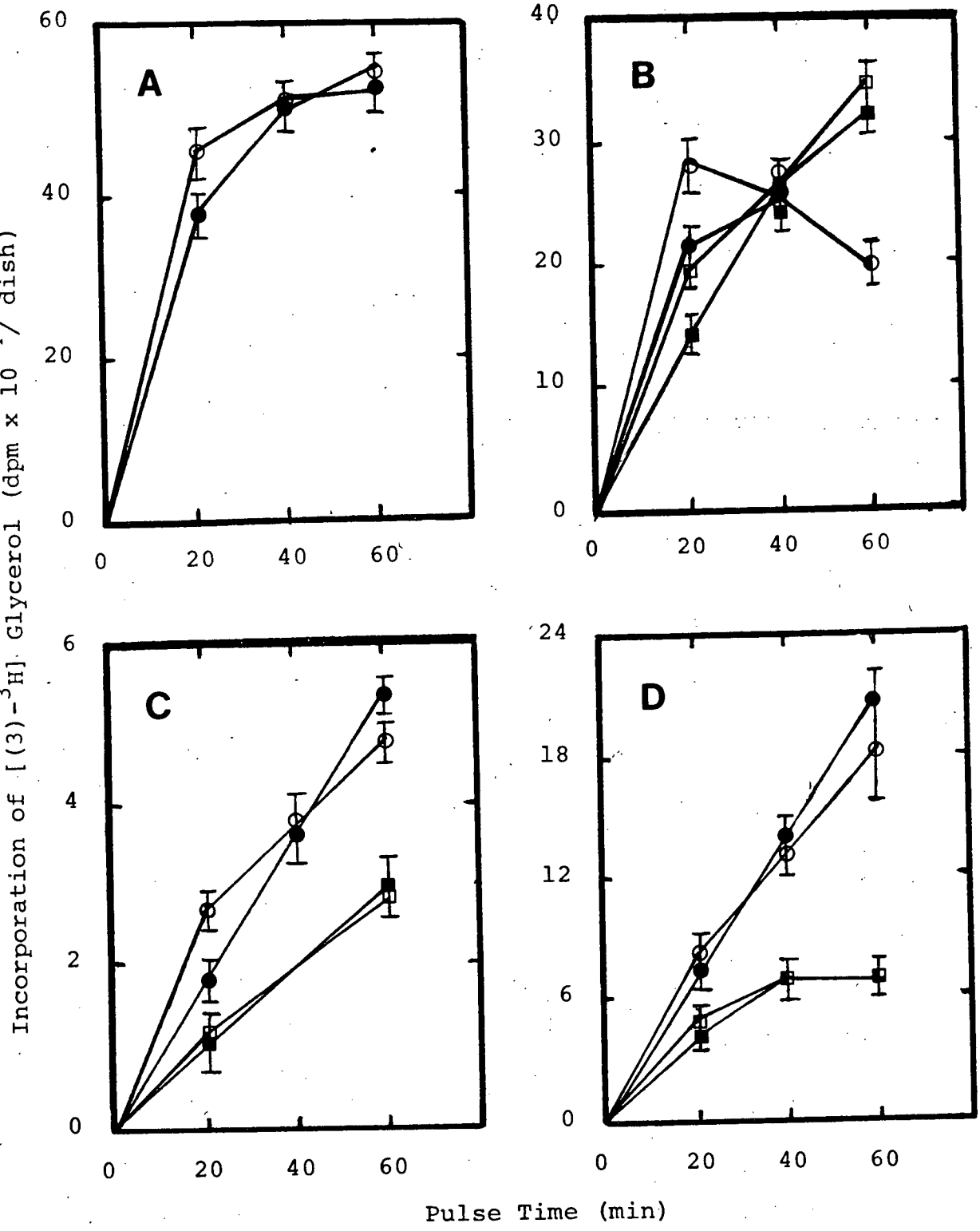
fatty acid away from TG synthesis in order to maintain the phospholipid and energy requirements of the cell.

3.13 The Effect of Glucagon on [^{3}H] Glycerol Incorporation into Hepatocyte Glycerolipids

A pulse experiment was performed with glycerol radiolabel to see if a glucagon effect on PC biosynthesis could be observed with this precursor. In figures 3.5a-d, the incorporation pattern of [1(3)-^{3}H] labeled glycerol into hepatocytes is shown,in the presence and absence of 100nM glucagon. The total incorporation plateaued within 30 minutes (3.5a), implying that the cells have sufficient endogeneous glycerol to maintain normal cell functions. The incorporation into lipid was approximately linear throughout the hour pulse (fig. 3.5b), while the label in the upper phase peaks and diminishes, as would be expected if the total uptake plateaus. Glucagon slightly inhibits the uptake at 15 minutes, but this is of doubtful physiological significance. The relative incorporation of glycerol label into the various lipid components is unaffected by glucagon.

The incorporation of glycerol into phospholipids proceeds linearly throughout the pulse period (figure 3.5c). After one hour, 100nM glucagon exerted a slight stimulatory effect on the incorporation PC (15%, $p<.001$), but no significant effect on the incorporation into PE.This result is consistent with the hypothesis that DG is preferentially channelled into PL under a glucagon load, although the stimulation is so small that it lends no real support to this theory. Geelen et al.(141), demonstrated an increase in the incorporation of

Figure 3.5 The effect of glucagon on the incorporation and metabolism of [(3)-^{3}H] glycerol by cultured rat hepatocytes. Cultured rat hepatocytes were pulse labeled for various times in culture media with 200uM [(3)-^{3}H]glycerol and without glucagon (open symbols), or with 100nM glucagon (closed symbols). The cells were harvested, and the radioactivity was quantitated in (A) total cellular metabolites (B) aqueous phase metabolites (circles) and total lipid metabolites (squares); (C) phosphatidylcholine (circles) and phosphatidyethanolamine (squares); and (D) triglycerides (circles) and diglycerides (squares). Each point represents the mean of three dishes, and SE is indicated by bars.


A
B
C
D
Incorporation of [(3)-^{3}H] Glycerol (dpm x 10^{-4}/ dish)
Pulse Time (min)
0
20
40
60
0
10
20
30
40
0
2
4
6
0
6
12
18
24

glycerol label into PC and PE of about 25% after one hour. This inconsistency may be explained by considering several experimental differences. The concentration of media glycerol was 0.5mM in his study, and this could be significant as there is some indication that glycerol can stimulate PE and PC synthesis (54). Also, Geelen had no insulin in his glucagon containing media, which should act to enhance a glucagon mediated effect. Lastly, Geelen used 10uM ethanolamine in his media, and this could be neccesary to see an increase in PE synthesis . It could be that the concentration of ethanolamine is rate limiting for PE biosynthesis at very low media concentrations of ethanolamine. This is supported by the observation (73) that hepatocytes incorporate pulse ethanolamine into PE very fast when the specific activity is high enough. Thus any increase expected in the incorporation into PE in this experiment could be masked by the lack of substrate. The difference between these experiments emphasizes the need to determine the effect of various substrates on phospholipid synthesis in order to properly interpret experimental observations.

In figure 3.5d the incorporation pattern of [^{3}H] glycerol into neutral lipids is shown. It is shown that the label in DG peaks within 30 minutes. This is to be expected as the label in the upper phase precursors peaked within 20 minutes. Glucagon had no effect on the amount of label recovered in DG. As it is likely that glucagon decreases the pool size of DG (140), this result implies that the pool size of glycerol phosphate available to lipid biosynthesis is coordinately regulated with the DG pool. In other words, the glucagon induced decrease in the rate of DG synthesis from glycerol phosphate and acyl-CoA would be coordinated with a decrease in the pool size of

glycerol phosphate. Thus, in glucagon treated cells, the amount of label in DG would remain constant although the specific activity would increase. Measurement of the DG and glycerol phosphate pool sizes could be done to confirm this postulate. Another way to test this hypothesis would be to use higher media concentrations of glycerol, to increase experimentally the glycerol phosphate pool size. A glucagon effect on DG label incorporation should then be seen, as the inhibition of glycerol phosphate acyltransferase and PA phosphatase by glucagon could then be observed (discussed in section 1.5). The observation that the incorporation of glycerol into TG was not influenced by glucagon confirms Geelen's (142) study, as well as the palmitate study presented above. Both the palmitate and glycerol label studies are consistant with the hypothesis that most TG is produced from DG arising from the PA-phosphatase pathway.

The contrast between these experiments, as well as the palmitate experiments described above, emphasize the need to do future studies that vary the concentrations of lipid precursors in the presence and absence of glucagon. Specifically, the effect of glucagon on the incorporation of choline into phospholipid should be examined as a function of glycerol concentration, to see if glycerol has a compensating effect on the inhibition, as fatty acid does. Also, the effect of glucagon on the <u>de novo</u> pathway of PE synthesis as a function of fatty acid and glycerol should examined. This would allow a comparison of the regulation of the <u>de novo</u> pathways of PE and PC synthesis, and this could help elucidate the mechanisms by which these pathways respond differently to fasting. The palmitate and glycerol studies presented above should be repeated as a function of media

fatty acid and glycerol concentrations, in the presence of normal serum concentrations of ethanolamine and choline. The results from these experiments would greatly contribute to our understanding of how substrate supply and hormonal control mechanisms coordinately adjust the biosynthesis of hepatic glycerolipids to the nutritional state of the organism *in vivo*.

3.14 The Effect of Glucagon on the Enzyme Activities Responsible for de novo PC biosynthesis

The enzyme activities of choline kinase, CTP:phosphocholine cytidylyltransferase, and choline phosphotransferase from control and glucagon treated hepatocytes were determined in an attempt to correlate the rate changes observed with labeled precursors with changes in enzyme activities. Previous studies (53) with cAMP analogs suggested that the inhibition of the cytidylyltransferase was caused by protein phosphorylation. In the work up of the hepatocytes, kinases and phosphatases could be active which would preclude any attempt to observe a glucagon dependent inhibition. To circumvent this possibility, harvests were carried out in the presence of 10mM NaF in order to prevent the action of phosphatases, and 10mM EDTA to inhibit protein kinases. This insures that any change in the amount of protein phosphorylation will be maintained throughout the work up.

Glucagon inhibited CTP:phosphocholine cytidylyltransferase by 35% ($p<0.001$) after one hour, in both the cytosol and the microsomes (table 3.2). This correlates well with the inhibition observed with [*Me*-^{3}H] choline. Choline kinase activity is also inhibited

Table 3.2 The effect of glucagon on the activities of enzymes responsible for phosphatidylcholine biosynthesis. Cultured rat hepatocytes were treated with culture media or 100nM glucagon for 1 hour. Values are the average of 15 dishes ± SD.

Enzyme Assayed	Control Activity (nmol/min/mg) ±SE	Activity with 100nM Glucagon (nmol/min/mg) ±SE	
Choline Kinase	0.68±.04	.56±.05	*
Cytosolic CTP:phosphocholine Cytidylyltransferase without Phospholipid	.047±.014	.031±.004	**
Cytosolic CTP:phosphocholine Cytidylyltranferase with Phospholipid	.52±.04	.54±.03	
Microsomal CTP:phosphocholine Cytidylyltranfersase	.57±.2	.32±.07	**
Cholinephosphotranferase	.031±.003	.024±.002	*

* $p<0.01$
** $p<0.001$

marginally by 18% ($p<0.01$). It is possible that this increases the amount of choline which is oxidized, by leaving more choline to be acted on by choline oxidase. cAMP analogs were shown to have a similar inhibitory effect on the hepatic cytidylyltransferase when cells were harvested in this manner (53). The addition of phospholipid to the phosphocholine cytidylyltransferase assay supresses the inhibition and this was also found with cAMP analogs. Choline phosphotransferase was slightly inhibited but this is not statistically significant. Thus, this data supports the generally accepted notion of glucagon as a starvation signal, which inhibits key biosynthetic enzymes by elevating cAMP levels.

In vitro studies with rat liver cytosol (54) have shown that the activity of CTP:phosphocholine cytidylyltransferase can be inhibited in a time and concentration dependent manner by Mg-ATP and calcium (see section 1.25). It was also shown that the activation of cytosolic cytidylyltransferase at $4^{\circ}C$ can be inhibited simply by preincubation with NaF at $4^{\circ}C$. The inhibition can be supressed by addition of protein kinase inhibitors isolated from rabbit skeletal muscle. Moreover, studies have shown that the inhibition of hepatocyte phosphocholine cytidylyltransferase observed with cAMP analogs can not be seen if NaF is not present during the harvest (53). All of these typesof inhibition can be reversed by phospholipid, and suggest that they operate bya common mechanism involvingphosphorylation of CTP:phosphocholine cytidylyltransferase. However, a complete proof that the cytidylyltransferase is phosphorylated must await purification of the enzyme so that kinase-induced phosphorylation may be demonstrated directly. Moreover, as was discussed in section 1.2,

the identity of the kinase remains unknown. This presumptive phosphorylation of cytidylyltransferase inhibits activity by increasing the Km for CTP, and also inhibits lipid induced aggregation (54). It seems likely that glucagon inhibits the enzyme through the same mechanism, the results are consistant with the phosphorylation/translocation/activation model presented in the introduction (see section 1.2). It would be worthwhile to repeat the glucagon experiment presented here without NaF in the harvesting media and see if the inhibition of the cytidylyltransferase is diminished, as it was with cAMP analogs.

A model for the effect of fasting on PC biosynthesis can now be presented. During starvation, glucagon inhibits the synthesis of PC bycausing a phosphorylation of the cytidylyltransferase. This has the effect of increasing the Km for CTP, and translocating some of the microsomal enzyme to the cytoplasm, away from DG micro-domains. However, when the fatty acid or acidic phospholipid concentrations in the cell rise, the inhibition of PC synthesis by glucagon can be counteracted. This effectively keeps the synthesis of PC to a minumum during fasting. However, the true inhibition under these conditions is only marginal as a constant level of PC is vital to cell function. But if the intracellular CTP levels drop during fasting, PC synthesis is decreased much more readily. When the animal is fed again, glucagon levels decrease, and the phosphorylation level of the enzyme decreases. Simultaneously, intracellular fatty acid concentrations decrease, and DG levels increase. The CTP:phosphocholine cytidylyltransferase tends to reassociate with the microsomes, but the net activity remains fairly constant.

3.2. Studies on the Effect of Calcium on Phospholipid Biosynthesis Using Ionophore A23187

The effect of calcium on phospholipid biosynthesis was studied in view of the wide regulatory properties of calcium, and the failure of attempts to demonstrate a cAMP dependent inhibition of choline cytidylyltransferase *in vitro* (54). Ionophore A23187 was chosen as a calcium adjuvant because most other methods of varying calcium in the cell activate adenylate cyclase.

3.21 Pulse-Chase Studies with [^{3}H] Serine

As it has been known since the 1950's that calcium stimulates the serine synthase enzyme *in vitro* (76) it was felt that using a serine label to study the effect of IPA would serve as a positive control for all subsequent studies. First, it was neccesary to determine good pulse conditions for serine label, as there was no data in the literature on the incorporation of serine into hepatocyte phospholipids. MEM with the nonessential amino acids deleted was used for the pulse media in order to increase the uptake of serine label. Figure 3.6 shows the incorporation of 15uCi of [3-^{3}H] serine into phospholipid as a function of time. It can be seen that the incorporation is fairly linear into all lipid classes examined. It was felt that the one hour pulse point was a good time to begin a chase as there are sufficient counts to detect changes in flux yet there are still 2.5 times as many counts in PS as there are in PE.

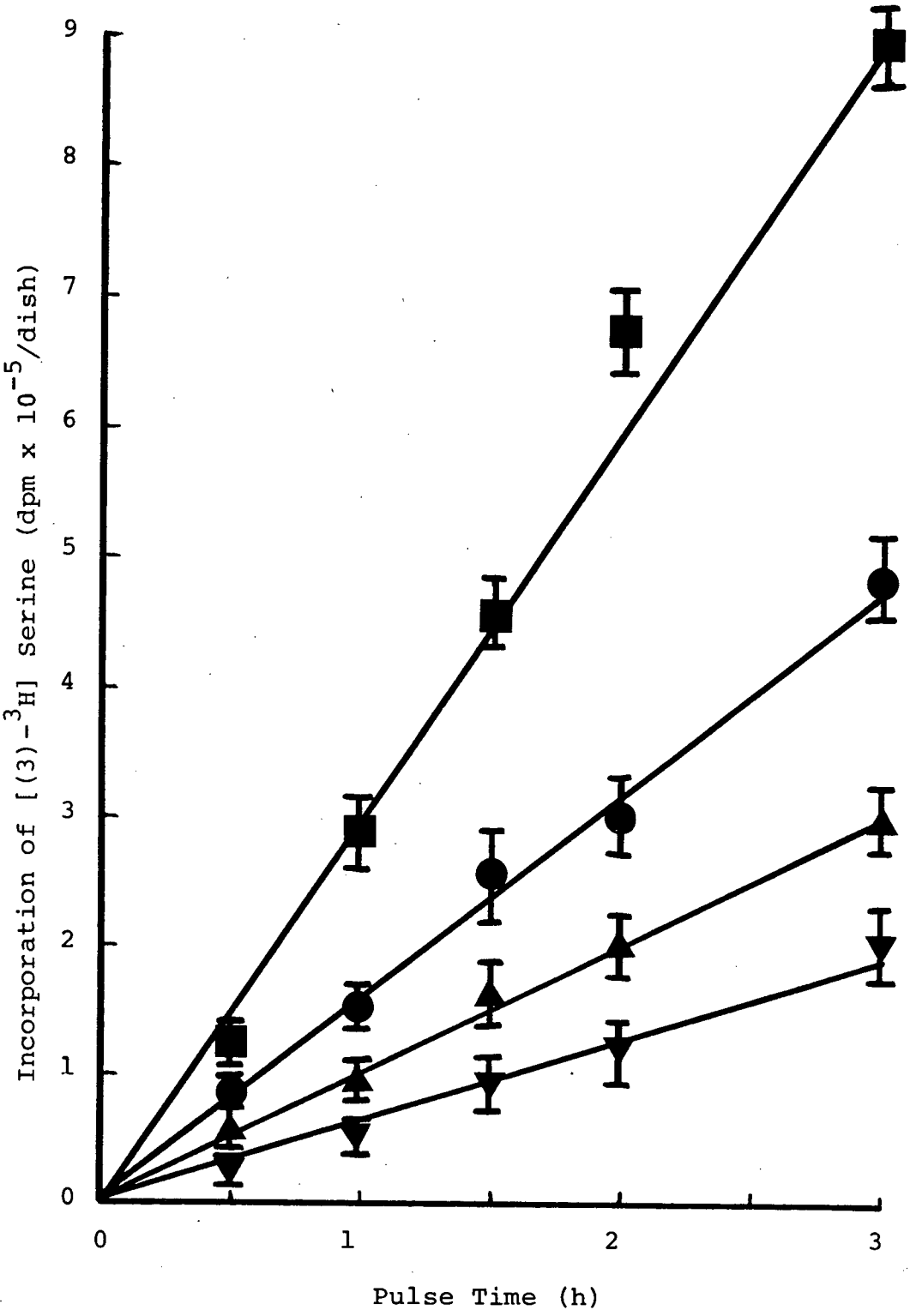
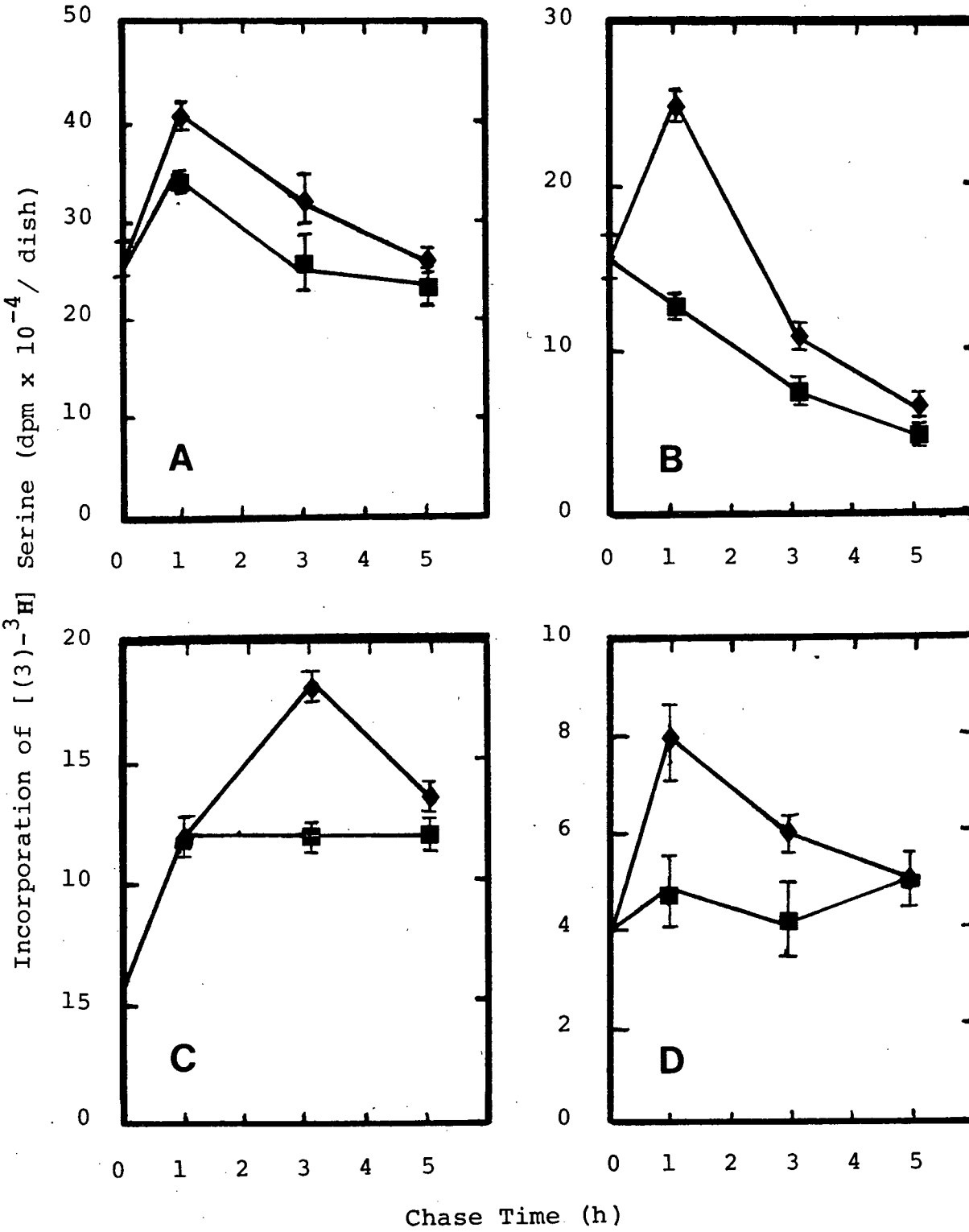


Figure 3.6 The incorporation of [3-^{3}H] serine into phospholipids. Cultured rat hepatocytes were incubated for various lengths of time with 15uCi [3-^{3}H] serine in MEM minus nonessential amino acids containing 100nM insulin. The cells were harvested and the radioactivity was quantitated in total lipid (■), phosphatidylserine (●), phosphatidylethanolamine (▲), and phosphatidylcholine (▼). Each point represents three dishes and the SD of each point is indicated by bars.

In the pulse chase studies, the IPA was added in MEM with normal calcium and insulin during the chase only, as it is known that IPA enhances serine uptake (143). IPA was added for one hour, and then the cells were chased with control media. In figure 3.7a, the effect of IPA on the incorporation of 15uCi [3-^{3}H] serine into hepatocyte phospholipids is shown as a function of time. It can be seen that IPA stimulated incorporation. The lipids were separated and the results are shown in figures 3.7b-d. Virtually all the PS label is rapidly decarboxylated to PE. This implies that the PS formed due to the calcium stimulation is rapidly transported to the mitochondria to be decarboxylated. The further incorporation of label into PC by N-methylation is fast as well. This would require the PE synthesized in the mitochondria to be rapidly transported back to the endoplasmic recticulum. It appears that not all of the PE made at the mitochondria is methylated to PC. It is known that PE made by the <u>de novo</u> PE pathway is committed to PC biosynthesis, at least at low ethanolamine concentrations (73). These results suggest that mitochondrial PE has another fate, as the counts lost from PE do not all end up in PC (fig 3.7d). This could be explained if the PE was being converted to lyso-PE by the phospholipase A_2 in the mitochondria, a reaction which is also stimulated by calcium (144). This experiment suggests that the turnover of serine in phospholipid is much faster than choline and ethanolamine.

A consideration of the topology of the enzymes involved shows that the PS made in the endoplasmic recticulum must be transported to the mitochondria to be decarboxylated, and then back to the endoplasmic recticulum to be methylated to PC. The fact that this

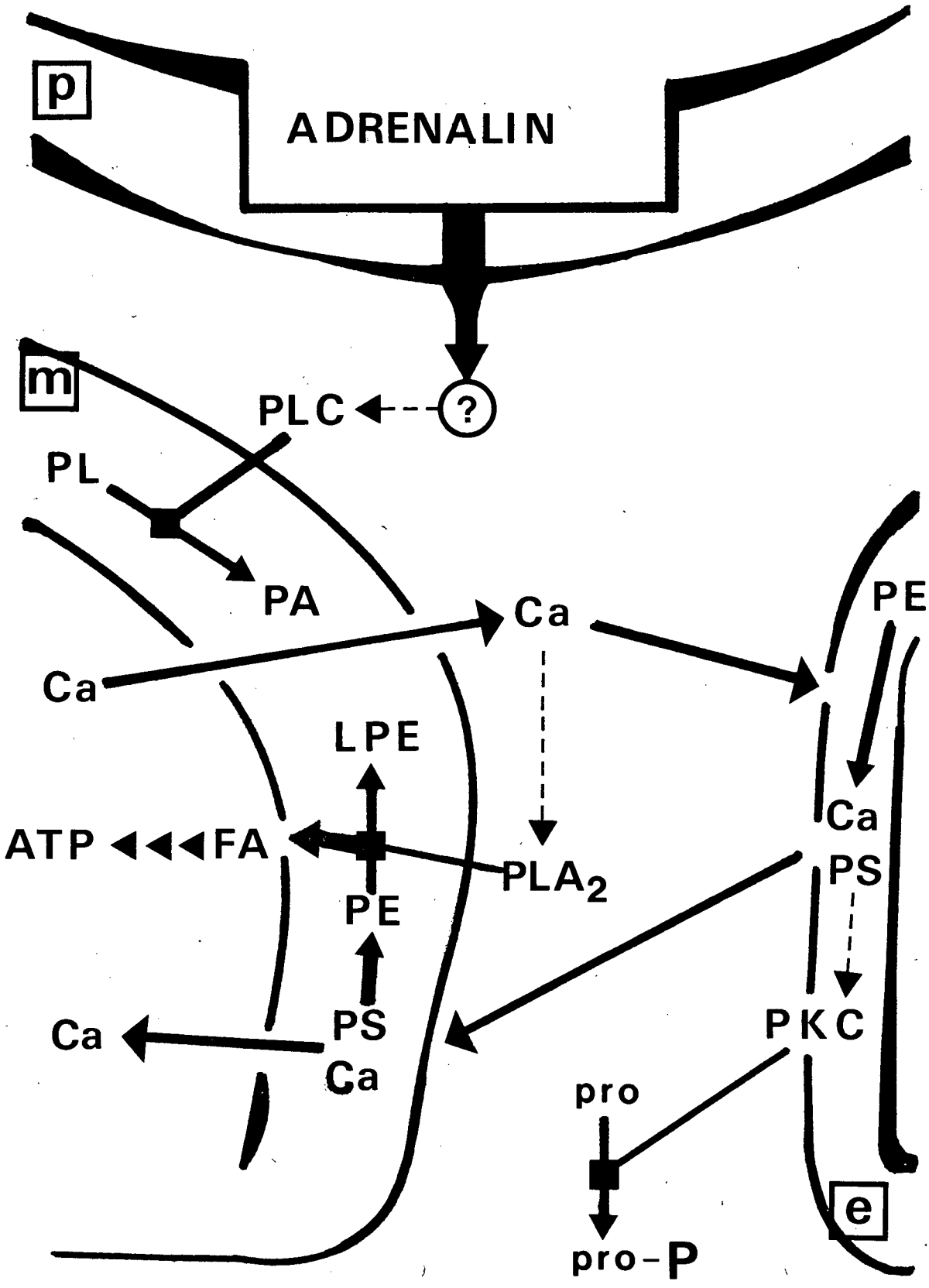
Figure 3.7 The effect of ionophore A23187 on the incorporation of [3-^{3}H] serine into phospholipids. Cultured rat hepatocytes were incubated for one half hour with 15uCi [3-^{3}H] serine in MEM$^-$ minus nonessential amino acids containing 100nM insulin. Some cells were harvested at this point. The rest of the cells were rinsed with culture media and then incubated for one hour in MEM$^-$ with 100nM insulin, and with (diamonds) or without (squares) 1uM ionophore IPA. Some cells were harvested at this time. The rest of the cells were rinsed with media and incubated for 2 or 4 hours in MEM$^-$ containing 100nm insulin, and then harvested. The lipids were extracted and the radioactivity was quantitated in (A) total lipid, phosphatidylserine (B), phosphatidylethanolamine (C), and phosphatidylcholine (C). Each point represents three dishes and the SD of each point is indicated by bars.


A
B
C
D
Incorporation of [(3)-^{3}H] Serine (dpm x 10^{-4}/ dish)
Chase Time (h)

process is calcium stimulated is interesting in regard to other reports in the literature. It is known that when hepatocytes are stimulated with adrenalin, calcium is released from the mitochondria concommitantly with the activation of mitochondrial phospholipase A_2 (90). The mitochondrial phospholipase A_2 is known to work predominantly on PE (144), generating lyso-PE and fatty acid. The calcium ends up in the endoplasmic recticulum (124), where it presumably activates PS synthase , phospholipases, and protein kinase C. The observation made here that most of the PS made in the endoplasmic recticulum goes right to the mitochondria and is turned into PE provides a way to regenerate the PE lost in the mitochondria (figure 3.8), and is analogous to the PI cycle operating on the plasma membrane of many cells. This form of intracellular regulation could have important implications in the regulatory scheme of the hepatocyte. One possible consequence of this calcium activation is to channel DG away from TG and toward β-oxidation, through phospholipid intermediates. Furthermore, this mechanism can channel arachadonate into prostaglandin synthesis when calcium release is stimulated in the mitochondria. Also, by the activation of protein kinase C by the calcium-PS, this mechanism provides a means of coupling protein phosphorylation to hormonal response.

This process (designated as the PS cycle), can conceivably be extended to other metabolic situations. It is known that mitochondria release calcium when their cytochrome system is oxidized (125). This would activate PS synthesis from PE in the endoplasmic recticulum. The ethanolamine released from the PS base exchange would rapidly be remade into PE, assuming that the concentration of

Figure 3.8 The PS cycle hypothesis. See text for discussion. [p] = plasma membrane, [m] = inner mitochondrial membrane, [e] = endoplasmic recticulum, PLC= phospholipase C, PL = phospholipid, Ca = calcium, PlA_2 = phospholipase A_2, PKC = protein kinase C, pro = protein, pro-P = phosphoprotein. ($-\overset{2}{=}\!\!\rightarrow$) denotes activation, (—■) denotes catalysis, (⟶) denotes a reaction. Other abbrieviations as in text.


p
ADRENALIN
m
PLC
?
PL
PA
Ca
Ca
PE
LPE
ATP
FA
PLA2
PE
Ca
PS
Ca
PS
Ca
PKC
pro
pro-P
e

ethanolamine is rate limiting to de novo PE synthesis. This would effectively channel DG away from TG into PS. The PS synthesized would then be transported to the mitochondria where it would be decarboxylated to PE, and further acted on by phospholipase to generate free fatty acid at the mitochondria. This could then be used for β-oxidation and reduction of the cytochrome system. Thus the activation of the PS cycle would effectively transport fatty acid to the mitochondria required for oxidative phosphorylation and prostaglandin biosynthesis via phospholipid intermediates. This system is novel in that all mitochondrial fatty acid transport systems reported to date utilize carnitine fatty acid. In this regard it is interesting that acyl-carnitine inhibits protein kinase C (152); this may represent another regulatory feature of this system. Additionally, it would provide a way to rapidly channel DG away from TG synthesis without inhibiting DG-acyltransferase, or DG synthesis, as the cAMP pathway does. It is also an example of intracellular communication between the mitochondria and the endoplasmic recticulum, which could effectively coordinate β-oxidation and TG synthesis to the oxidation state of the cell. If this autoreguation model operates in vivo, it would intuitively seem that calcium release from the mitochondria could be adjusted continuously to a rate optimal for both the endoplasmic recticulum and the mitochondria, as opposed to the rapid release of calcium induced by adrenalin. If this were the case, it would be useful to consider the relative amount of calcium in the endoplasmic recticulum and the mitochondria as a measure of the oxidation state. Thus the mitochondria would be 'loaded' with calcium when they are energized, and deficient in calcium when they require

more fuel.

In light of the above discussion, one would intuitively expect the calcium to return to the mitochondria to complete the cycle, or when sufficient PS has reached the mitochondria. In this regard it could be relevant that calcium and PS form colcleate structures *in vitro* (145), effectively sequestering both the calcium and the PS into local domains. One could postulate then that the PS and calcium return to the mitochondria together *in vivo*. To test this hypothesis, it would be interesting to do dual labeling studies with with calcium and serine to see if the calcium returns to the mitochondria, and see if this return is correlated with the PS decarboxylation. Such experiments are difficult to perform in practice, and it is difficult to say *a priori* whether such experiments would work. As a preliminary investigation, *in vitro* reconstitution studies with respiring mitochondria and microsomes could be carried out, with calcium and serine label. One could then increase the oxidation state of the mitochondria and do a timecourse on the label metabolism. With this system it might be possible to observe calcium release from the mitochondria, calcium uptake by the endoplasmic recticulum, stimulated synthesis of PS, and transport of calcium and PS to the mitochondria, in a temporal sequence. It is possible that cytoplasmic factors such as phospholipid exchange proteins are neccesary components of the cycle.

One could also test for an second messenger of adrenalin with this system. Plasma membranes could be isolated and adrenalin could be added to them *in vitro*. The membranes could be spun down and the

supernatant could be assayed for its ability to cause calcium release from *in vitro* preparations of mitochondria. Alternatively, one could propose a coupling scheme involving decreased GTP levels. As was mentioned in section 1.5, adrenalin coupling with the adenylate cyclase system increases GTP hydrolysis, in contrast to glucagon (102). Adrenalin also causes calcium efflux from the mitochondria in addition to elevating cAMP levels (124). It could be that the decrease in GTP levels causes mitochondrial calcium efflux by inhibiting CDP-DG acyltransferase, as it inhibits it *in vitro* (61). If this enzyme were to be inhibited, the mitochondrial PA levels would almost certainly rise, as the formation of CDP-DG from PA is a crossover point in the synthesis of PI and CL. The increase in PA could increase mitochondrial calcium efflux by virtue of its calcium ionophore properties. One could test this hypothesis simply by varying the GTP concentration of a [^{45}Ca] loaded mitochondrial preparation and measuring calcium release. Measurements could also be made of GTP levels *in vivo* as a functon of mitochondrial calcium efflux to see if there is a positive correlation. Such experiments could contribute greatly to our knowledge of the regulatory schemes dealt with in this thesis.

3.22 The Effect of Calcium on Choline Uptake

Before studying the effect of calcium and IPA on PC biosynthesis, it was felt that it should be determined if calcium had any effect on choline uptake, as it does with amino acid uptake (143). The effect of a one hour preincubation with 1uM IPA on the uptake of 28uM [*Me*-^{3}H]

labeled choline (119mCi/mmol) was studied in hepatocytes, as a function of calcium concentration. A one hour pulse time was used. The concentration of IPA used in this study was taken from Kelley et al. (143), who performed a similar study on amino acid uptake. Figure 3.9 shows that IPA inhibits choline uptake in a calcium dependent manner. The maximal inhibition occurred at a calcium concentration of 200mg/l or greater, and amounted to about 40% relative to control. Figure 3.9 also shows that the uptake of choline can be increased relative to control if the calcium is removed from the media. This inhibition is different to the effect on amino acid uptake, which is stimulated by calcium in the presence of IPA. This difference is in keeping with what is known about hepatocyte physiology and the general effects of calcium (see section 1.6) : hepatocytes respond to the stressful conditions mediated by calcium by increasing gluconeogenesis and hence increase uptake of amino acids. However, it is sensible that the hepatocyte would leave serum choline for other tissues which cannot make significant PC from PE and PS.

In figure 3.10, a Lineweaver-Burke Plot is presented for the effect of calcium and IPA on saturatable choline uptake. From this data it appears that the Vmax decreased from 500 to 200 pmol/min/dish in the presence of calcium. The Km value of 14uM compares well with previously observed values (54). It has been shown that cAMP analogs inhibit choline uptake by inhibiting the Vm (146), and thus it is possible that the two effectors operate through the same final pathway. Such a mechanism is difficult to postulate though, as it is known that calcium decreases the hormonally stimulated level of cAMP

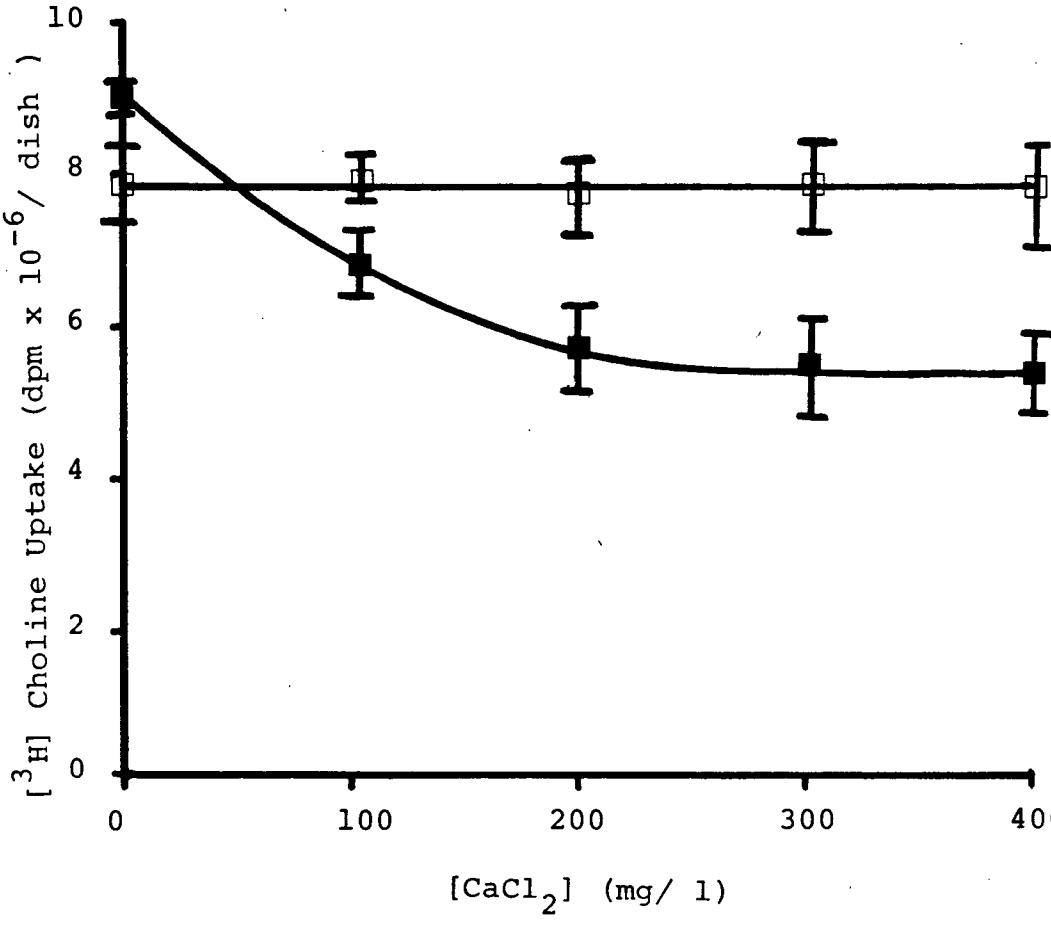


Figure 3.9 The effect of calcium and Ionophore A23187 on choline uptake in cultured rat hepatocytes. Cells were pulsed for one hour with 28uM [Me-^{3}H] choline (119mCi/mmol) containing various amounts of calcium in the presence (■) or absence (□) of 1uM Ionophore A23187. Cells and media were collected and the total uptake of choline was determined. Each point represents three dishes and the bars indicate the SE.

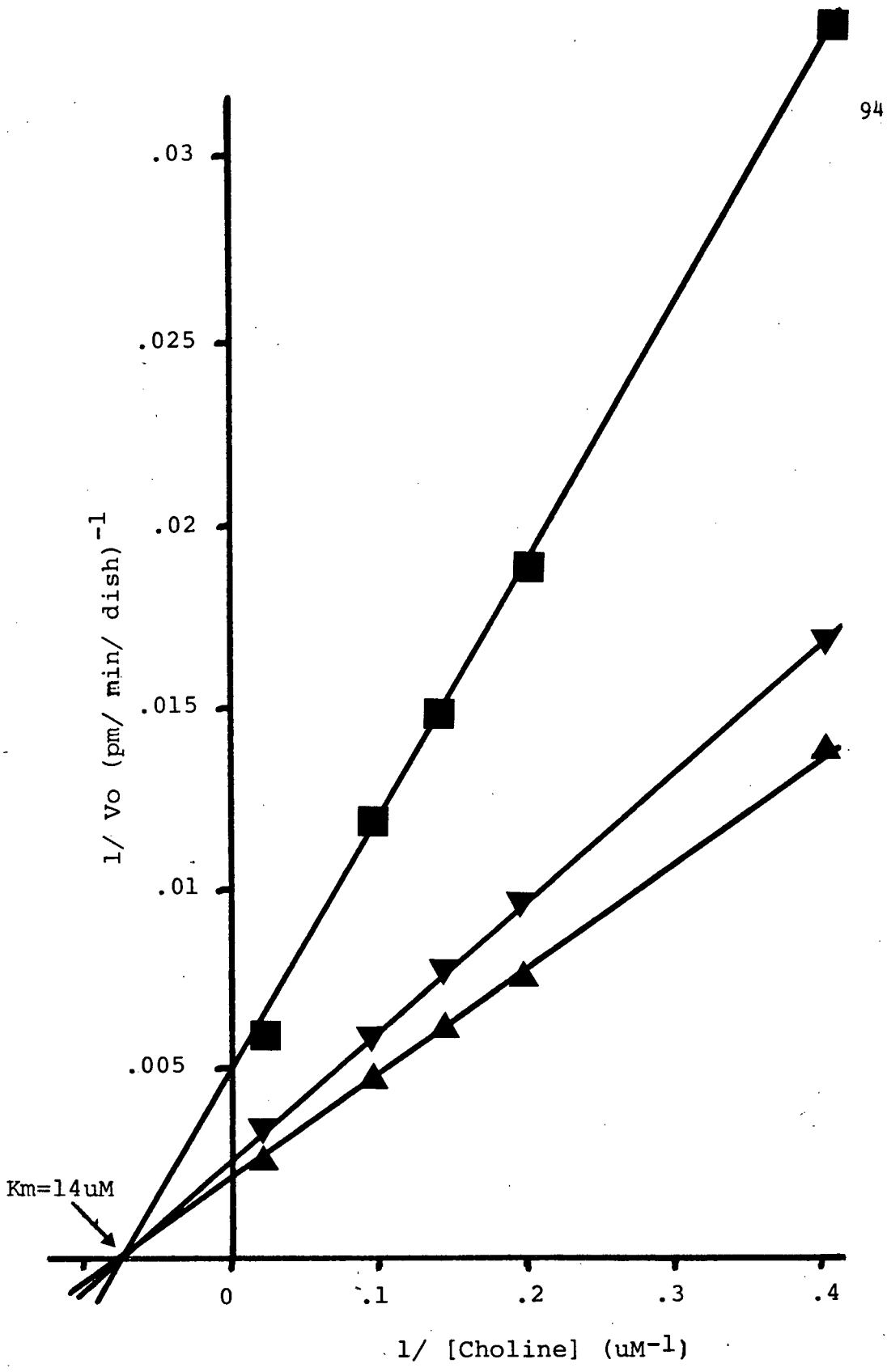


Figure 3.10 A Lineweaver-Burke Plot of saturatable choline uptake. Cells were pulsed with 2.5-100uM [Me-^{3}H] choline for one half hour in the presence (■,▼) or absence(▲) of 200mgs/ml calcium chloride and in the presence (■,▲) or absence (▼) of ionophore A23187.

in hepatocytes (143), and glucagon, a well known stimulator of cAMP, has no observable effect of calcium levels (124). One possibility is that the two effectors phosphorylate a common protein, and thus exert the same effect. Phosphorylase a is activated by both calcium and cAMP (109), and could be responsible for the inhibition of choline uptake. Further experiments would be needed to determine this.

3.23 The Effect of Calcium and IPA on PC Biosynthesis

It was of interest to see if calcium had an effect on PC biosynthesis, as it has on many other pathways. As it had previously been determined that calcium with IPA inhibits choline uptake, IPA was added during the chase only. Cells were pulsed with [Me-^{3}H] choline for one hour prior to treatment with IPA in the chase media. The hepatocytes took up the choline label efficiently, and the incorporation pattern was similar to previous studies (45). Figure 3.11 shows the effect of adding 1uM IPA on the incorporation of label into PC and choline phosphate. There was only a marginal decrease in the rate of PC biosynthesis (20% after a three hour chase). However, it would be premature to conclude that calcium has no significant effect on PC biosynthesis *in vivo*. It is known that calcium activates phospholipases (24), and hence generates fatty acid and lyso-PE. Both are known to activate phosphocholine cytidylyltransferase (54). Thus it is possible that the increase in fatty acid and lysolipids overcomes any inhibition caused by calcium. In view of the model proposed in section 1.3, the maintainance of PC synthesis by lyso-PE when under a calcium load would be equivalant to the effect of fatty acid under a glucagon load. However, many more studies are required to

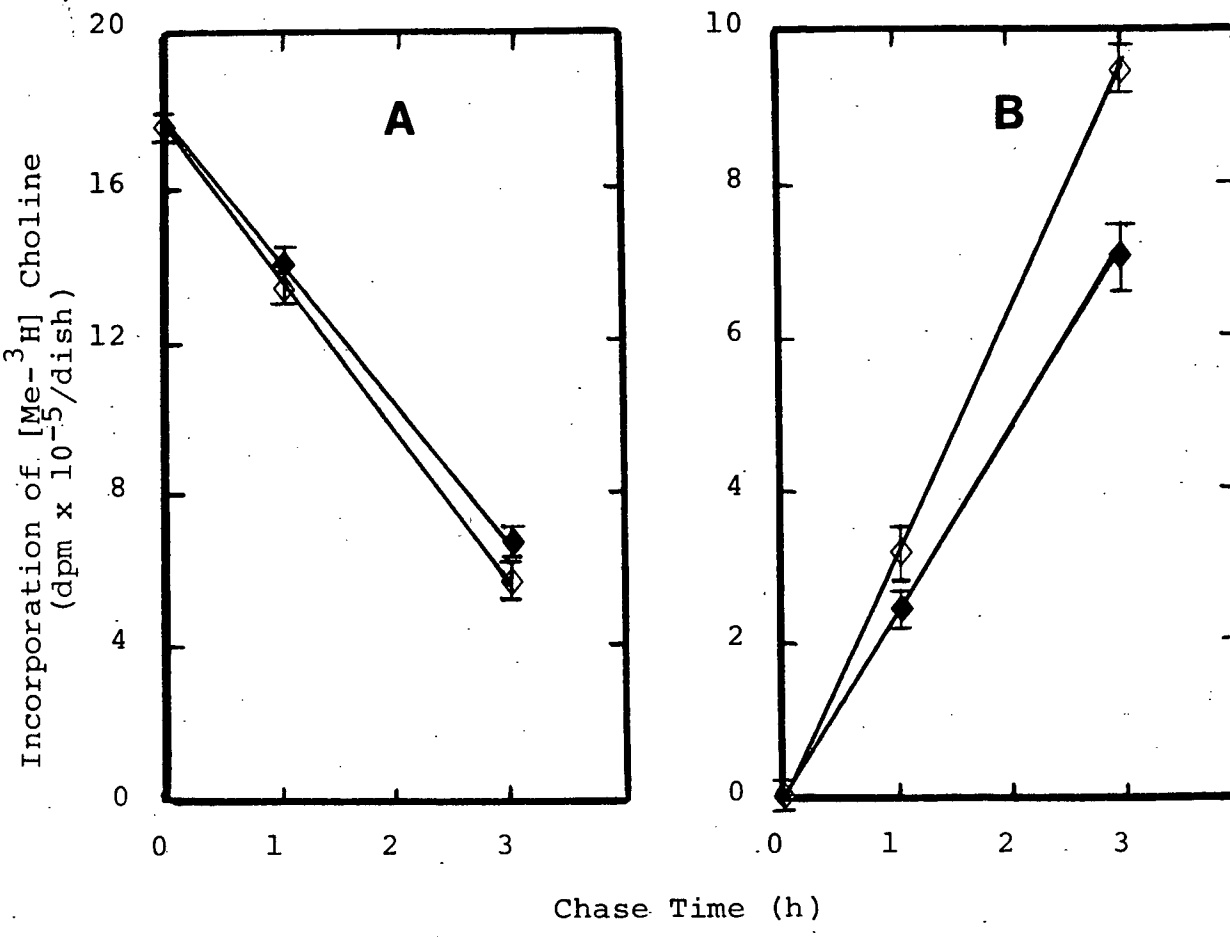


Figure 3.11 The effect of Ionophore A23187 on the disappearance of [Me-^{3}H] choline in cellular phosphocholine and its its accumulation ito phosphatidylcholine. Cultured rat hepatocytes were pulse labeled for 30 minutes with 10uCi [Me-^{3}H] choline. The cells were washed and fresh medium with (◇) or without (◆) 1uM Ionophore A23187 was added. At various time, the cells and media were collected, and the radioactivity was determined in (A) phosphocholine; and (B) phosphatidtylcholine. Each point represents three dishes and the SE is indicated by bars.

accertain this.

In an experiment analogous to the choline experiment above, an attempt was made to observe the effects of calcium on the methyltranferase pathway, by using an ethanolamine label. Control cells rapidly incorporated label into PE, with only trace amounts recovered in the upper phase precursors. During the chase period, label was transferred to PC, such that only 20% remained in PE after a three hour chase. This incorporation pattern is similar to observations reported previously (73). As shown in figure 3.12, addition of 1uM IPA to the chase media had no significant effect on the incorporation of label into hepatocyte phospholipids. Subsequent to these experiments, attempts were made to observe an effect by increasing the IPA concentration incrementally to 25uM. No effect was seen on this pathway at any IPA concentration. However, concentrations of IPA greater than about 10uM killed most of the cells as observed with trypan blue. Also, the concentration of insulin was adjusted to 1, 10, 100, and 1000 nM and again no effect of 1uM IPA was seen. Lastly, media calcium chloride levels were incrementally varied from to 500mgs/l and again 1uM IPA had no effect. At this point, it was decided that any possible effect that calcium has on PC synthesis might be easier to observe <u>in vitro</u>, to avoid the draw backs of labeling experiments and to avoid compensation effects operating in intact hepatocytes.

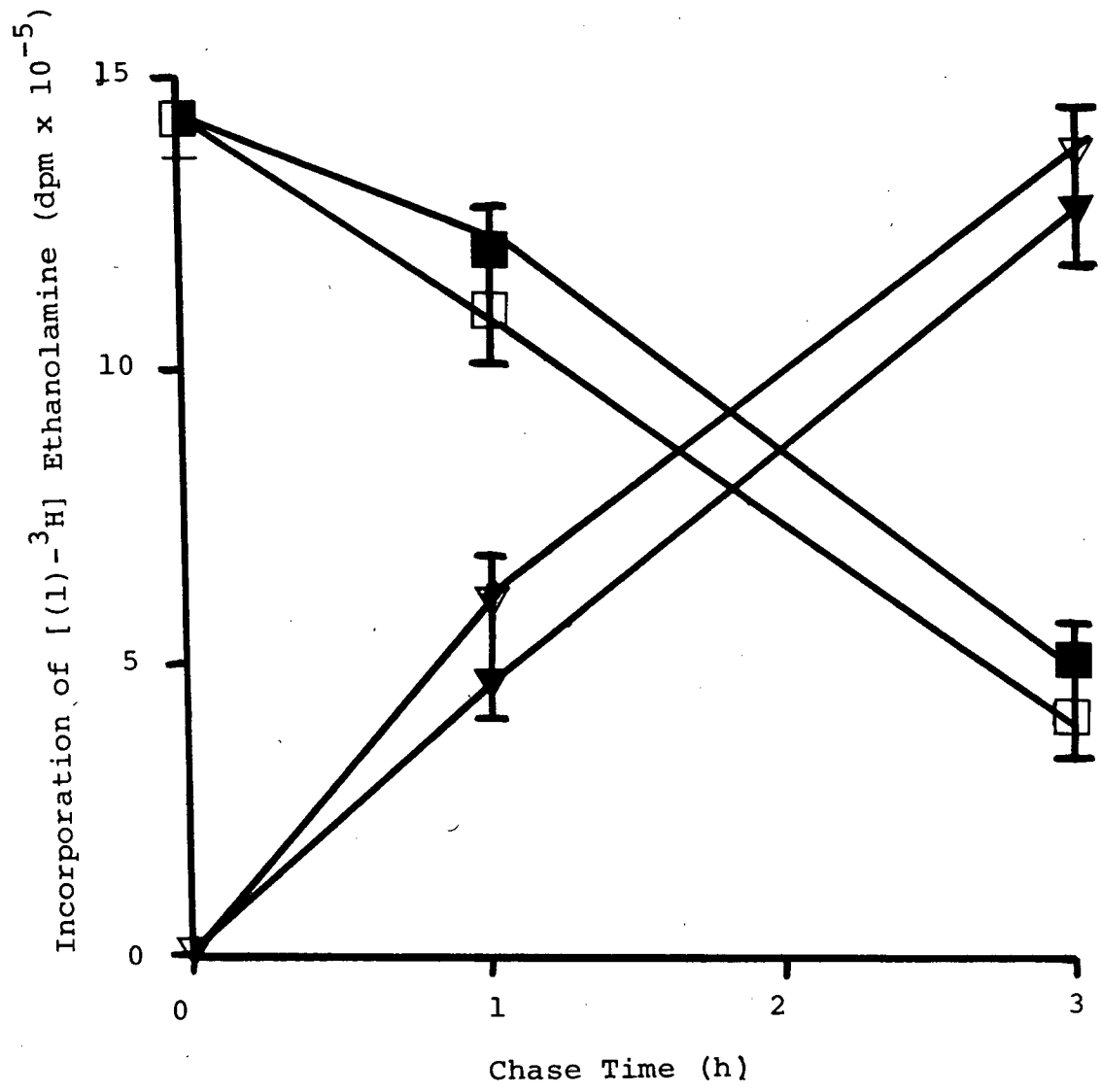


Figure 3.12 The effect of Ionophore A23187 on the disappearance of [(1)-^{3}H] ethanolamine in phosphatidylethanolamine and its accumulation ito phosphatidylcholine. Cultured rat hepatocytes were pulse labeled for 90 minutes with 15uCi [(1)-^{3}H] ethanolamine. Cells were washed and fresh medium with (closed symbols) or without (open symbols) 1uM Ionophore A23187 was added. At various time, the cells and media were collected, and the radioactivity was determined in (■, □) phosphatidylethanolamine; and (▼, ▽) phosphatidtylcholine. Each point represents three dishes and the SE is indicated by bars.

3.3 The Effect of CaM Preparations on CTP:Phosphocholine Cytidylyltransferase in vitro:

Calcium and CaM are have been shown to have extensive regulatory effects on many enzymes. Previous studies have shown that phosphocholine cytidylyltransferase in cytosol can be inhibited by Mg-ATP or 2mM calcium in a time dependent fashion (54). This raised the possibility that there may be a CaM dependent protein kinase or activator operating on the enzyme in vivo. In this set of studies, the effect of CaM on cytosolic CTP:phosphocholine cytidylyltransferase was investigated.

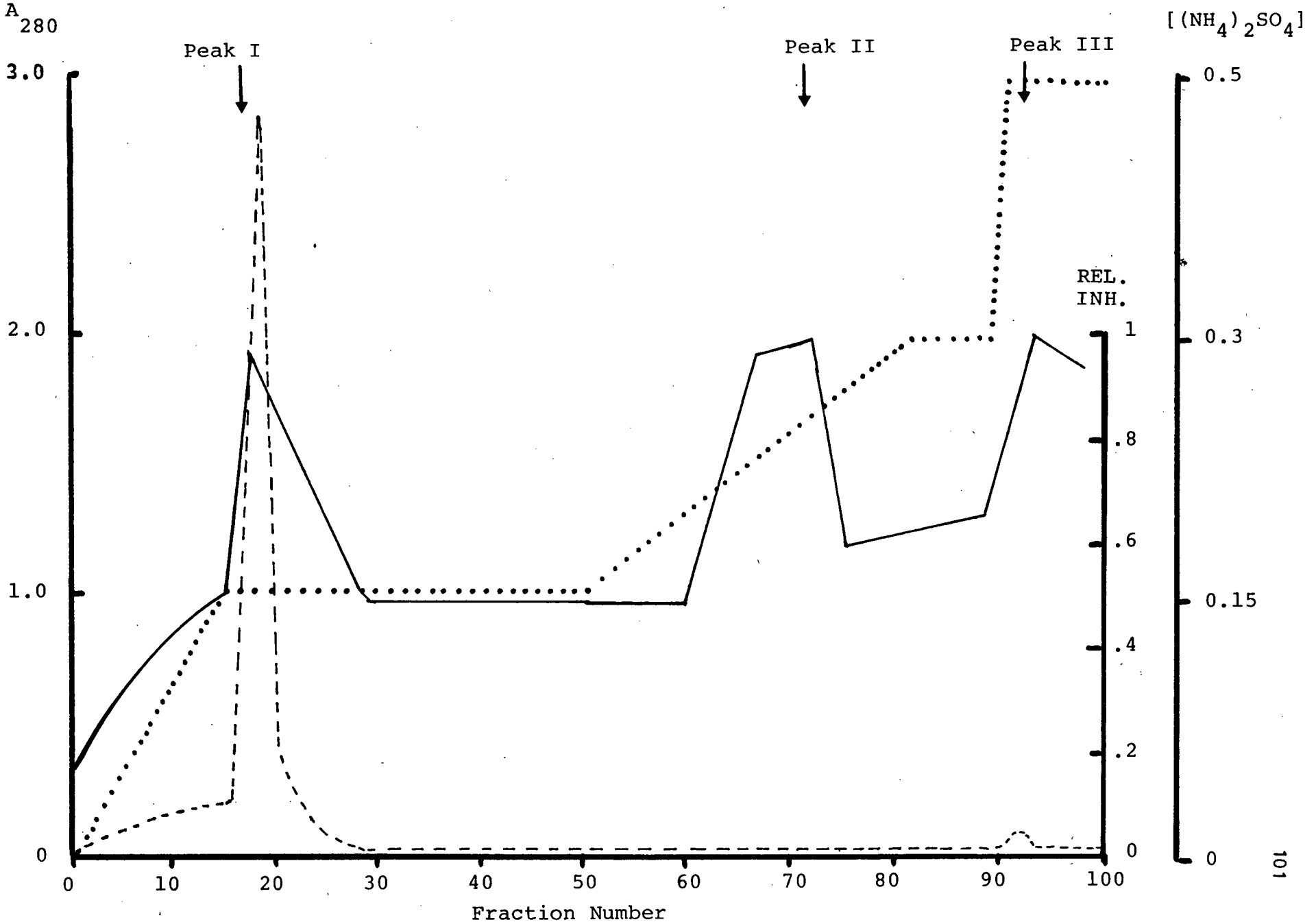
3.31 The Partial Purification of Rat Liver CaM and its Effect on CTP:Phosphocholine Cytidylyltransferase

An attempt to purify rat liver CaM was made by using a method similar to the protocol of Cheung et al. (105), for the purification of CaM and calcium binding proteins from rat brain (see section 2.5). A boiled, dialized liver homogenate was assayed for cytidylyltransferase inhibition. A two fold inhibition was obtained at protein concentrations of 4ug/ml (see table 3.4). The dialysate was applied to a DEAE Sepharill column, and the activity was eluted off with an ammonium sulfate gradient. Each 4.5 ml fraction collected was assayed for cytidylyltransferase inhibition, OD_{280}, and conductance to estimate the salt concentration (see figure 3.13). Three peaks of cytidylyltranferase inhibition were obtained. Peak two eluted close to

Table 3.3 Inhibition of cytidylyltranferase activity by a boiled rat liver homogenate.The ability of a boiled rat brain homogenate to inhibit cytosolic CTP:phosphocholine cytidylyltranferase was determined. Assays were done as described in the methods section.

SAMPLE	ENZYME ACTIVITY (nmol/min/mg)
Control	0.35
With 4ug/ml dialysate	0.17
With 20ug/ml dialysate	0.25

Figure 3.13 DEAE Sepharill Chromatography of a boiled rat liver homogenate. 30mls of a boiled rat liver dialysate (see section 2.52) was applied to a DEAE Sepharill column (bed volume 40ml). The major protein fraction was eluted with a buffer containing 20mM $MgSO_4$, 20mM Tris-HCl, pH 7.4, and .15M NH_4SO_4. After running 200mls of this buffer, the concentration of NH_4SO_4 was increased linearly to .3M over a range of 150ml, and then maintained at .3M for an additional 150ml. The remaining protein was eluted with 30ml of buffer with .5M NH_4SO_4. 100 fractions of 4.5ml were taken, and assayed for cytidylyltransferase inhibition (——), OD_{280} (----), and conductance. the conductance was standardized to NH_4SO_4 (•••••).


A280
Peak I
Peak II
Peak III
[(NH4)2SO4]
3.0
2.0
1.0
0
REL. INH.
1
.8
.6
.4
.2
0
0.5
0.3
0.15
0
0
10
20
30
40
50
60
70
80
90
100
Fraction Number

where CaM normally elutes. The three peaks were assayed for their ability to inhibit cytidylyltransferase in the presence of phospholipid. In table 3.5 , it can be seen that only peaks 2 and 3 were able to do this. Because of the large amount of protein in peak 1, it was assumed that peak 1 inhibition was due to nonspecific protein binding. This effect has been observed previously (47), and was found to be reversible by phospholipid. The inhibition from peak one was not further characterized. Peaks two and three were dialized against homogenizing buffer to remove the ammonium sulfate, and then assayed again. Peak three inhibition disappeared, while peak two inhibition was diminished but still significant (see table 3.6). The peak 2 inhibition properties were studied as a function of concentration and the results are shown in figure 3.14. It can be seen only that protein concentrations of only 40ng/ml are required to elicit a maximal response. The calcium dependence of the inhibition was next examined by adding EGTA to an assay system containing only 17uM calcium. 0.2mM EGTA did not reverse the inhibition. In view of the fact that this inhibitor was not calcium dependent, no further attempt was made to purify it. However, it should be noted that the inhibitory activity was destroyed by ultrafiltration on an amicon concentrator, implying that the inhibitor is a protein. Moreover, the observation that the inhibitor can even reverse lipid activation suggests that the mechanism of inhibition is different from the inhibition observed by phosphorylation, LPC or salt inhibition (54). One of the major impurities found in CaM preparations obtained in this way are what are known as CaM binding proteins (47). These proteins have have the propertyof diminishing the activity of CaM.

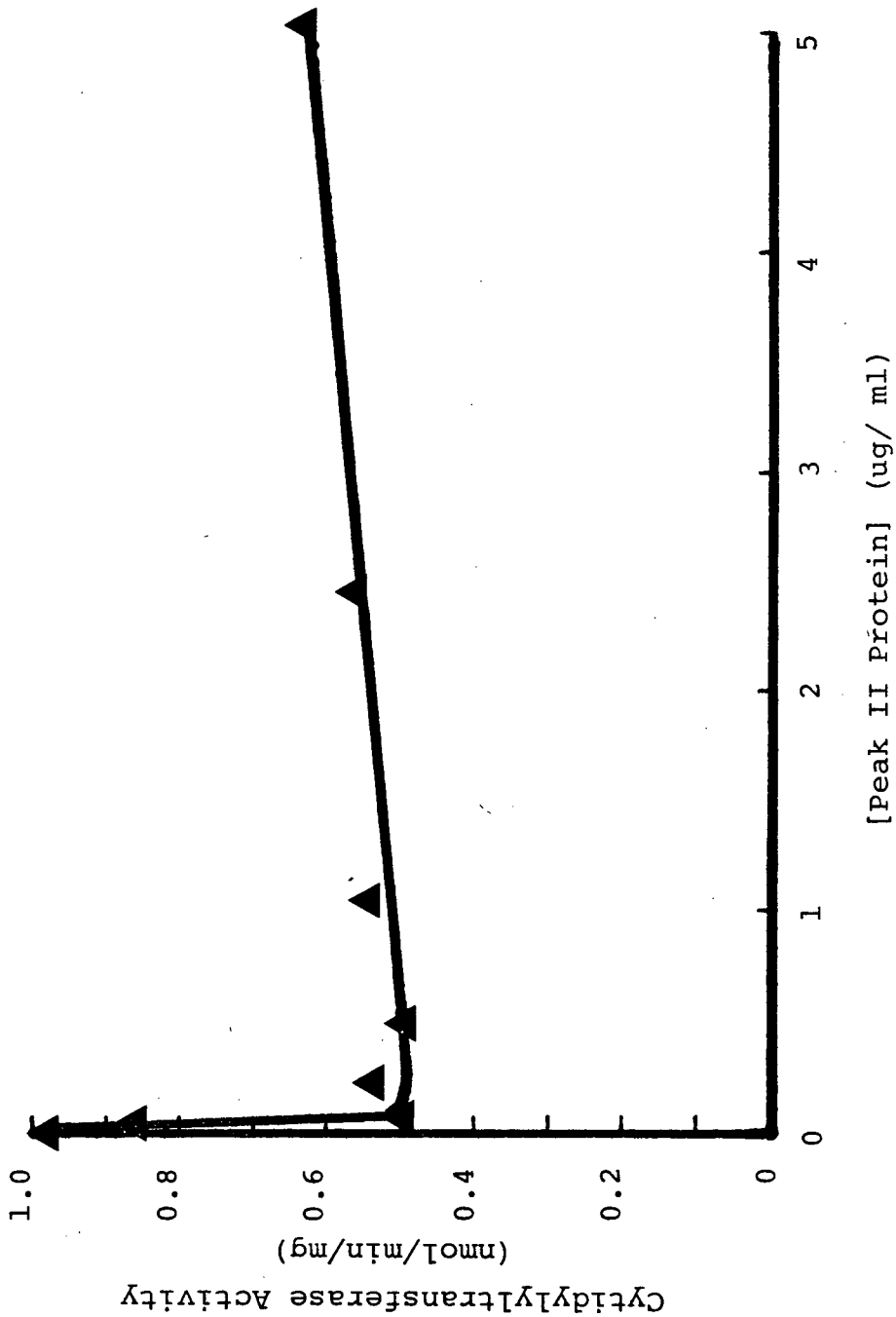
Table 3.5 The inhibition of CTP:phosphocholine cytidylyltransferase by DEAE sepharill fractions and the effect of phospholipid.

SAMPLE	ENZYME ACTIVITY (nmol/min/mg)
Control	0.33
4ug/ml Peak II Dialysate	0.23
4ug/ml Peak III Dialysate	0.30

Table 3.6 The inhibition of CTP:phosphocholine cytidylyltranferase by DEAE sepharill fractions after dialysis.

SAMPLE	ENZYME ACTIVITY (nmol/min/mg)
Control	0.38
Control with Phospholipid	0.97
With Peak I (4ug/ml)	0.26
With Peak I (4ug/ml) Plus Phospholipid	0.89
With Peak II (4ug/ml)	0.04
With Peak II (4ug/ml) Plus Phospholipid	0.18
With Peak III (4ug/ml)	0.08
With Peak III (4ug/ml) Plus Phospholipid	0.3

Figure 3.14 Inhibition of CTP:phosphocholine cytidylyltranferase activity by a dialyzed DEAE sepharill fraction. Peak II from the DEAE sepharill column was dialyzed and its ability to inhibit cytosolic cytidylyltransferase was examined as a function of concentration. Each point is an avearage of two determinations.



3.32 Studies with Pure CaM

It was possible that the CaM in the above preparation was inhibiting the cytidylyltransferase in a calcium independent manner. To test this hypothesis, the ability of pure calmodulin from Calbiochem to inhibit cytosolic CTP:phosphocholine cytidylyltransferase was investigated. No effect was observed at concentrations of CaM exceeding 10ug/ml (figure 3.15). In a control study, this preparation was found to stimulate rat brain cAMP phosphodiesterase (148), confirming that it had normal biochemical activity. Thus it may be concluded that CaM does not inhibit cytosolic CTP:phosphocholine cytidylyltransferase.

Table 3.7 The effect of pure calmodulin on the activity of cytosolic CTP:phosphocholine cytidylyltransferase.

Sample	Cytidylyltransferase Activity (nmol/min/mg)
Control	0.92
With .25ug/ml CaM	0.94
With 1ug/ml CaM	1.10
With 5ug/ml CaM	0.91
With 10.25ug/ml	1.10

GENERAL CONCLUSIONS

Hepatic phospholipid metabolism is coordinated to the physiological state of an animal through a variety of control mechanisms. Our understanding of these processes has increased dramatically in recent years, but is at an elementary level. This thesis has investigated some of the regulatory mechanisms which operate in rat hepatocytes. It was shown that glucagon inhibits the rate of PC biosynthesis by inhibiting the activity of CTP:phosphocholine cytidylyltransferase. This finding is in accord with the widely accepted view that glucagon inhibits key biosynthetic enzymes in order to adapt the liver to starvation conditions. It is likely that this hormone acts on the cytidylyltransferase via a phosphorylation cascade system. Future studies in this area should focus on the mechanism by which this inhibition occurs, as well as the role of otherstarvation signals (eg. glycerol, fatty acid) in the regulation of PC synthesis.

The role of calcium in the regulation of phospholipid metabolism was also investigated. It was found that calcium slightly inhibits PC synthesis in hepatocytes, but not through a mechanism involving CaM. It seems possible that protein kinase C or phosphorylase a are involved. Calcium also inhibits the uptake of choline, by decreasing the Vm of the uptake system. Lastly, it was shown that calcium stimulates the incorporation of serine into phospholipid and the tranfer of lipid to the mitochondria. In the next few years it is likely that research in lipid metabolism will establish that phospholipids are of critical importance in many regulatory systems.

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