CONTROL OF PHOSPHOLIPID METABOLISM IN MAMMALIAN TISSUES

By

KARMIN O

A thesis submitted to the Faculty of Graduate Studies

in partial fulfillment of the requirements for the degree of

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TO MY PARENTS

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ABSTRACT

Phosphatidylcholine is the principal phospholipid in mammalian tissues. The majority of phosphatidylcholine is synthesized *de novo* via the CDP-choline pathway with choline as precursor. The enzymes involved in this pathway are choline kinase, CTP:phosphorylcholine cytidylyltransferase and CDP-choline 1,2-diacylglycerol cholinephosphotransferase. Both choline kinase and cytidylyltransferase have been purified and extensively studied in various mammalian tissues. Cholinephosphotransferase catalyzes the conversion of CDP-choline and diacylglycerol for the formation of phosphatidylcholine. The enzyme is tightly associated with the endoplasmic reticulum, and the mechanism for its modulation was largely unknown. In the present study, cholinephosphotransferase was solubilized from hamster liver microsomes by Triton QS-15 and partially purified by DEAE-Sepharose chromatography and Sepharose 6B chromatography. The microsomal and partially purified enzymes displayed similar pH profile, and both showed absolute requirement for Mg^{2+} or other divalent cations. The K_m values for CDP-choline were similar between microsomal and partially purified enzymes, whereas the K_m value for diacylglycerol was substantially lowered when the enzyme was partially purified. Ethanolaminephosphotransferase was cosolubilized with cholinephosphotransferase from hamster liver microsomes by Triton QS-15. Partial separation of these two activities was achieved by ion-exchange chromatography.

Studies on the characteristics of cholinephosphotransferase and ethanolaminephosphotransferase suggest that they are separate enzymes in hamster liver microsomes.

The regulation of cholinephosphotransferase by endogenous metabolites was investigated. An inhibitor was isolated from hamster liver cytosol and later identified as argininosuccinate. Kinetic studies revealed that the inhibition of argininosuccinate on cholinephosphotransferase activity was essentially non-competitive. The physiological role of argininosuccinate in the modulation of phosphatidylcholine biosynthesis was investigated. The level of argininosuccinate was increased in the liver of the fasted hamster with a concomitant decrease in phosphatidylcholine biosynthesis. Analysis of choline containing metabolites after pulse labelling with radioactive choline revealed that the conversion of CDP-choline to phosphatidylcholine was attenuated. The level of argininosuccinate was higher in the cytosol of the fasted animal, which caused a higher degree of inhibition of cholinephosphotransferase than the control. Hence, the accumulation of argininosuccinate in the liver of the fasted hamster may be one of the biological factors regulating phosphatidylcholine biosynthesis.

The principal pathway for phosphatidylcholine catabolism is through the hydrolytic action of phospholipase A. In this study, the effects of ethanol and the combined

effect of ethanol and vitamin E (DL-α-tocopherol) on phospholipid metabolism were studied. When the isolated rat heart was perfused with 1% ethanol for 4 h, the major cardiac phospholipids were not altered but a 60% increase in lysophosphatidylcholine level was observed. Studies on the lysophosphatidylcholine metabolic enzymes revealed that phospholipase A (both phospholipase A_1 and A_2) activity was enhanced in the ethanol-perfused heart, but the lysophospholipase and acyltransferase activities were unaffected by ethanol treatment. When the heart was perfused with 1% ethanol in the presence of 50-100 μM vitamin E, the ethanol-induced lysophosphatidylcholine accumulation was completely abolished. Assay of phospholipase A in the presence of ethanol resulted in a biphasic response, with maximum stimulation of enzyme activity at 1% ethanol concentration. When phospholipase A was assayed in 1% ethanol and 25-100 μM vitamin E, its activity was inhibited by vitamin E in a dose-dependent manner. Kinetic study revealed that at low substrate concentrations, ethanol was inhibitory to the reaction, whereas at high substrate concentrations, the reaction was enhanced by ethanol. Vitamin E (50 μM) completely abolished the ethanol-induced enhancement of enzyme activity in a noncompetitive manner. Our results indicate that cardiac phospholipase A can be modulated by ethanol and vitamin E, and the modulation of the enzyme is an important mechanism for the regulation of lysophosphatidylcholine levels in the rat heart.

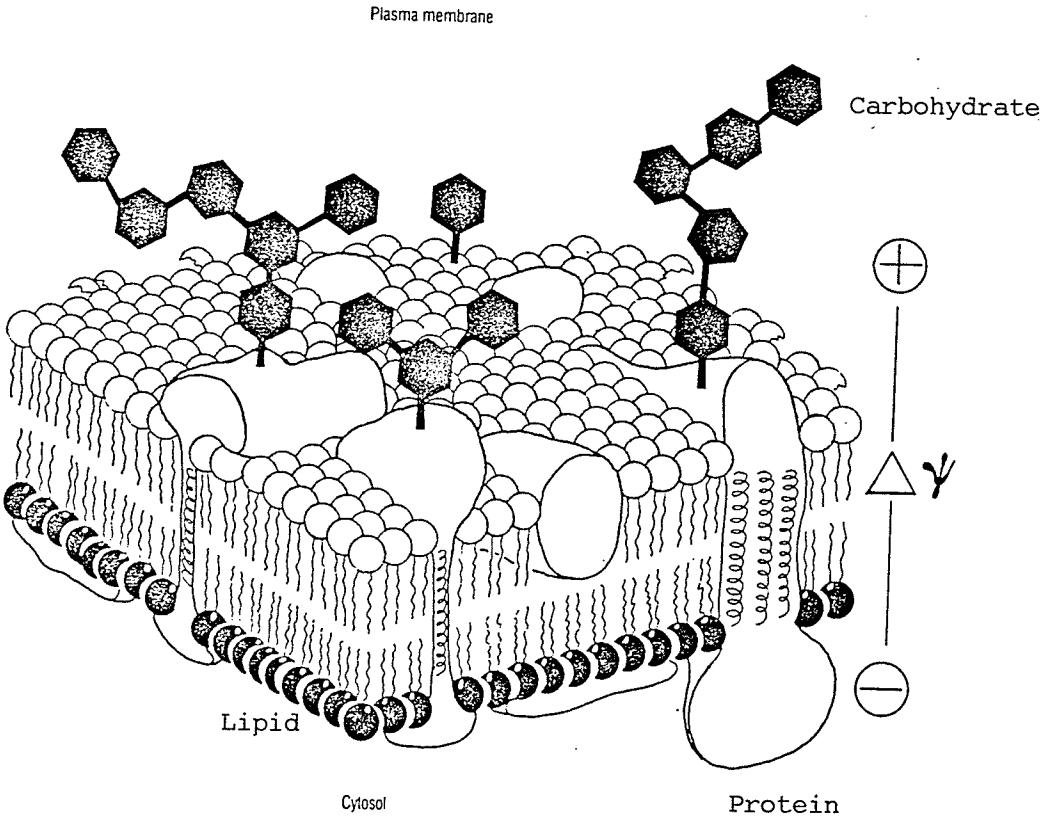
INTRODUCTION

I. BIOLOGICAL MEMBRANE AND PHOSPHOLIPIDS

The plasma membrane and the internal membranes of eucaryotic cells are collectively known as biological membranes. The development of the plasma membrane is a key step in the generation of the earliest forms of life (Alberts *et al.* 1989). Biological membranes are involved in a number of cellular functions. The plasma membrane defines the geographical limit of the cell and provides a selective barrier to the outside environment. It is also a selective filter that maintains the concentration gradient of ions on either side of the membrane and allows nutrients to enter and waste products to leave the cell (Alberts *et al.* 1989). Eucaryotic cells also contain an extensive set of internal membranes which form closed boundaries resulting in different compartments within the cells, known as cell organelles. There are six major types of organelles present in most eucaryotic cells: endoplasmic reticulum, Golgi apparatus, nucleus, mitochondria, lysosomes, and peroxisomes. Each organelle has specialized functions that are carried out by the unique enzymes within the organelle.

The structure of all biological membranes can be interpreted by the "fluid mosaic model" first proposed by Singer and Nicholson (1972). Fig. 1 shows the topography of membrane lipid, protein and carbohydrate in the fluid mosaic model of a typical

FIG. 1



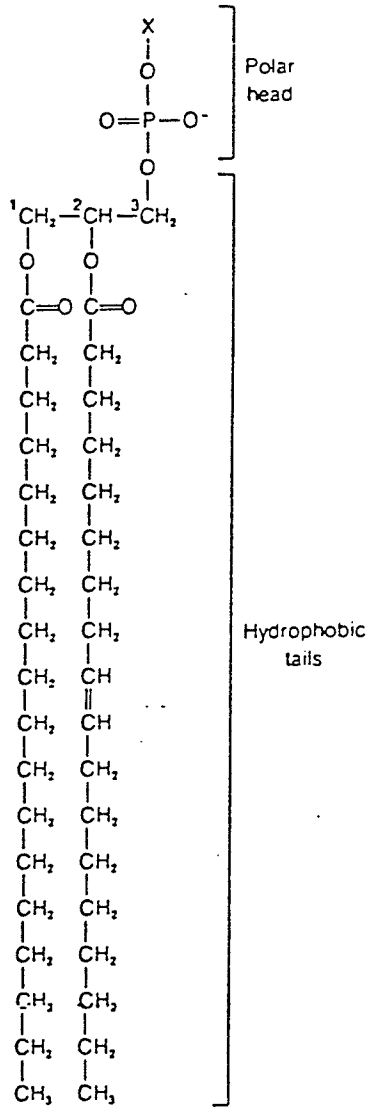
Fluid mosaic model of eucaryotic plasma membrane

eucaryotic plasma membrane. The membrane lipids and proteins are associated via non-covalent assemblies. Carbohydrate moieties linked to lipids and proteins face the extracellular space. Lipids constitute about 50% of the mass of mammalian cell plasma membrane (Alberts *et al.* 1989). The ability of the lipids to form the basic bilayer organization is a result of their amphipathic character, i.e. they contain a polar (hydrophilic) head group and a nonpolar (hydrophobic) region. As shown in Fig. 2, the typical phospholipid molecule has a polar head group attached to the sn-3 position of the glycerol backbone by a phosphodiester linkage, and two hydrophobic acyl groups at sn-1 and sn-2 positions. The length of the acyl groups (tails) normally varies from 14 to 24 carbon atoms, and the acyl group at sn-2 position usually contains one or more double bonds. The differences in chain length and degree of unsaturation of acyl groups are important in influencing the fluidity of the membrane.

1. Membrane Lipids

There are three major classes of lipids in eucaryotic membrane bilayer: phospholipids, cholesterol and glycolipids (Cullis and Hope, 1985). The lipid compositions can be different between the inner and outer monolayers, organelles, and various cell types. Phospholipids are the most abundant lipids in the membrane. Glycerol-based phospholipids are predominant, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and cardiolipin (Fig. 3). Sphingosine-based lipids, including sphingolipids and the glycosphingolipids, constitute a major fraction in neural tissues. Cholesterol is also an important

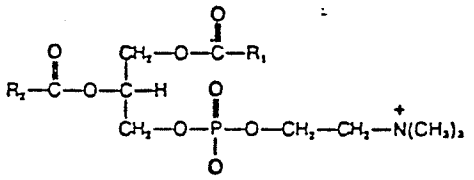
FIG. 2



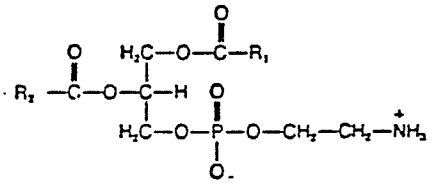
General structure of phospholipid

X = head alcohol (i.e. ethanolamine, choline, serine, inositol)

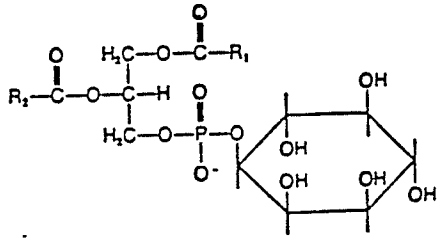
FIG. 3



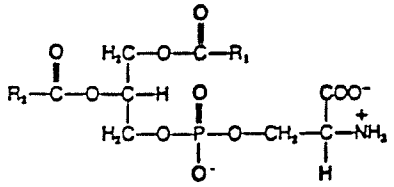
Phosphatidylcholine



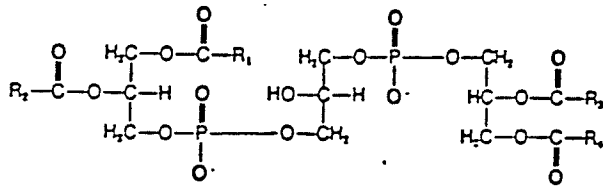
Phosphatidylethanolamine



Phosphatidylinositol



Phosphatidylserine



Cardiolipin

R_1: fatty acid attached to sn-1 position of glycerol moiety

R_2: fatty acid attached to sn-2 position of glycerol moiety

Classification of phosphopholipids

component in eucaryotic membrane, generally accounting for approximatelly equimolar proportion with phospholipid. The major functions of cholesterol include the maintenance of membrane fluidity and the enhancement of the mechanical stability of the membrane. The glycolipids play major roles as cell surface-associated antigens and recognition factors in eucaryotes.

2. Membrane Proteins

Although the basic structure of biological membrane is provided by the lipid bilayer, the proteins associated with the membrane also play specific roles, i.e. serving as specific receptors, enzymes, transport proteins etc. They constitute about 50% by mass in some membrane (Alberts *et al.* 1989). They can be classified into two groups, namely integral membrane proteins and peripheral membrane proteins. Integral membrane proteins extend their hydrophobic regions across the bilayer as a single α helix or as multiple α helices and interact with the hydrophobic tails of the lipid molecules in the interior of the bilayer. Peripheral membrane proteins are attached to the surface of the membrane bilayer by noncovalent interaction with other membrane proteins, or by covalent interaction with either a fatty acid chain (i.e. myristic acid) in the cytoplasmic monolayer or an oligosaccharide in the noncytoplasmic monolayer. Peripheral proteins can be isolated from the membrane by relatively gentle extraction procedures, i.e. exposure to solutions of very high or low ionic strength, extreme pH, sonication, etc. The release of integral membrane proteins requires disrupting the membrane bilayer with detergents or organic

solvents. The detergents are small amphiphilic molecules whose hydrophobic ends can bind to the hydrophobic regions of the membrane proteins, thereby displacing the lipid molecules. However, detergent solubilization of membrane proteins is not a routine procedure, and finding a suitable detergent for a particular situation is a process of trial and error.

3. Membrane Carbohydrate

All eucaryotic cells have carbohydrate on their surface. Both oligosaccharide and polysaccharide chains are covalently bound to membrane proteins or lipids (Alberts *et al.* 1989). The total carbohydrate in plasma membranes constitutes 2-10% of total weight of the membrane. Some membrane carbohydrates are involved in cell-cell and cell-matrix recognition processes (Alberts *et al.* 1989).

4. Phosphatidylcholine

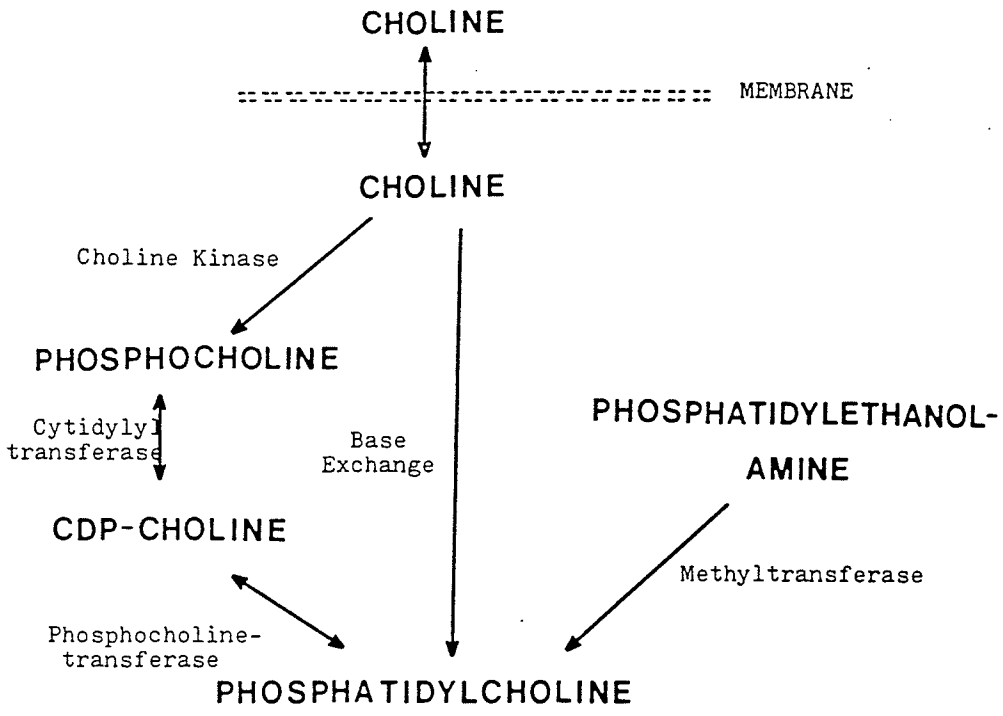
Among the different classes of phospholipids, phosphatidylcholine (lecithin) is predominant in eucaryotic cell membrane and is generally absent in procaryotic cells. Endoplasmic reticulum (ER) is the major quantitative site for phosphatidylcholine biosynthesis in mammalian cells (Vance and Vance 1988). Beyond its structural role as a principal membrane component, phosphatidylcholine also serves many important functions in the cells. A portion of phosphatidylcholine synthesized in the liver is incorporated into lipoprotein, and the dipalmitoyl-phosphatidylcholine

synthesized in the lung is secreted as surfactant. Phosphatidylcholine is also involved in the regulation of certain membrane-bound enzymes. Recently, the participation of phosphatidylcholine in signal transduction has been implicated (Exton 1990; Pelech and Vance 1989). The hydrolysis of phosphatidylcholine by phospholipase A_2 is an important source of arachidonic acid which is a precursor for eicosanoid biosynthesis. By the action of phospholipase C, phosphatidylcholine is hydrolyzed to yield diacylglycerol which may be responsible for activation of protein kinase C in mammalian tissues (Exton 1990; Pelech and Vance 1989).

II. PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN MAMMALIAN TISSUES

The pathways for phosphatidylcholine biosynthesis were elucidated in the 1950s, largely by Kennedy and co-workers (Kennedy 1989). There are three known pathways for the formation of phosphatidylcholine in mammalian tissues (Fig. 4). These are the CDP-choline pathway, the progressive methylation of phosphatidylethanolamine, and the Ca^{2+}-mediated base exchange pathway (Vance and Choy 1979). The contribution of each pathway to total phosphatidylcholine biosynthesis appears to be tissue specific. For example, the methylation of phosphatidylethanolamine accounts for 20% to 40% of phosphatidylcholine synthesized in the liver (Vance and Choy 1979; Vance 1990) and 2.5% phosphatidylcholine formed in the heart (Zelinski *et al.* 1980), whereas the base exchange pathway is a minor pathway for the biosynthesis of phosphatidylcholine in both tissues. The major pathway for phosphatidylcholine biosynthesis occurs via the CDP-choline pathway (Vance and Choy 1979; Zelinski *et*

FIG. 4



Pathways for the biosynthesis of phosphatidylcholine

al. 1980).

1. CDP-Choline Pathway

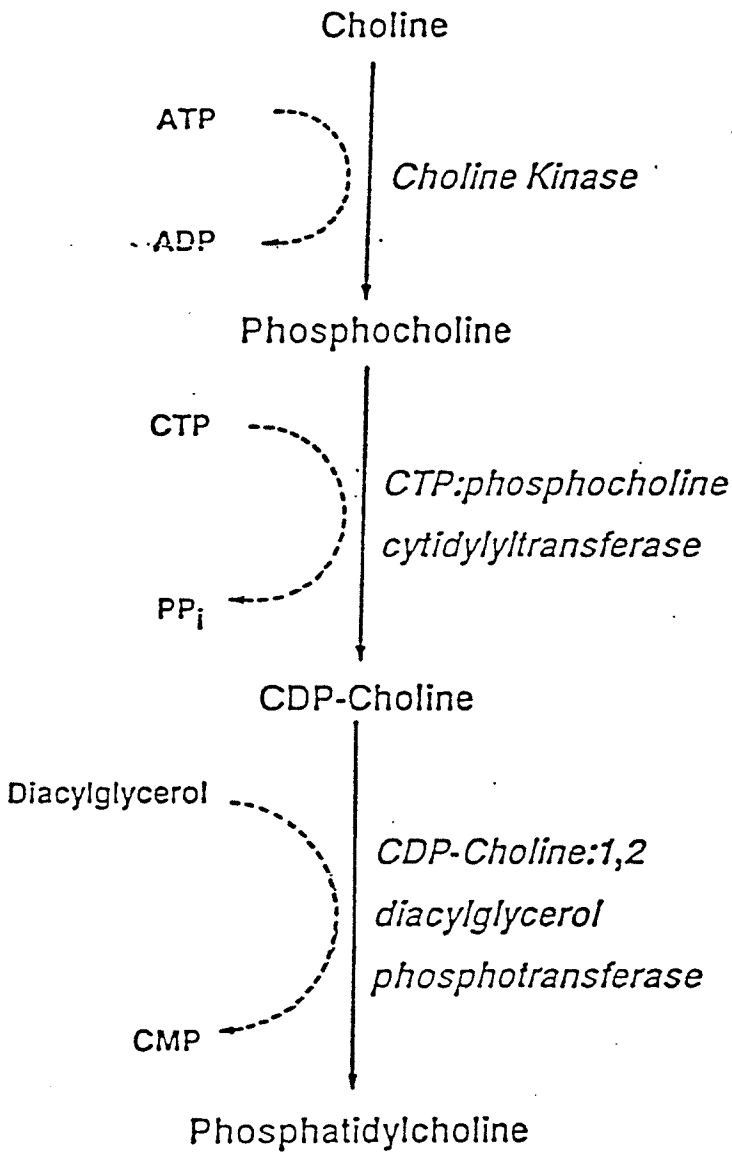
In the CDP-choline pathway (Fig. 5), choline is first phosphorylated to phosphorylcholine in the presence of ATP. This step is catalyzed by choline kinase (EC 2.7.1.32). Phosphorylcholine is then converted to CDP-choline in the presence of CTP. This is the rate-limiting step in the pathway and is catalyzed by CTP:phosphorylcholine cytidylyltransferase (EC 2.7.7.15). The final step in this pathway is the formation of phosphatidylcholine from CDP-choline and diacylglycerol, catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2). Both choline kinase and cytidylyltransferase have been purified to homogeneity from various tissues (Ishidate *et al.* 1984; Weinhold *et al.* 1986; Feldman and Weinhold 1987).

(1) Choline Kinase (EC 2.7.1.32)

Choline kinase catalyses the phosphorylation of choline in the presence of ATP and Mg^{2+}. This step commits choline to the CDP-choline pathway for the biosynthesis of phosphatidylcholine. The enzyme appears to be present in the cytosol of various tissues (Pelech and Vance 1984). A few exceptions are the reports of the enzyme activity associated with the membranes of the anaerobic protozoan E. *caudatum* (Bygrave and Dawson 1976) and rat brain (Ishidate 1989). The enzyme has been

FIG. 5

CDP-CHOLINE PATHWAY



Phosphatidylcholine biosynthesis via the CDP-choline pathway

highly purified from yeast, rat liver, monkey lung, rabbit brain, etc. with molecular weight in the range of 67,000 - 166,000 Da (Ishidate 1989). Complete purification of choline kinase from rat kidney cytosol has been achieved by Ishidate *et al.* with choline-Sepharose affinity chromatography (Ishidate *et al.* 1984). According to the report, the purified enzyme has a minimum molecular weight of 42,000 Da and its intact form is composed of two subunits with a molecular weight of 75,000 - 80,000 Da. The enzyme has an absolute requirement for Mg^{2+} and a pH optimum between 8.0 and 9.5 (Ishidate 1989). The apparent K_m values of choline kinase are 31 μM for choline and 8.3 - 10.8 μM for ATP-Mg^{2+} (Ishidate 1989).

Utilizing immunoprecipitation techniques, multiple forms of choline kinase have been identified in various tissues (Ishidate 1989). In addition, choline kinase and ethanolamine kinase appear to be separate enzymes (Vance 1985). For example, there are at least two distinct kinases responsible for the phosphorylation of choline and ethanolamine in primate liver (Ishidate 1989). In other case, choline kinase and ethanolamine kinase activities have been found to reside on the same protein but do not share a common active site (Ishidate 1989). Recently, the structural gene for choline kinase has been isolated from yeast and the translation product exhibits phosphorylation activity for both choline and ethanolamine (Hosaka *et al.* 1988). The physiological importance of multiple forms of choline kinase remains to be explored.

(2) CTP:Phosphorylcholine Cytidylyltransferase (EC 2.7.7.15)

Cytidylyltransferase catalyzes the conversion of phosphorylcholine to CDP-choline. This reaction has been regarded as the rate-limiting step in the CDP-choline pathway (Vance and Choy 1979). The definitive evidence for the rate-limiting role of this reaction has been obtained from a set of pulse-chase experiments in rat hepatocytes (Pritchard and Vance 1981), and isolated hamster heart (Zelinski *et al.* 1980). For example, when isolated hamster heart was perfused with [Me-^{3}H]choline, the majority of radioactivity taken up by the heart was quickly converted to phosphorylcholine. Subsequently, as the radioactivity disappeared from the phosphorylcholine, it was transiently associated with CDP-choline and immediately converted into phosphatidylcholine (Zelinski *et al.* 1980). Cytidylyltransferase is located in both cytosolic and microsomal compartments of various tissues (Vance 1989). It is generally accepted that cytidylyltransferase associated with the microsomes is the active form of the enzyme (Vance 1989). The enzyme in rat liver cytosol has been purified to homogeneity by Weinhold and co-workers (Weinhold *et al.* 1986; Feldman and Weinhold 1987). The purified enzyme appears to contain equal molar of two nonidentical proteins, with molecular weights of 38,000 and 45,000. The 45,000 protein contains the catalytic activity and the functional role of the 38,000 protein as part of a enzyme complex is not clear (Feldman and Weinhold 1987). When the activity of cytidylyltransferase was determined in the presence of saturating amount of phosphatidylcholine:oleic acid (1:1) vesicles, the K_m values of the purified enzyme are 0.22 mM for phosphorylcholine and 0.24 mM for CTP (Vance 1989).

Recently, a cDNA of cytidylyltransferase has been cloned from rat liver by oligonucleotide-directed polymerase chain reaction (Kalmar *et al.* 1990). The activity of the transfected cytidylyltransferase is lipid-dependent. The entire sequence lacks any signals for covalent lipid attachment and lacks a hydrophobic domain long enough to span a bilayer. However, it contains a potential 58-residue amphipathic α-helix, encompassing three homologous 11-residue repeats. It has been proposed that the interaction of cytidylyltransferase with membrane phospholipids is mediated by hydrophobic residues of this amphipathic helix lying on the surface of the protein (Kalmar *et al.* 1990).

(3) CDP-Choline:1,2-Diacylglycerol Cholinephosphotransferase (EC 2.7.8.2)

The final step in the CDP-choline pathway is the formation of phosphatidylcholine and CMP from diacylglycerol and CDP-choline, catalyzed by cholinephospho-transferase. The enzyme appears to be located on the cytoplasmic side of the endoplasmic reticulum (Vance *et al* 1977; Ballas and Bell 1980). However, it has been reported that cholinephosphotransferase exists in mitochondria as well as in microsomes of liver and lung of guinea pig and rat (Sikpi 1987; Ghosh *et al.* 1990). The activity of the enzyme is lower in mitochondria than it is in microsomes. The role of mitochondrial cholinephosphotransferase in phosphatidylcholine biosynthesis remains to be clarified.

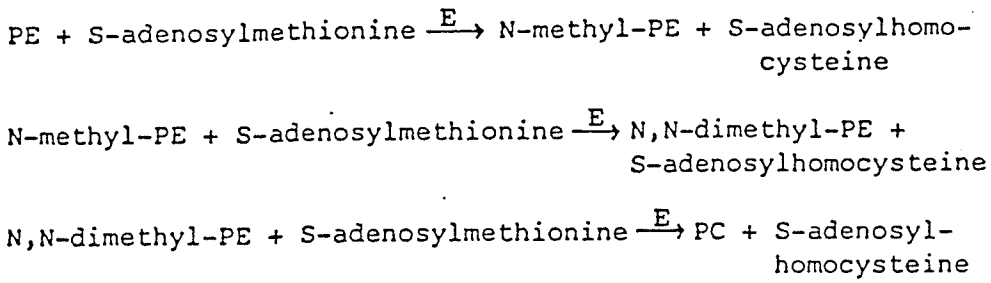
The cholinephosphotransferase in the hamster liver and heart has a broad pH profile with optimal pH of 7.5 - 8.5 (O *et al.* 1989). The enzyme has an absolute divalent cation requirement (Mg^{2+} or Mn^{2+}) for the activity and the activation of the enzyme by Mg^{2+} is more prominent. The apparent K_m values for CDP-choline range from 10 μM to 104 μM (Cornell 1989).

Recently, a cholinephosphotransferase gene in yeast has been isolated and the amino acid sequence of the enzyme elucidated (Hjelmstad and Bell 1990). Cholinephosphotransferase from the yeast has 407 amino acid with a predicted molecular weight of 46,305. The presence of seven transmembrane helices has been predicted from the sequence which is in agreement with the enzyme being tightly associated with membranes. The cloning of cholinephosphotransferase from mammalian sources has not been reported.

2.Methylation of Phosphatidylethanolamine

Methylation of phosphatidylethanolamine is quantitatively significant in liver (Pelech and Vance 1984). This pathway is responsible for 20-40% of phosphatidylcholine biosynthesis in the liver, whereas the methylation activity in other organs is very low (Vance 1990). Successive transfer of methyl groups from S-adenosyl-L-methionine generates the intermediates phosphatidyl-N-monomethylethanolamine and phosphatidyl-N,N-dimethylethanolamine, and finally phosphatidylcholine (Fig. 6). Phosphatidylethanolamine-N-methyltransferase (PE N-methyltransferase) is

FIG. 6



E: Phosphatidylethanolamine-N-methyltransferase

Progressive methylation of phosphatidylethanolamine

responsible for the three transmethylation reactions converting phosphatidyl-ethanolamine to phosphatidylcholine. The enzyme is located on the cytosolic side of the microsomal membranes and has been recently purified from rat liver microsomes (Ridgway and Vance 1987). The purified enzyme protein has a molecular mass of 18,300 Da and this single protein catalyzes the methylation of phosphatidyl-ethanolamine, phosphatidyl-N-monomethylethanolamine and phosphatidyl-N,N-dimethylethanolamine. The major control mechanism of the reaction appears to be the supply of phosphatidylethanolamine and S-adenosylmethionine (Vance 1990). The supply of methionine influences the concentration of S-adenosylmethionine which, when elevated, stimulates the methylation of phosphatidylethanolamine. There is a reciprocal regulation of the CDP-choline and phosphatidylethanolamine methylation pathways (Vance and Ridgway 1988). For example, treatment of hepatocytes with deazaadenosine caused a marked inhibition of the methylation of phosphatidylethanolamine. The loss of phosphatidylcholine synthesis via the methylation of phosphatidylethanolamine was compensated by increasing the activity of cytidylyltransferase in the CDP-choline pathway. It has been proposed that when the energy supply is not limiting, the inhibition of one pathway might be compensated by an increase in the other pathway. However, when the energy supply is reduced, both pathways might be attenuated (Vance 1990).

3. Base Exchange Reaction

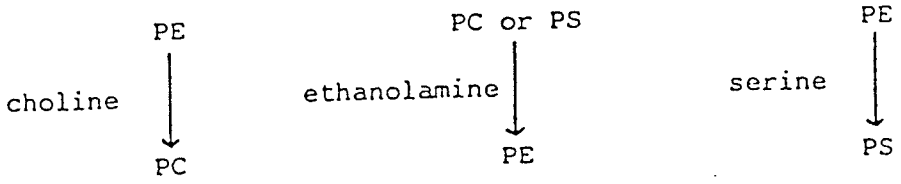
In base exchange reaction (Fig. 7), serine, ethanolamine and choline are incorporated into their respective phospholipids by exchanging with the headgroup of a preexisting phospholipid (Kanfer 1980). In animals, phosphatidylserine is synthesized via a base-exchange reaction (Vance 1985). The base exchange reactions are energy independent, but require Ca^{2+} (Kanfer 1980, 1989). These reactions have been detected in plants and animal tissues. It has been proposed that the major function of the base exchange reactions is to remodel preexisting membrane phospholipids (Kanfer 1989).

III. REGULATION OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS VIA THE CDP-CHOLINE PATHWAY

1. Choline Uptake

It is generally accepted that choline is taken up by a low-affinity, Na^+-independent transport system in non-neural tissues (Ishidate 1989). In the last two decades, a number of compounds including hemicholinium-3 (Diamond and Kennedy 1969), benzylcholine and chlorocholine (Haeffner 1975) were found to be potent inhibitors of choline uptake. Recently, ethanolamine, a structural analogue of choline, was found to inhibit choline uptake in a competitive manner (Zelinski and Choy 1984). Glycine and neutral amino acids were found to enhance choline uptake in the isolated hamster heart (Hatch and Choy 1986; Hatch *et al.* 1989). Although the short term inhibition or enhancement of choline uptake by ethanolamine and amino acids,

FIG. 7



PE: Phosphatidylethanolamine

PC: Phosphatidylcholine

PS: Phosphatidylserine

Base exchange reaction

respectively, do not have immediate effect on phosphatidylcholine biosynthesis, prolonged effect of those metabolites on phosphatidylcholine biosynthesis remains to be investigated.

2. Regulation of CTP:Phosphorylcholine Cytidylyltransferase

Cytidylyltransferase catalyzes the rate-limiting step in the CDP-choline pathway (Vance 1990). Over last two decades, research has focused on the mechanisms by which this enzymatic reaction is regulated (Vance and Choy 1979; Pelech and Vance 1984; Vance 1989). In mammalian tissues, cytidylyltransferase is located in both cytosolic and microsomal fractions (Vance and Choy 1979). It is generally accepted that the microsomal cytidylyltransferase is the active form of the enzyme (Sleight and Kent 1983a, 1983b, 1983c), and the translocation of the enzyme between these two subcellular compartments presents a plausible mechanism for the regulation of enzyme activity (Pelech and Vance 1984). In addition, direct activation of the enzyme has been observed with the enzyme from rat hepatocytes (Pelech *et al.* 1983) and hamster heart (Mock *et al.* 1986).

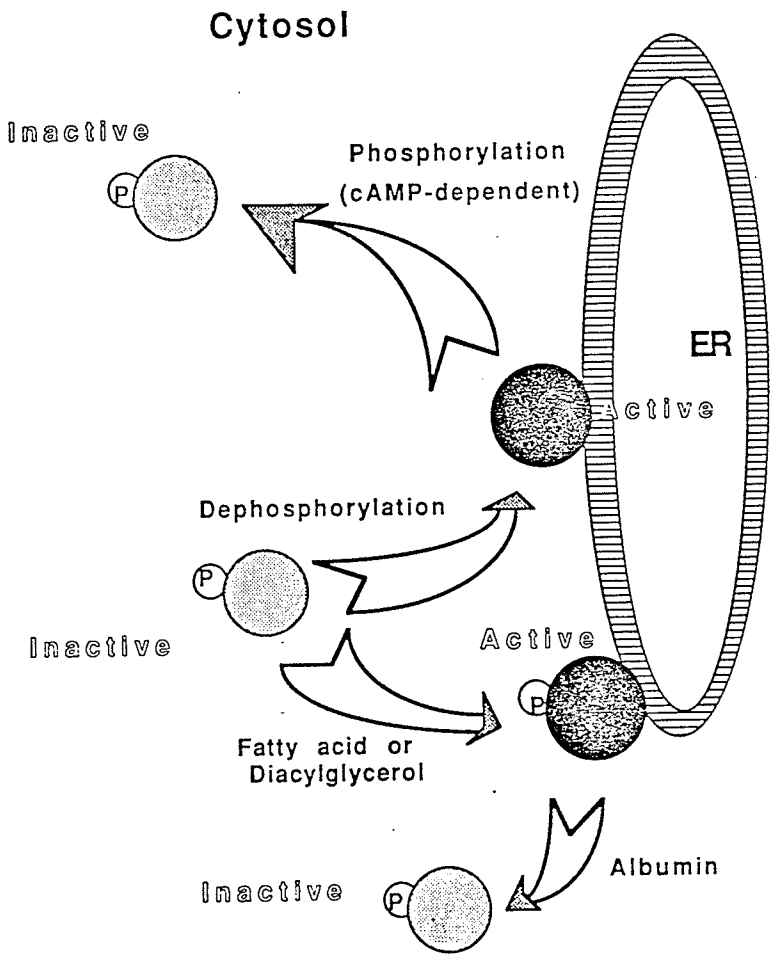
(1) The Translocation Hypothesis

Movement of cytidylyltransferase on and off the endoplasmic reticulum has been proposed as the major mechanism for the regulation of phosphatidylcholine biosynthesis (Vance and Pelech 1984). Indirect evidence for the translocation of

cytidylyltransferase has been obtained in HeLa cells (Pelech *et al.* 1984), rat liver (Pritchard *et al.* 1981) and chick embryonic myoblasts (Sleight and Kent 1980). It has been demonstrated that a decrease in the activity of cytidylyltransferase on microsomal fraction correlates with a decrease in phosphatidylcholine biosynthesis (Pelech *et al.* 1981). Proposed mechanism for the translocation of cytidylyltransferase between two subcellular compartments is illustrated in Fig. 8 (Vance 1989a). Fatty acids promote the translocation of cytidylyltransferase from cytosol to membranes where the enzyme is activated by certain phospholipids in the membranes. It has been shown in HeLa cells and rat lung that the effect of fatty acids on binding of cytidylyltransferase to membranes is reversible (Weinhold *et al.* 1984; Cornell and Vance 1987a, 1987b). Recently, diacylglycerol has been suggested as a mediator of cytidylyltransferase translocation from cytosol to membranes (Vance 1989a).

Another mechanism for enzyme translocation is the reversible phosphorylation-dephosphorylation of cytidylyltransferase (Pelech and Vance 1984; Vance 1989a). It has been shown *in vitro* that cytidylyltransferase is a substrate of cAMP-dependent kinase and phosphorylation of cytidylyltransferase by cAMP-dependent protein kinase inhibits the binding of the enzyme to membranes (Sanghera and Vance 1989). Recently, the dephosphorylation of the enzyme by phosphatase has been shown to correlate with increased binding of cytidylyltransferase to membrane (Hatch *et al.* 1990). However, phosphorylation of the enzyme *in vivo* as a result of cAMP treatment and the phosphorylated sites on the enzyme have not been identified.

FIG. 8



Proposed mechanism for translocation of cytidylyltransferase between cytosol and ER

(2) Activation of Cytidylyltransferase by Fatty Acids

The direct activation of fatty acids on cytidylyltransferase in lung (Weinhold *et al.* 1984), heart (Mock *et al.* 1986), and liver (Pelech *et al.* 1983; Vance 1990) has been demonstrated. However, there are some major difference in the regulation of phosphatidylcholine biosynthesis by fatty acids between tissues. For example, only stearic acid was found to be effective in hamster hearts (Mock *et al.* 1986), whereas in rat hepatocytes stimulatory effects were produced by all fatty acids examined (Pelech *et al.* 1983). When isolated hamster hearts were perfused with exogenous stearic acid (50 μM), phosphatidylcholine biosynthesis was stimulated and such stimulation was attributed to a corresponding 2.3-fold increase in the activity of the microsomal cytidylyltransferase (Mock *et al.* 1986). However, no translocation of cytidylyltransferase from the cytosolic to the microsomal fractions was detected. Although the importance of the microsomal form of cytidylyltransferase in the regulation of phosphatidylcholine biosynthesis in the heart has been documented (Zelinski *et al.* 1980), there is no direct evidence to show that the heart cytidylyltransferase is regulated by translocation of the enzyme (Hatch *et al.* 1989).

(3) Lipid Requirements for the Activation of Cytidylyltransferase

It has been shown that the activity of cytidylyltransferase can be stimulated by phospholipids (Choy and Vance 1978). Studies with pure enzyme showed that cytidylyltransferase could be activated by phosphatidylcholine, phosphatidylethanolamine, fatty acids, lysophosphatidylcholine and

lysophosphatidylethanolamine (Weinhold *et al.* 1986; Feldman and Weinhold 1987). Since phospholipids are usually found in the cellular membranes, the requirement of phospholipids for the enzyme activity suggests that the membrane-associated cytidylyltransferase is the active form of the enzyme (Vance 1989).

3. Regulation of Cholinephosphotransferase

(1) Selection of Molecular Species of Diacylglycerol

Cholinephosphotransferase catalyzes the last reaction which is the condensation of the lipoidal moiety (diacylglycerol) with CDP-choline in the CDP-choline pathway. The majority of phosphatidylcholine synthesized in mammalian tissues contains a saturated fatty acid group at the sn-1 position and an unsaturated fatty acid group at the sn-2 position of the glycerol moiety (Vance 1985; Choy and Arthur 1989a). The composition of the fatty acid groups have been found to vary from one tissue to another (Choy and Arthur 1989). The selection of the desired molecular composition of phosphatidylcholine can be mediated by the selectivity of cholinephosphotransferase for diacylglycerol, or by the deacylation-reacylation cycle. It was shown in rat liver that microsomal cholinephosphotransferase had a preference for the 1-palmitoyl 2-linoleoyl and 1-palmitoyl 2-arachidonoyl diacylglycerols (Holub 1978). In rat lung, 1-palmitoyl 2-oleoyl-sn-glycerol was the preferred substrate for the enzyme (Possmayer *et al.* 1977). The specificity of the enzyme was also investigated in the isolated hamster heart (Arthur and Choy 1984). Maximum enzyme activity

was obtained with diacylglycerol containing a monoenoic acyl group at the sn-2

position, regardless of the acyl content at the sn-1 position. The relative contribution of cholinephosphotransferase to the selection of appropriate acyl groups appears to be tissue specific (Arthur and Choy 1984). Recently, it has been suggested that there is a preferred pool of diacylglycerol utilized by cholinephosphotransferase *in vivo* (Binaglia *et al.* 1982; Rustow and Kunze 1985). According to those investigators, there are two different pools of diacylglycerol in the membrane, designated the *de novo* (diacylglycerol synthesized from glycerol-3-phosphate) and endogenous (existing diacylglycerol in membrane) pools. Both pools of diacylglycerol are accessible to cholinephosphotransferase, but the *de novo* pool generated from glycerol-3-phosphate is preferentially utilized by the enzyme. It has been proposed that the enzymes involved in phosphatidylcholine biosynthesis from glycerol-3-phosphate are organized in a multienzyme complex, in which the metabolic intermediates are channelled directly from one enzyme active site to the next without mixing with the endogenous membrane lipid (Rustow and Kunze 1987).

(2) Regulation by Inhibitors and Activators

The divalent cations including Mg^{2+} and Mn^{2+} are cofactors of cholinephosphotransferase (Cornell 1989). In the presence of Mg^{2+}, the enhancement of the enzyme activity is substantially higher (O, *et al.* 1989). The inhibition of the enzyme by Ca^{2+} has been observed in various tissues and the nature

of inhibition is competitive toward Mg^{2+} and Mn^{2+} (Taniguchi *et al.* 1986; Cornell 1989). The mechanism of the cofactor inhibition has been proposed that Ca^{2+} competes for complexation with CDP-choline, and the resulting complex is unfavourable as a substrate (Cornell 1989).

Lysophosphatidylcholine has been found to have biphasic effect on the activity of cholinephosphotransferase (Parthasarathy and Baumann 1979). The enzyme activity is stimulated by lower level (< 0.5 mM) of lysophosphatidylcholine and inhibited by higher level (> 0.5 mM) of lysophosphatidylcholine. The regulation of cholinephosphotransferase activity by lysophosphatidylcholine has been implicated as an important mechanism for the control of phosphatidylcholine biosynthesis (Parthasarathy and Baumann 1979).

In the presence of phosphatidylcholine transfer protein, the incorporation of CDP-choline and diacylglycerol into phosphatidylcholine is markedly stimulated (Khan and Helmkamp 1990). This transfer protein is located in cytosol and catalyses the transport of phosphatidylcholine between membranes. The activation of cholinephosphotransferase by this transfer protein may have physiological significance in the coordination of phosphatidylcholine biosynthesis and transportation in mammalian tissues (Khan and Helmkamp 1990).

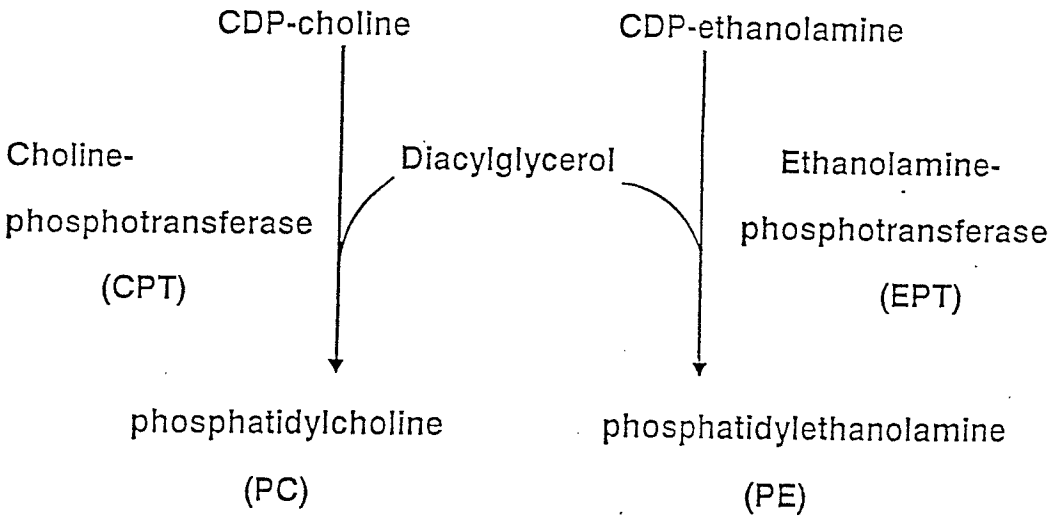
(3) Distinction from Ethanolaminephosphotransferase

Phosphatidylethanolamine is a major phospholipid in mammalian tissues. The CDP-ethanolamine pathway is an important pathway for the synthesis of this phospholipid (Kennedy 1962; Vance 1985). Ethanolaminephosphotransferase is responsible for the condensation of CDP-ethanolamine and diacylglycerol by a reaction similar to that catalyzed by cholinephosphotransferase (Kennedy 1962). Both phosphotransferase activities are located on the cytoplasmic side of the endoplasmic reticulum (Vance *et al.* 1977; Ballas and Bell 1980) and both reactions utilize diacylglycerol as a common substrate (Fig. 9). Indirect evidence indicates that the two activities are different in mammalian tissues. For example, two phosphotransferases display some differences in stability, acyl specificity for diacylglycerol, sensitivity to detergent treatment, etc. (Possmayer *et al.* 1977; Holub 1978; Kanoh and Ohno 1976; Morimoto and Kanoh 1978; O *et al.* 1989). The most definitive evidence for the distinction between these two enzymes comes from the isolation of mutant yeasts defective in either cholinephosphotransferase or ethanolaminephosphotransferase (Hjelmstad and Bell 1987; 1988). There are two separate genes which code for cholinephosphotransferase and ethanolaminephosphotransferase in yeast.

4. Supply of High Energy Nucleotides

Both ATP and CTP are required in the CDP-choline pathway and the availability of these high energy compounds may affect the rate of phosphatidylcholine biosynthesis (Vance and Choy 1979; Hatch *et al.* 1988). It was shown that an increase in cytoplasmic CTP in polio-infected HeLa cells caused an enhancement of

FIG. 9



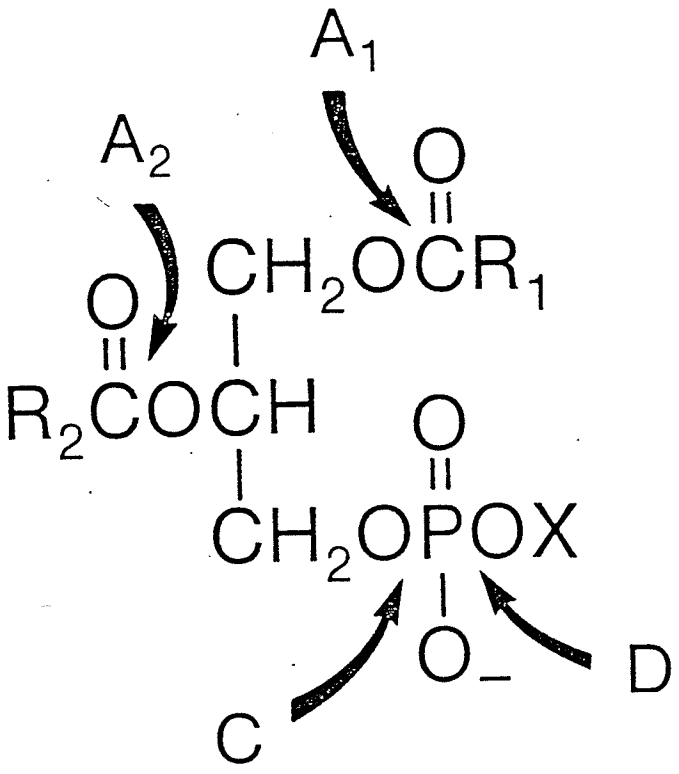
Cholinephosphotransferase and ethanolaminephosphotransferase

phosphatidylcholine biosynthesis, but the increase in ATP level did not affect the phosphorylation of choline (Choy *et al.* 1980). The role of CTP in phosphatidylcholine biosynthesis was also demonstrated in myopathic hamster heart (Choy 1982). A 34% decrease of CTP concentration and 33% decrease of ATP concentration were observed in the myopathic heart, but only the formation of CDP-choline was altered. Recently, the role of ATP and CTP was examined in the isolated hamster heart under hypoxic condition (Hatch and Choy 1990). The cardiac ATP and CTP were significantly decreased under hypoxia. However, the lowered ATP level did not affect the phosphorylation of choline to phosphocholine, but the lowered CTP level resulted in the decreased conversion of phosphocholine to CDP-choline. It is clear from these studies that the intracellular CTP concentration is an important factor for the maintenance of phosphatidylcholine biosynthesis in the heart.

IV. PHOSPHATIDYLCHOLINE CATABOLISM IN MAMMALIAN TISSUES

The catabolism of phosphatidylcholine is carried out by the action of phospholipases. Phospholipases are a group of enzymes which catalyze the hydrolysis of ester bonds in glycerophospholipids (Roberts and Dennis 1989). Each phospholipase has its own specificity. The classification of those phospholipases is based on the sites of ester bonds they hydrolyze (Fig. 10). Phospholipase A_1 hydrolyzes the sn-1 fatty acyl bond and phospholipase A_2 hydrolyzes the sn-2 fatty acyl bond of phosphatidylcholine. Lysophosphatidylcholine and free fatty acid are formed upon the action of phospholipase A. Phospholipase C hydrolyzes the glycerophosphate ester bond and

FIG. 10



Sites of action of the phospholipases

A_1: Phospholipase A_1

A_2: Phospholipase A_2

C: Phospholipase C

D: Phospholipase D

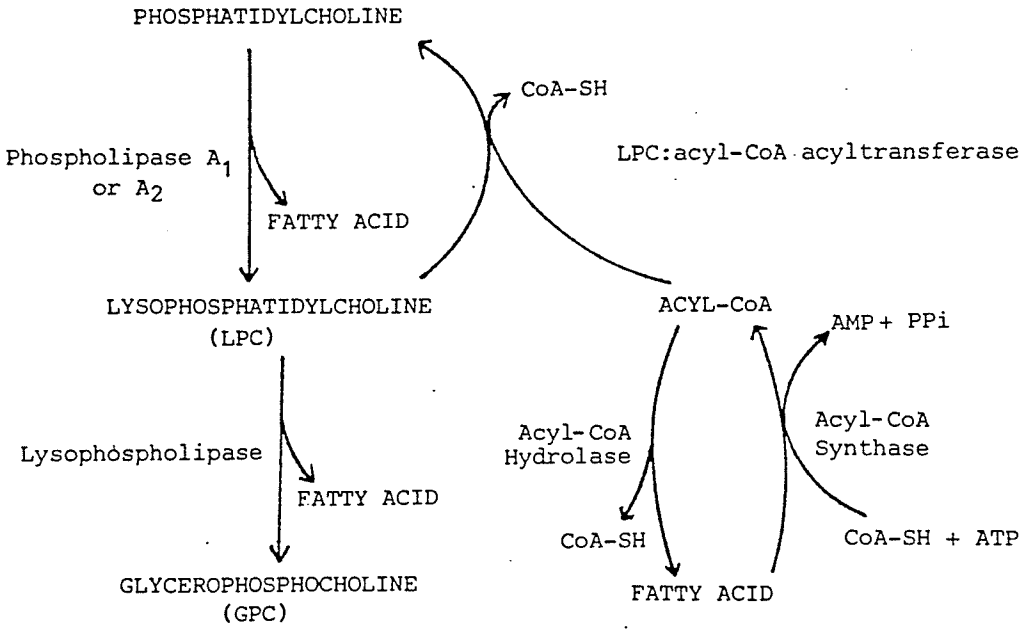
the products are diacylglycerol and phosphorylcholine. Phospholipase D hydrolyzes the choline phosphate ester bond and the products are phosphatidic acid and choline.

Although the catabolism of phosphatidylcholine can be initiated by any one of above four phospholipases, the principal pathway for its catabolism is through the hydrolytic action of phospholipase A (Brockerhoff and Jenson 1974). As shown in Fig. 11, the resultant lysophosphatidylcholine can be further deacylated by lysophospholipase, or alternatively, it can be reacylated back to phosphatidylcholine by lysophosphatidyl-choline:acyl-CoA acyltransferase (Lands 1960). Both phospholipase A_1 and A_2 are located in all subcellular fractions. In the heart, the highest activity for phospholipase A_1 has been found in microsomal and cytosolic fractions, and the highest activity for phospholipase A_2 in the microsomal fraction (Tam *et al.* 1984).

V. REGULATION OF PHOSPHATIDYLCHOLINE CATABOLISM IN MAMMALIAN HEART

Phosphatidylcholine metabolism in mammalian heart is in a dynamic state. Its level and composition in the cardiac membrane are closely regulated by the rate of biosynthesis and catabolism (Dawidowicz 1987). Over past decade, the regulation of phosphatidylcholine catabolism has been extensively investigated in mammalian heart as well as in other tissues (Choy and Arthur 1989; Roberts and Dennis 1989).

FIG. 11



The catabolism of phosphatidylcholine and the deacylation-reacylation pathway

1. Substrate Specificity of Phospholipase A

The majority of the early studies on phospholipase A were confined to the venom and pancreatic enzymes (Roberts and Dennis 1989). Little was known about the regulation of intracellular phospholipase A in mammalian tissues. Recently, phospholipase A has been purified from hamster heart cytosol (Cao *et al.* 1987b). The purified enzyme has a molecular weight of 140 kilodaltons (kDa) and is made up of 14 kDa identical subunits (Cao *et al.* 1987b). It has no absolute requirement for Ca^{2+} or other divalent metallic cations. The purified enzyme displays both phospholipase A_1 and A_2 activities, and is active towards phosphatidylcholine and phosphatidylethanolamine but not lysophosphatidylcholine or neutral phospholipids. Both phospholipase A_1 and A_2 in the heart display a high degree of specificity for the acyl groups (saturation and length) of phosphatidylcholine at both sn-1 and sn-2 positions (Tam *et al.* 1984; Cao *et al.* 1987b). It has been clearly demonstrated in the heart that the activity of phospholipase A_1 is not only dependent on the sn-1 acyl group of phosphatidylcholine, but also dependent on the acyl group at sn-2 position. Similarly, the activity of phospholipase A_2 is also dependent on the nature of acyl groups at both sn-1 and sn-2 positions. Phospholipase A_1 prefers a sn-1 stearoyl group, whereas phospholipase A_2 exhibits a higher activity when a sn-1 palmitoyl group is present. Both phospholipases favour a highly unsaturated acyl group at sn-2 position. Based on the fact that phospholipase A_1 and A_2 have a high degree of specificity towards the various species of phosphatidylcholine, it has been suggested that the use of a natural substrate (with mixed acyl groups) for the enzyme assay

would provide a better assessment of phospholipase A activity (Tam *et al.* 1984; Cao *et al.* 1987b).

Recently, a Ca^{2+}-dependent phospholipase A_2 has been solubilized and purified from rat liver mitochondria (Aarsoman *et al.* 1989). It has an estimated molecular weight of 14 kDa. In the separate study, a Ca^{2+}-independent, plasmalogen-selective phospholipase A_2 has been purified from canine myocardial cytosol (Hazen *et al.* 1990). The purified enzyme has absolute specificity for cleavage of the sn-2 acyl linkage in plasmalogen (1-O-alk-1-enyl-2-acyl-glycerophosphocholine). Although several types of phospholipase A have been purified from mammalian sources, the control of phospholipase A in phosphatidylcholine catabolism remains undefined.

2. Control of Lysophosphatidylcholine Level

Lysophosphatidylcholine, generated by the action of phospholipase A, is the immediate metabolite of phosphatidylcholine. Due to its detergent property, lysophosphatidylcholine is cytolytic at high cellular concentrations and its intracellular level is rigidly controlled (Weltzien 1979). The development of ventricular arrhythmias after the onset of myocardial ischemia is well documented (Elharrar and Zipes 1977; Wit and Rosen 1983). The elevated level of lysophosphatidylcholine has been found in the ischemic myocardium (Corr *et al.* 1982). Although the exact biochemical cause for the production of arrhythmias remains largely unknown, the accumulation of lysophosphatidylcholine in the ischemic heart has been suggested as

one of the biochemical factors for causing arrhythmias (Katz and Messineo 1981; Corr *et al.* 1982; Hatch *et al.* 1989). Owing to its potent cytolytic effect, the intracellular concentration of lysophosphatidylcholine must be under rigid control (Weltzein 1979). Control of intracellular lysophosphatidylcholine level can be achieved by the action of phospholipase A, lysophospholipase and acyltransferase.

Since lysophosphatidylcholine is produced by the action of phospholipase A, it has been postulated that activation of phospholipase A and the resultant accumulation of lysophospholipids are intimately related to the development of electrophysiological dysfunction in ischemic myocardium (Hazen *et al.* 1990). Recently, it has been reported that dietary vitamin E has a regulatory role on the activities of phospholipase A_1 and A_2 in rat heart (Cao *et al.* 1987a). In the rats fed with a vitamin E-deficient diet, the level of cardiac lysophosphatidylcholine was significantly increased, whereas the cardiac lysophosphatidylcholine level was decreased in the rats fed with a high vitamin E diet. The alterations in cardiac lysophosphatidylcholine level by dietary vitamin E were attributed to the changes in the activities of cardiac phospholipase A_1 and A_2. Vitamin E inhibited both phospholipase A_1 and A_2 activities in a non-competitive manner, but had no effect on the other major enzymes (lysophospholipase and acyltransferase) which were responsible for the metabolism of lysophosphatidylcholine. Hence, the tissue vitamin E content might be one of the underlying factor for the regulation of phosphatidylcholine catabolism.

VI. RESEARCH AIMS

The first part of my research was to study the control of phosphatidylcholine biosynthesis via the CDP-choline pathway. Our hypothesis was that cholinephosphotransferase might play an active role in regulation of phosphatidylcholine biosynthesis. Specifically, the work was focused on the CDP-choline:1,2-diacylglycerol cholinephosphotransferase. This part of the research can be divided into three sections: (1) solubilization and purification of cholinephosphotransferase; (2) differentiation of cholinephosphotransferase and ethanolaminephosphotransferase; (3) identification of endogenous metabolic regulator of cholinephosphotransferase.

The second part of my research was to study the control of phosphatidylcholine catabolism. In this study, ethanol and vitamin E were used as probes to perturb phosphatidylcholine catabolism; hence, the role of phospholipase A in the control of phosphatidylcholine catabolism was explored.

MATERIALS AND METHODS

A. MATERIALS

I. Experimental Animals

Male Syrian golden hamsters (110 ± 10 g) were used throughout the study of phosphatidylcholine biosynthesis. Male Sprague-Dawley rats (300 ± 30 g) were used for the study of phosphatidylcholine catabolism. Both hamsters and rats were maintained on Agway rodent chow, and tap water *ad libitum*, in a light-and temperature-controlled room.

II. Chemicals

Choline iodide, phosphorylcholine, ATP, CTP, CDP-ethanolamine, octyl glucoside, taurocholic acid, Triton QS-15, Triton X-100, Tween 20, synthetic vitamin E (DL-α-tocopherol), oleoyl-CoA, amino acid standards and argininosuccinic acid were obtained from Sigma Chemical Co. (St. Louis, MO). CDP-choline, 1,2-diacylglycerol (pig liver), phosphatidylcholine (pig liver), phosphatidylethanolamine (pig liver), lysophosphatidylcholine (pig liver), lysophosphatidylethanolamine (pig liver) and other lipid standards were purchased from Serdary Research laboratories (London,

Ont.). The purity of the above lipid standards was approximately 99% determined by the supplier based on chromatographic methods CHAPSO and CHAPS were purchased from Pierce Chemical Co. (Rockford, IL). DEAE-Sepharose (fast flow), and Mono Q HR 5/5 column for FPLC were the products of Pharmacia LKB Biotechnology (Dorval, Que.). CM-Cellulose (CM-11) and 1PS filter paper were the products of Whatman Ltd. (England). Thin-layer chromatographic plates Sil-G25 and Redi-Plate, Sil Gel G were obtained from Brinkmann Inc. and Fisher Scientific Co., respectively. Thin-layer chromatographic cellulose plates were obtained from Mandel Scientific Co. (Edmonton, Alb.). Ion-exchange resin (AG1-X8) was obtained from Bio-Rad Laboratories (Mississauga, Ont.). Centriflo membrane cones (type CF25A) were obtained from Amicon division of W.R.Grace & Co. (Beverly, MA). [Me-^{3}H]choline (80.0 mCi/mmol), CDP-[Me-^{14}C]choline (42.4 mCi/mmol) and CDP-[Me-^{14}C]ethanolamine (60.0 mCi/mmol) were obtained from NEN division, Dupont Co. (Dorval, Que.). 1-[1-^{14}C]palmitoyl lysophosphatidylcholine (58.5 mCi/mmol), 1-stearoyl-2-[^{14}C]arachidonyl-glycerophosphocholine (60.1 mCi/mmol) and aqueous counting scintillant were obtained from Amersham Canada Ltd. (Oakville, Ont.). All other chemicals were reagent grade and were obtained through the Canlab Division of Travenol Canada Inc. (Winnipeg, Man.). Phosphoryl[Me-^{3}H]choline was synthesized enzymatically from [Me-^{3}H]choline and ATP with yeast choline kinase (Vance *et al.* 1981). Phosphatidyl[Me-^{3}H]choline was prepared from [Me-^{3}H]choline as described previously (Tam *et al.*1984).

B. METHODS

I. Studies on Phosphatidylcholine Biosynthesis in Hamster Tissues

1. Preparation of Subcellular Fractions

Hamsters were sacrificed by decapitation, and the livers and hearts were excised and rinsed in ice-cold saline. The livers and hearts were cut into small pieces and homogenized in 0.25 M sucrose in 10 mM Tris-HCl (pH 7.4) to yield 15% homogenate. The homogenate was centrifuged at 10,000 g for 10 min and the resulting supernatant was recentrifuged at 100,000 g for 60 min. The supernatant obtained from high speed centrifugation formed cytosolic fraction. The microsomal pellet was resuspended in 10 mM Tris-HCl (pH 7.4). Choline kinase activity was determined in the cytosolic fraction, whereas the CTP:phosphorylcholine cytidylyltransferase activity was determined in both cytosolic and microsomal fractions. For cholinephosphotransferase assay, the microsomes were washed once with 10 mM Tris-HCl (pH 7.4) and the enzyme activity was determined in the washed microsomal fraction.

2. Solubilization of Cholinephosphotransferase

The washed microsomes were resuspended in 25 mM Tris-succinate (pH 6.0) - 2 mM ß-mercaptoethanol with 3% Triton QS-15. The mixture was homogenized with a Dounce homogenizer at 4 °C and then centrifuged at 100,000 g for 60 min. The resulting clear supernatant was employed as a source for further purification.

3. Partial Purification of Cholinephosphotransferase

The solubilized enzyme preparation (containing 200 - 250 mg protein) was applied to a DEAE-Sepharose (fast flow) column (1.5 x 15 cm) equilibrated with 25 mM Tris-succinate (pH 6.0) - 2 mM ß-mercaptoethanol. Subsequent to the application of the sample, the column was washed with 100 ml of 25 mM Tris-succinate (pH 6.0) - 2 mM ß-mercaptoethanol, followed by 0.5 M KCl in the same buffer. Fractions (5 ml) were collected after sample application and assayed for cholinephosphotransferase activity. The most active fractions (fraction 29 and 30) from the DEAE-Sepharose column were pooled (25 mg protein) and applied to a Sepharose 6B column (2.5 x 40 cm) which was equilibrated with 25 mM Tris-succinate(pH 6.0) - 0.5 mM KCl - 5% glycerol. The column was washed with the same buffer and fractions (3.5 ml) were collected. The fractions were assayed for enzyme activity.

4. Enzyme Assays

(1) Choline Kinase (EC 2.7.1.32)

The enzyme activity in the cytosol was determined with labelled choline as a substrate (Ishidate *et al.* 1980). The reaction mixture contained 100 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$ 10 mM ATP, 0.25 mM [Me-^{3}H]choline iodide and 0.5 - 1.0 mg cytosolic protein in a final volume of 0.5 ml. The reaction was initiated by the addition of labelled choline and the mixture was incubated at 37 °C for 20 min. The reaction was stopped by placing the assay tubes in a boiling water bath for 3 min. The mixture was then centrifuged at 5,000 g for 10 min and a portion (0.25 ml) of the supernatant was applied to a Dowex AG 1-X8 (OH^-) column (0.5 x 2 cm). The column was first washed with 2.5 ml 5 mM choline chloride and then with 6 ml water. Phosphorylcholine was eluted from the column with 0.5 ml 1.0 M NaOH followed by 1.5 ml 0.1 M NaOH and fractions were collected. Acetic acid (0.1 ml) was added to the pooled fractions and the radioactivity associated with phosphorylcholine was determined by liquid scintillation counting.

(2) CTP:Phosphorylcholine Cytidylyltransferase (EC 2.7.7.15)

Enzyme activity in the microsomal and cytosolic fractions was determined as described by Vance *et al* (1981). The reaction mixture contained 100 mM Tris-succinate (pH 8.0), 12 mM magnesium acetate, 2.5 mM CTP, 1.0 mM phosphoryl-[Me-^{3}H]choline and 0.5 - 1.0 mg microsomal or cytosolic proteins in a final volume of 0.1 ml. The reaction was initiated by the addition of labelled phosphorylcholine and the mixture was incubated at 37 °C for 15 min. The reaction was stopped by placing the reaction tubes in boiling water bath for 3 min. The reaction mixture was centrifuged at 5,000 g for 10 min and an aliquot of the supernatant was applied to a thin-layer chromatographic plate (Sil G25). The plate was developed in the solvent containing CH_3OH:0.6%NaCl:NH_4OH (50:50:5, v/v). The location of CDP-choline on the plate was determined with Bioscan System 200 Imaging Scanner. The silica gel was removed and placed into a scintillation counting vial, and 1 ml water was added. Acetic acid (0.1 ml) was added to prevent chemiluminescence and the radioactivity associated with CDP-choline fraction was then determined by liquid scintillation counting.

(3) CDP-Choline:1,2-Diacylglycerol Cholinephosphotransferase (EC 2.7.8.2)

The reaction mixture for the determination of cholinephosphotransferase activity contained 100 mM Tris-HCl (pH 8.5), 10 mM magnesium chloride, 1 mM EDTA, 0.4 mM CDP-[Me-^{14}C]choline (1.0 μCi/μmol), 1.0 mM diacylglycerol (prepared in

0.015% Tween 20 by sonication) and 0.1 - 0.5 mg microsomal protein in a final volume of 1.0 ml. The reaction was initiated by the addition of labelled CDP-choline and the mixture was incubated at 37 °C for 15 min. The reaction was terminated by the addition of 3 ml of chloroform:methanol (2:1, v/v) to the mixture. Water (0.5 ml) was added to the mixture to cause phase separation. The lower (organic) phase was washed twice with 2 ml of 40% methanol and the solvent in the lower phase was subsequently evaporated. The radioactivity associated with phosphatidylcholine was determined with liquid scintillation counting. Analysis by thin-layer chromatography revealed that over 98% of the radioactivity in the lower phase was in the phosphatidylcholine fraction.

(4) CDP-Ethanolamine:1,2-Diacylglycerol Ethanolaminephosphotransferase (EC 2.7.8.1)

The reaction mixture for the determination of ethanolaminephosphotransferase activity contained 100 mM Tris-HCl (pH 8.5), 10 mM manganese chloride, 1 mM EDTA, 0.2 mM CDP-[1,2-^{14}C]ethanolamine (1.0 μCi/μmol), 1.0 mM diacylglycerol (prepared in 0.015% Tween 20) and 0.1 - 0.5 mg microsomal protein in a final volume of 1.0 ml. The reaction was initiated by the addition of labelled CDP-ethanolamine and the mixture was incubated at 37 °C for 15 min. The reaction was terminated by the addition of 3 ml of chloroform:methanol (2:1, v/v) to the mixture.

Phase separation was obtained by the addition of water (0.5 ml). The radioactivity of phosphatidylethanolamine in the lower phase was determined in the same manner as in the cholinephosphotransferase assay.

5. Isolation of Cholinephosphotransferase Inhibitor

The hamster liver cytosol was incubated at 100 °C for 10 min and subsequently centrifuged at 5,000 g for 30 min. The resulting supernatant was filtered through an Amicon centriflo cone (type CF25A). The filtered sample (containing 2.06 mg protein) was loaded onto a FPLC Mono Q HR 5/5 anion-exchange column, equilibrated with 10 mM Tris-HCl (pH 9.5). The column was first washed with 10 ml of 10 mM Tris-HCl (pH 9.5) and subsequently, a linear pH gradient (pH 9.5 to pH 7.5) of 10 mM Tris-HCl was applied. A flow rate of 1 ml per min was maintained. Fractions (1 ml) were collected and an aliquot was used to test the inhibitory activity on cholinephosphotransferase. The fractions containing inhibitory activity were pooled and the volume was reduced by lyophilization. An aliquot of the lyophilized sample was applied to a cellulose chromatographic plate with authentic amino acids and amino acid derivatives as standards. The plate was developed in the solvent containing butanol:formic acid:water (75:15:10, v/v). The locations of amino acids and amino acid derivatives were visualized with ninhydrin spray reagent. The Rf value of the sample containing inhibitory activity was identical to that of

argininosuccinic acid. The cellulose gel fraction containing inhibitory activity was removed and placed into a test tube and the inhibitor was extracted with 3 ml water three times. The extract was passed through a 0.2 μm filter unit and the volume of the extract was reduced by lyophilization. The lyophilized sample was then dissolved in 0.5 ml of 0.2 M lithium citrate buffer (pH 2.8) and the nature of the inhibitor was further confirmed by an automatic amino acid analyzer. A high resolution Ultropac 8 resin cation exchange column (LKB) was used for the analysis. After applying an aliquot of the lyophilized sample, the column was washed with lithium citrate buffer for 204 min. A series of the eluting buffers were used in the following order: (1) Buffer I: 0.2 M lithium citrate, pH 2.80 (0 - 17 min); (2) Buffer II: 0.3 M lithium citrate, pH 3.0 (17 - 65 min); (3) Buffer III: 0.6 M lithium citrate, pH 3.02 (65 - 93 min); (4) Buffer IV: 1 M lithium citrate, pH 3.45 (93 - 141 min); (5) Buffer V: 1.65 M lithium citrate, pH 3.55 (141 - 204 min). Post-column modification of the sample by ninhydrin was utilized in the Analyzer. Absorbance of the reacted sample was monitored at 440 and 570 nm. The retention profile of the sample containing the inhibitor was compared with that of standard argininosuccinic acid. This part of the work was done at the Department of Clinical Chemistry by the courtesy of Dr. L. Seargeant.

6. Perfusion of Hamster Liver

Hamster was anaesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg) with 700 U of heparin. An inflow cannula was placed in the portal vein and an outflow cannula was placed in the thoracic segment of the inferior vena cava (IVC). The hepatic artery and the IVC above the renal vein were ligated. The liver was perfused (non-recirculating) with Krebs-Henseleit buffer saturated with 95% O_2/5% CO_2 at a flow rate of 2 ml per min. The Krebs-Henseleit buffer (1000 ml) was prepared freshly prior to perfusion, which contained 100 ml of solution A (containing 70.1 g/l sodium chloride, 21.0 g/l sodium bicarbonate and 9.91 g/l dextrose), 10 ml of solution B (containing 3.55 g/100 ml potassium chloride, 2.94 g/100 ml magnesium sulfate and 1.63 g/100 ml sodium phosphate, monobasic), 5 ml of solution C (containing 3.73 g/100 ml calcium chloride, dihydrate) and 885 ml distilled water. After an initial period of stabilization (the liver was perfused with Krebs-Henseleit buffer for 10 min), the liver was then perfused with 80 ml of Krebs-Henseleit buffer containing 50 μM [Me-^{3}H]choline (1 μCi/ml). All perfusions were carried out at 37 °C. Subsequent to perfusion, the liver was cut into small pieces and homogenized in chloroform:methanol (1:1, v/v) to yield 15% homogenate. The homogenate was centrifuged at 1,000 g for 10 min and the resulting pellet was washed twice with chloroform:methanol (1:1, v/v). The supernatants were pooled and an aliquot was taken for the determination of total uptake of radioactivity.

7. Analysis of Phospholipids

Phase separation of the pooled tissue extract was achieved by adding chloroform and water until a chloroform:methanol:water ratio of 4:2:3 (v/v) was obtained. The solvent in the upper phase was removed by evaporation *in vacuo*. The content was dissolved in 1 ml water and stored at -20 °C. The solvent in the lower phase (organic) was removed by evaporation *in vacuo*. The content was resuspended in 3 ml of chloroform:methanol (2:1, v/v) and the solution was filtered through Whatman 1PS filter paper and placed into a test tube. The solvent was then removed by evaporation under nitrogen. The content was resuspended in 1 ml chloroform and stored at -20 °C. Phosphatidylcholine and lysophosphatidylcholine in the organic phase were analyzed by thin-layer chromatographic plate (Redi-Plate, Sil Gel G) with standard phospholipids as carriers. The plate was developed in the solvent containing chloroform:methanol:acetic acid:water (70:30:2:4, v/v). Phosphatidylcholine and lysophosphatidylcholine on the plate were visualized by exposure of the plate to iodine vapor in a closed chamber. The silica gel fractions containing phosphatidylcholine or lysophosphatidylcholine was scraped into a scintillation counting vial and 1 ml of water was added. Acetic acid (0.1 ml) was added to prevent chemiluminescence. The radioactivity was determined by liquid scintillation counting.

8. Analysis of Choline-containing Metabolites

Choline-containing metabolites in aqueous phase were analyzed by thin-layer chromatography (Sil-G25 plate) with a solvent containing chloroform:0.6% sodium chloride:ammonium hydroxide (50:50:5, v/v). The positions of choline, phosphorylcholine and CDP-choline on the plate were localized with Bioscan System 200 Imaging Scanner. Silica gel in the choline and phosphocholine fractions was scraped into scintillation counting vials and the radioactivity associated with each metabolite was then determined by liquid scintillation counting.

It has been demonstrated that substantial amount of choline taken up by the cells is oxidized into betaine in the liver (Pelech and Vance 1984; O *et al.* 1988). CDP-choline and betaine have similar Rf values after thin-layer chromatography with the above solvent. CDP-choline can be separated from betaine with Norit A charcoal chromatography. In this study, CDP-choline and betaine were extracted from the silica gel twice with 2 ml of 2% ethanol after thin-layer chromatography. The extracts were pooled and loaded onto a Norit A charcoal column (0.5 x 3 cm, with a ratio of charcoal to celite 1:2) equilibrated with 2% ethanol. Betaine was eluted from the column with 20 ml of 2% ethanol, whereas CDP-choline was subsequently eluted with 10 ml of 40% ethanol containing 1% ammonium hydroxide. The solvent in the CDP-choline fraction was evaporated by air, and the radioactivity associated with CDP-choline was determined by liquid scintillation counting.

II. Studies on Phosphatidylcholine Catabolism in Rat Heart

1. Perfusion of Rat Heart

The isolated rat heart was perfused in the Langendorff mode with Krebs-Henseleit buffer (pH 7.4) saturated with 95% O_2/5% CO_2 at a flow rate of 4 ml per min. After an initial period of stabilization (the heart was perfused with Krebs-Henseleit buffer for 10 min), the heart were then perfused with the same buffer containing 0.5 - 2.0% ethanol in a recirculated manner for 4 h. In another set of experiment, the hearts were perfused with 1% ethanol and 25 - 100 μM vitamin E in Krebs-Henseleit buffer under the same conditions. After perfusion, one half of the tissue was used for the determination of phospholipids, while the other half was used for the preparation of subcellular fraction (Choy *et al.* 1989).

2. Preparation of Subcellular Fractions

One half of the rat heart (0.5 - 0.6 g) was cut into small pieces and homogenized in 5 ml buffer containing 20 mM Tris-HCl (pH 7.4), 0.25 M sucrose and 1 mM EDTA. The homogenate was centrifuged at 600 g for 10 min to pellet nuclei, cell debris and unbroken cells. The resulting supernatant was centrifuged at 10,000 g for 10 min. The precipitate represented the mitochondrial fraction, whereas the post mitochondrial

supernatant was used for the preparation of microsomal and cytosolic fractions as by the method described in the previous section.

3. Separation and Determination of Phospholipids

Another half of the rat heart (0.5 - 0.6 g) was homogenized in 6 ml chloroform:methanol (1:1, v/v) and the homogenate was centrifuged at 1,000 g for 10 min. The precipitate was re-extracted once with the same solvent and the supernatants were pooled. Chloroform and water were added to the supernatant to cause phase separation. The upper phase was re-extracted twice with chloroform and the extracts were pooled with the lower phase. The solvent in the pooled lower phase was removed by evaporation *in vacuo*, and the phospholipid classes were separated by thin-layer chromatography (Redi-Plate, Sil Gel G) with a solvent containing chloroform:methanol:acetic acid:water (70:30:2:4, v/v). The lipid phosphorus content of each phospholipid class was determined by the procedure of Bartlett (1958).

4. Preparation of Labelled Phosphatidylcholine

Phosphatidyl-[Me-^{3}H]choline was prepared by perfusion of the isolated rat heart in the Langendorff mode with labelled choline as described previously (Tam *et al.* 1984). After an initial period of stabilization (the heart was perfused with Krebs-

Henseleit buffer for 10 min), the isolated rat heart was perfused Krebs-Henseleit buffer containing 5 μM of [Me-^{3}H]choline (6 μCi/ml) for 60 min in a recirculating manner. The flow rate was maintained at 4 ml per min. Subsequent to perfusion, the heart was cut into small pieces and homogenized in chloroform:methanol (1:1, v/v). The homogenate was centrifuged at 1,000 g for 10 min and the resulting pellet was washed twice with the same solvent. Chloroform and water were added to the pooled supernatant to a final ratio of chloroform:methanol:water 4:2:3 (v/v). After phase separation, the solvent in the lower (organic) phase was removed by evaporation and the lipid content was resuspended in chloroform:methanol (2:1). Phosphatidylcholine was separated from the other lipids by thin-layer chromatography (Redi-Plate, Sil Gel G) with a solvent containing chloroform:methanol:acetic acid:water (70:30:2:4, v/v). The labelled phosphatidyl[Me-^{3}H]choline was eluted from the silica gel and the specific radioactivity was 8,000 - 10,000 dpm/nmol.

5. Phospholipase A Assay

Phospholipase A activity in the microsomes, mitochondria and cytosol was assayed with phosphatidyl[Me-^{3}H]choline as a substrate (Cao *et al.* 1987b). The reaction mixture (0.5 ml) contained 20 mM Tris-HCl (pH 8.5), 5 mM calcium chloride and 1.0 μmol phosphatidyl[Me-^{3}H]choline dispersed in 0.2 ml water by sonication. When the assay was performed with the cytosolic or microsomal fractions, the presence of

lysophospholipase activity in these fractions might affect the assay. Hence, 200 nmol of unlabelled lysophosphatidylcholine was included in the reaction mixture. As demonstrated previously (Tam *et al.* 1984), the presence of lysophosphatidylcholine effectively inhibited the further hydrolysis of lysophosphatidyl[Me-^{3}H]choline formed, but did not significantly inhibited the hydrolysis of phosphatidylcholine. The assay was initiated by the addition of the enzyme preparation containing 0.2 - 0.5 mg protein, and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 1.5 ml chloroform:methanol (2:1, v/v). Water was added to the mixture to cause phase separation. The solvent in the lower phase was removed by evaporation under nitrogen. The labelled lysophosphatidylcholine (product) was separated from the labelled phosphatidylcholine (substrate) by thin-layer chromatography (Redi-Plate, Sil Gel G) with a solvent containing chloroform:methanol:acetic acid:water (70:30:2:4, v/v).

In order to discriminate the activities of phospholipase A_1 from A_2, 1-palmitoyl-2-[^{14}C]arachidonoyl glycerophosphocholine was used as a substrate (Tam *et al.* 1984). The condition of the assay was identical to that described in the preceding section. Subsequent to the reaction, fatty acid, lysophosphatidylcholine and glycerophosphocholine were separated by thin-layer chromatography with a solvent system containing chloroform:methanol:acetic acid:water (70:30:2:4, v/v). Phospholipase A_1 activity was calculated from the ^{14}C counts in the

lysophosphatidylcholine fraction, whereas phospholipase A_2 activity was estimated from the ^{14}C counts in the fatty acid fraction. The ^{3}H counts in the lysophosphatidylcholine fraction represented the sum of both phospholipase activities.

6. Lysophospholipase Assay

Lysophospholipase activity in the microsomal and cytosolic fractions was assayed with 1-[1-^{14}C]palmitoylglycerophosphocholine as substrate (Cao *et al.* 1987a). The reaction mixture (0.5 ml) contained 20 mM Tris-HCl (pH 7.0), 0.2 mM labelled lysophosphatidylcholine (1,200 dpm/nmol) and the enzyme preparation containing 0.3 - 0.5 mg protein. The reaction was initiated by the addition of labelled substrate. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 1.5 ml chloroform:methanol (2:1, v/v). Water was added to the mixture to cause phase separation. The solvent in the lower phase was removed by evaporation under nitrogen. The labelled fatty acid released by the reaction was separated from the labelled lysophosphatidylcholine (substrate) by thin-layer chromatography (Cao *et al.* 1987a).

7. Lysophosphatidylcholine:acyl-CoA Acyltransferase Assay

The enzyme activity in the microsomal fraction was assayed with labelled acyl-CoA (Arthur and Choy 1986). The assay mixture (0.7 ml) contained 50 mM Tris-HCl (pH 8.5), 0.1 μmol lysophosphatidylcholine (pig liver), 0.1 μmol of [1-^{14}C]oleoyl CoA and the enzyme preparation containing 0.3 - 0.5 mg protein. The reaction was initiated by the addition of the labelled oleoyl CoA and was incubated at 25 °C for 30 min. The reaction was terminated by the addition of 1.5 ml chloroform:methanol (2:1, v/v), and water was added to cause phase separation. The labelled phosphatidylcholine in the lower phase was separated from other labelled materials by thin-layer chromatography and the enzyme activity was calculated from the radioactivity associated with labelled phosphatidylcholine.

III. Other Procedures

1. Protein Assay

Protein concentrations of subcellular fractions were determined by the modified method of Lowry *et al* (1951). Bovine serum albumin (5-160 μg) was used as standard. Subcellular fractions (5 - 10 μl) were incubated in 1.5 ml of 0.66 N NaOH at 37 °C for 1 h. Subsequently, 1.5 ml of Reagent A (containing 1 ml of 2% $CuSO_4$, 1 ml of 4% sodium potassium tartrate and 33 ml of 13% Na_2CO_3) was added to each

tube and mixed thoroughly. After 10 min, 0.5 ml of 2 N phenol reagent was added. The reaction mixture was incubated at 25 °C for 30 min. Absorbance was measured at 625 nm with a Bausch & Lomb Spectronic 2000 spectrophotometer.

2. Determination of Lipid Phosphorus by Acid Digestion

The lipid phosphorus content in the sample was determined by the method of Bartlett (1959). An aliquot of the sample was taken and the solvent was evaporated under nitrogen. Inorganic phosphorus was used as standard (0-20 μg/tube). Perchloric acid (1.1 ml) was added to each sample and the mixture was incubated at 160 °C for 2 h. The mixture was then allowed to cool to the room temperature. Subsequently, 8 ml water and 0.8 ml of 5% ammonium molybdate were added and mixed thoroughly and then 0.2 ml of ANSA reagent was added. These tubes were placed in a boiling water bath for 10 min. After cooling to the room temperature, the absorbance was measured at 830 nm. The ANSA reagent was freshly prepared by dissolved 0.025 g of 1-amino, 2-naphthol, 4-sulphonic acid, 1.462 g of sodium bisulfite and 0.05 g of sodium sulfite in 10 ml of warm distilled water with constant stirring.

3. Radioactivity Determination

Radioactivity was determined by a LKB liquid scintillation counter (1211 MiniBeta) and the counting efficiency was calculated by channels' ratio calibration method.

4. Statistical analysis

The student's t-test was used for statistical analysis. The level of significance was at $P < 0.05$.

EXPERIMENTAL RESULTS

A. REGULATION OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS

- Cholinephosphostransferase

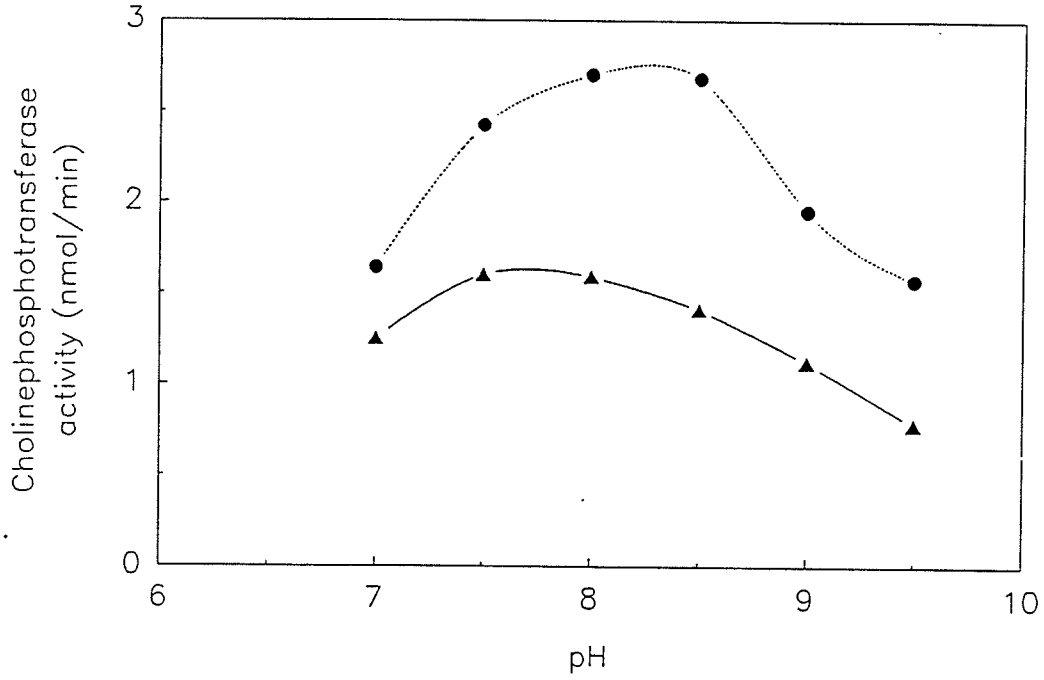
I. Solubilization and Purification of Cholinephosphotransferase

The majority of phosphatidylcholine in mammalian tissues is synthesized *de novo* via the CDP-choline pathway (Vance 1985). The final step in the CDP-choline pathway is the conversion of CDP-choline to phosphatidylcholine, catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (Vance 1985). In the present study, we solubilized and partially purified cholinephosphotransferase from hamster liver microsomes. The enzyme in the hamster heart was also characterized and compared with the liver enzyme.

1. Cholinephsophotransferase in Hamster Liver and Heart

The characteristics of cholinephosphotransferases in the microsomes of hamster liver and heart were compared. The enzymes from both sources displayed broad pH profiles and the optimal pH was between the range of 7.5 - 8.0 (Fig. 12). Both enzymes had absolute requirement for magnesium or manganese. Maximum enzyme

FIG. 12



The pH profiles of cholinephosphotransferase

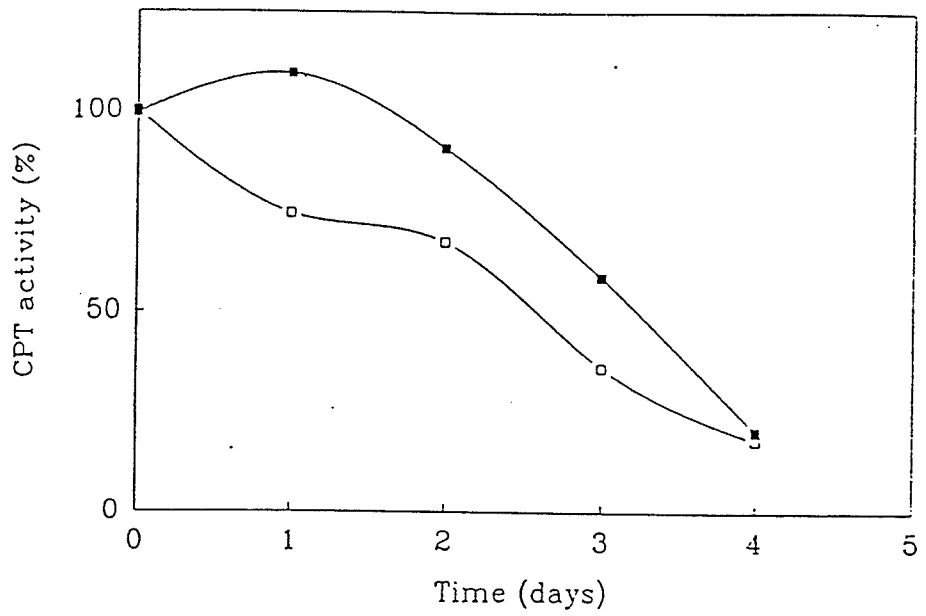
The pH profiles of cholinephosphotransferase were determined in the microsomal (▲) and partially purified fractions (●). Each point represents the mean of three separate determinations.

activity was obtained in the presence of 30 mM magnesium. The stability of the liver cholinephosphotransferase was quite different from the heart enzyme. Upon storage at 4 °C for 24 h, the activity of the liver enzyme was slightly elevated, whereas a 26% loss of activity was observed in the heart enzyme (Fig. 13). Kinetic studies were carried out with the cholinephosphotransferase prepared from the liver and heart. The enzyme activities were determined in the presence of different concentrations of CDP-[Me-^{3}H]choline or diacylglycerol. As shown in Table 1, the apparent K_m values for CDP-choline were similar between the liver and heart enzymes, whereas the apparent K_m values for diacylglycerol were different between two enzymes.

2. Solubilization of Cholinephosphotransferase

Since cholinephosphotransferase is tightly bound to the microsomal membrane, initial attempts were made to solubilize the enzyme in both liver and heart microsomes with detergents. A number of neutral and ionic detergents were employed for this study, including octyl glucoside, CHAPS, CHAPSO, taurocholate, Triton X-100 and Tween 20. These detergents were found to be ineffective for the solubilization of the enzymes from both sources. Alternatively, cholinephosphotransferase from hamster liver microsomal fraction was solubilized by Triton QS-15 (zwitterionic detergent). In this study, the liver microsomes were suspended in 3% Triton QS-15 (with a detergent to protein ratio of 1:1.5, mg/mg), and the mixture was centrifuged at

FIG. 13



Stability of cholinephosphotransferase in hamster liver and heart microsomes

Hamster liver (■) and heart (□) microsomes (3 mg/ml) were stored at 4 °C for various time periods. Enzyme activities were determined, and were expressed as percentage of activity obtained from fresh microsomes. Each point was the mean of two separate experiments, each determined in duplicate.

TABLE 1

K_m values of CDP-choline and diacylglycerol for cholinephosphotransferase of hamster liver and heart

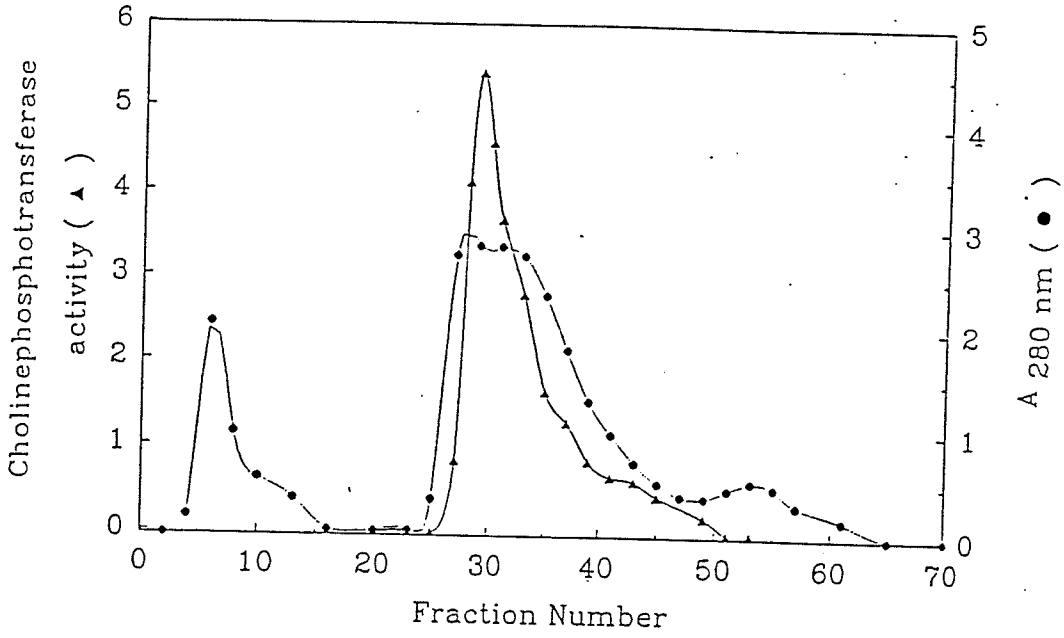
	Km	
	CDP-Choline	Diacylglycerol
	μM	μM
Liver microsomes	94	460
Heart microsomes	70	280
Partially purified enzyme from liver	85	160

100,000 g for 60 min. A substantial amount (20%) of the original activity in the microsomal fraction was found in the supernatant after centrifugation. However, Triton QS-15 was not effective in the solubilization of the enzyme from hamster heart microsomes. Only 1 - 4% of the original activity in the heart microsomal fraction was found in the supernatant after Triton QS-15 treatment followed by ultracentrifugation.

3. Partial Purification of Cholinephosphotransferase from Hamster Liver

The solubilized enzyme preparation from the hamster liver was subjected to further purification. An aliquot of the enzyme preparation (supernatant), containing 200 - 250 mg protein, was applied to a DEAE-Sepharose column (1.5 x 15 cm) equilibrated with 25 mM Tris-succinate (pH 6.0) - 2 mM ß-mercaptoethanol. The column was washed with 100 ml of the same buffer followed by 0.5 M KCl in 25 mM Tris-succinate (pH 6.0) - 2 mM ß-mercaptoethanol. The activity of cholinephospho-transferase was eluted from the column after the application of the buffer containing 0.5 M KCl (Fig. 14). The fractions containing highest cholinephosphotransferase activity (fractions 29 and 30) were pooled and applied to a Sepharose 6B column (2.5 x 40 cm) equilibrated with 25 mM Tris-succinate (pH 6.0) - 0.5 M KCl - 5% glycerol. The enzyme activity was eluted from the column near the void volume (Fig. 15). Further purification of cholinephosphotransferase by other chromatographic

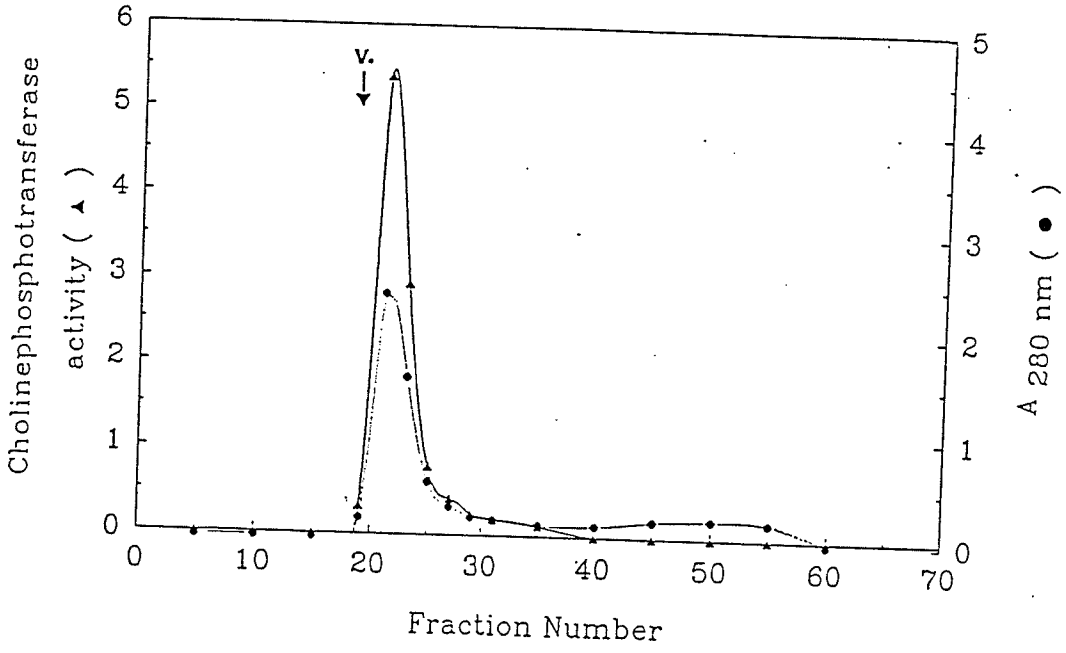
FIG. 14



Elution profile of cholinephosphotransferase from hamster liver microsomes by DEAE-Sepharose chromatography

Solubilized hamster liver microsomes were applied to a DEAE-Sepharose column (1.5 x 15 cm) equilibrated with 25 mM Tris-succinate (pH 6.0) - 2 mM ß-mercaptoethanol. The column was washed with 100 ml of the same buffer and subsequently washed with 0.5 M KCl in the same buffer. Fractions (5 ml) were collected and the enzyme activity was expressed as nmol of product formed per min.

FIG. 15



Elution profile of cholinephosphotransferase from hamster liver microsomes by Sepharose 6B chromatography

The active fraction obtained from DEAE-Sepharose chromatography were pooled (25.20 mg protein) and applied to a Sepharose 6B column (2.5 x 40 cm) equilibrated with 25 mM Tris-succinate (pH 6.0) - 0.5 M KCL - 5% glycerol. Fractions (3.5 ml) were collected and the enzyme activity was expressed as nmol of product formed per min.

techniques was not successful. A summary of the purification is depicted in Table 2.

4. Characterization of Partially Purified Cholinephosphotransferase

The activity of partially purified cholinephosphotransferase displayed an absolute requirement for magnesium or other divalent cations, but not phospholipids. The optimal pH for enzyme activity was 7.5 - 8.0. Kinetic studies revealed that the K_m value of CDP-choline was similar to that of liver microsomal enzyme (Table 1), but the K_m value for diacylglycerol was substantially lowered when the enzyme was partially purified.

TABLE 2

Purification of cholinephosphotransferase from hamster liver

	Total activity nmol/min	Protein mg	Specific activity nmol/ min/mg	fold
Microsomes	299.00	598.00	0.50	—
Solubilized microsomes	59.50	238.00	0.25	0.50
DEAE-Sepharose[a] chromatography	47.88	25.20	1.90	3.80
Sepharose 6B[b] chromatography	16.78	4.56	3.68	7.36

[a]After DEAE-Sepharose chromatography, only fractions 29 and 30 (containing 25.20 mg protein) were pooled and the total enzyme activity was calculated from this pooled sample.

[b]After Sepharose 6B chromatography, the total enzyme activity was calculated from fractions 21 and 22 (containing 4.56 mg protein).

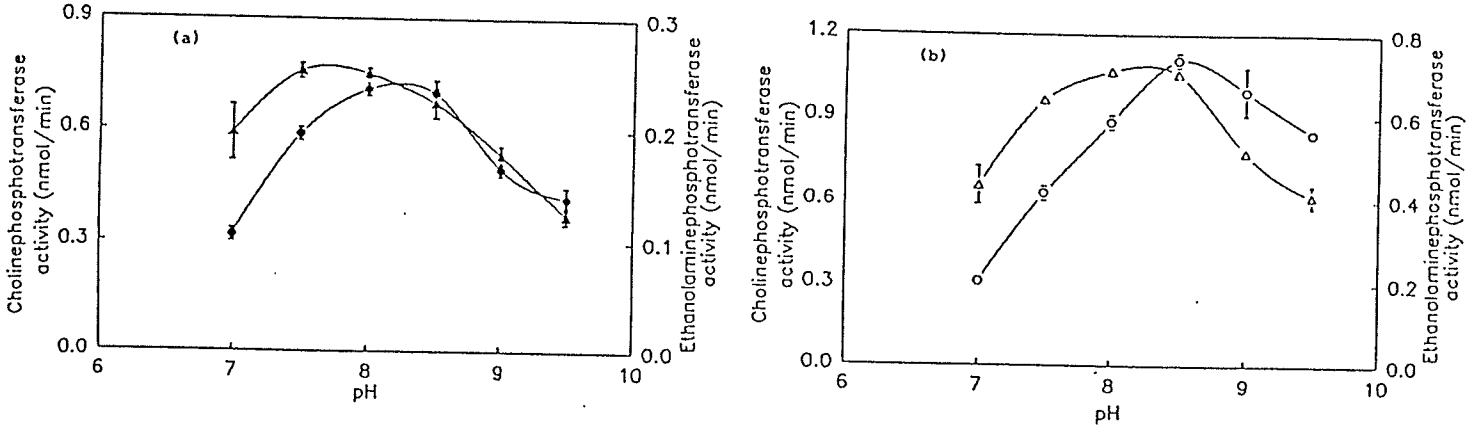
II. Differentiation of Cholinephosphotransferase and Ethanolamine-phosphotransferase

1. Cholinephosphotransferase and Ethanolaminephosphotransferase in Hamster Liver Microsomes

Separate genes which encode for cholinephosphotransferase and ethanolamine-phosphotransferase, respectively, have been identified in yeast (Hjelmstad and Bell 1988, 1990). At present, there is no direct evidence to demonstrate the existence of two distinct enzymes in mammalian tissues. In this study, both phosphotransferases were solubilized from hamster liver microsomes and separated from each other by ion-exchange chromatography.

The effects of pH, divalent cations, lipids and heat treatment on microsomal cholinephosphotransferase and ethanolaminephosphotransferase were examined. Although both enzyme activities exhibited broad pH profiles, the optimal pH for cholinephosphotransferase was not the same as ethanolaminephosphotransferase (Fig. 16a). The optimal pH range was between 7.5 - 8.0 for cholinephospho-transferase and 8.0 - 8.5 for ethanolaminephosphotransferase. Both enzyme activities displayed an absolute requirement for Mg^{2+} or Mn^{2+}, but in the presence of Mg^{2+} the enhancement of cholinephosphotransferase activity was substantially higher than

FIG. 16



The pH profiles of cholinephosphotransferase and ethanolaminephosphotransferase.

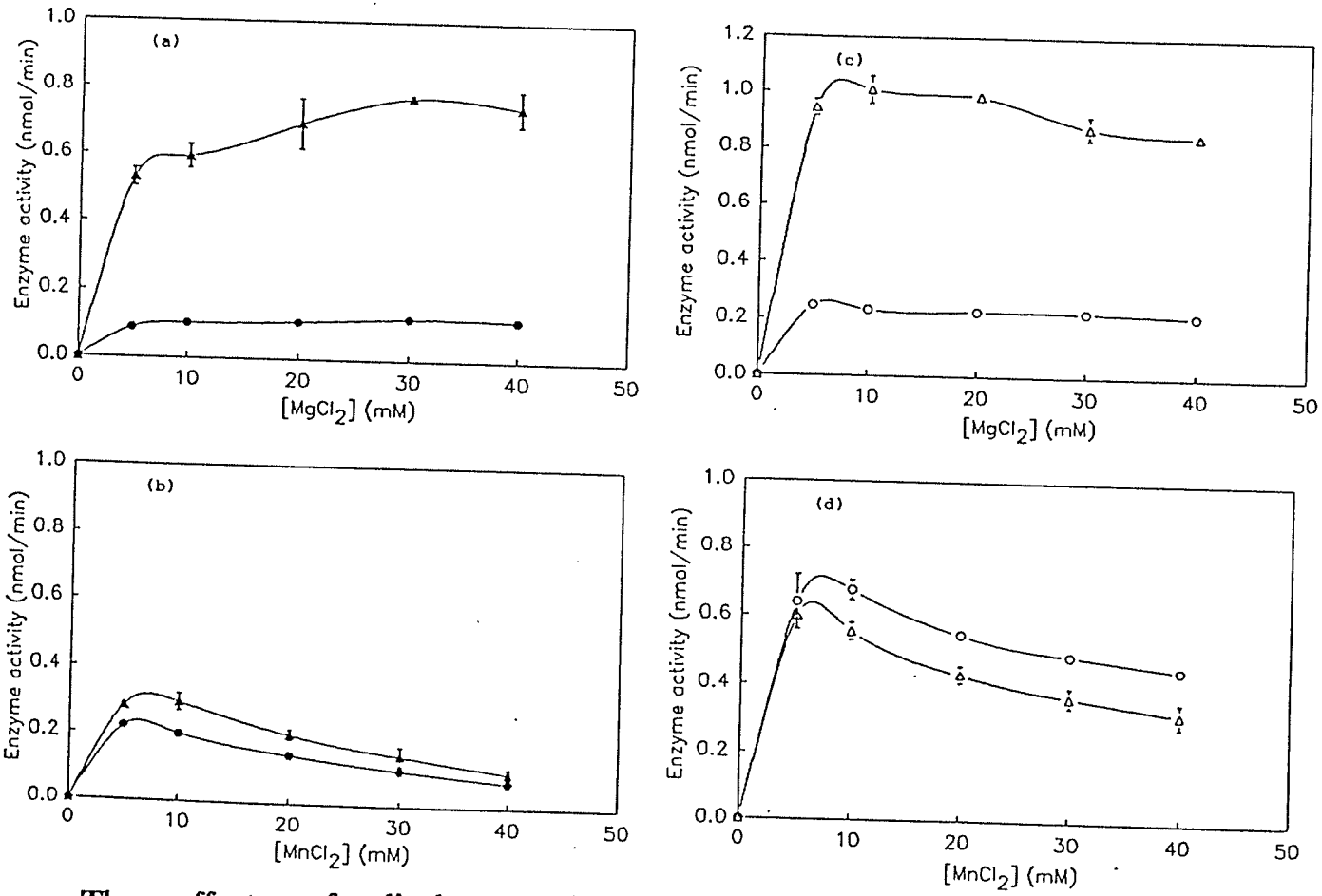
The pH profiles of microsomal cholinephosphotransferase (▲) and ethanolaminephosphotransferase (●) were determined and are depicted in *a*. The pH profiles of the partially purified choline-phosphotransferase (△) and ethanolaminephosphotransferase (○) were also determined and are depicted in *b*. Tris-HCl buffer was used for all the assays. Each point is the mean of three separate experiments. Standard deviations greater than 5% of the mean value is shown by vertical bars.

that obtained from ethanolaminephosphotransferase (Fig. 17a). The activation of ethanolaminephosphotransferase by Mn^{2+} was more prominent compared with that by Mg^{2+} (Fig. 17b).

The effect of phospholipids on both phosphotransferase activities were investigated. Hamster liver microsomes were preincubated with different phospholipids (0.2 mg), including phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine and lysophosphatidylethanolamine for 30 min. Subsequently, the activities of cholinephosphotransferase and ethanolaminephosphotransferase were determined as described in the Materials and Methods section. As shown in Table 3, addition of exogenous phospholipids to the microsomal preparation had similar effects on both enzyme activities. In the presence of exogenous phospholipids, the changes in both enzyme activities were less than 25%.

The effect of high temperature on the activities of microsomal cholinephospho-transferase and ethanolaminephosphotrnasferase were investigated. Microsomes were preincubated at 55 °C for different time periods and subsequently, the activities of both phosphotransferases were determined. As shown in Fig. 18a, two enzyme activities were inactivated to the same extents by this treatment. After 1 min of the incubation at 55 °C, more than 90% of both enzyme activities were lost.

FIG. 17



The effects of divalent cations on cholinephosphotransferase and ethanolaminephosphotransferase activities

The effects of Mg^{2+} and Mn^{2+} on microsomal cholinephospho-transferase (▲) and ethanolaminephosphotransferase (●) activities are depicted in *a* and *b*. The effects of Mg^{2+} and Mn^{2+} on the partially purified cholinephosphotransferase (△) and ethanolaminephospho-transferase (○) activities are depicted in *c* and *d*. Each point is the mean of three separate experiments. Standard deviations greater than 5% of the mean value are shown by vertical bars.

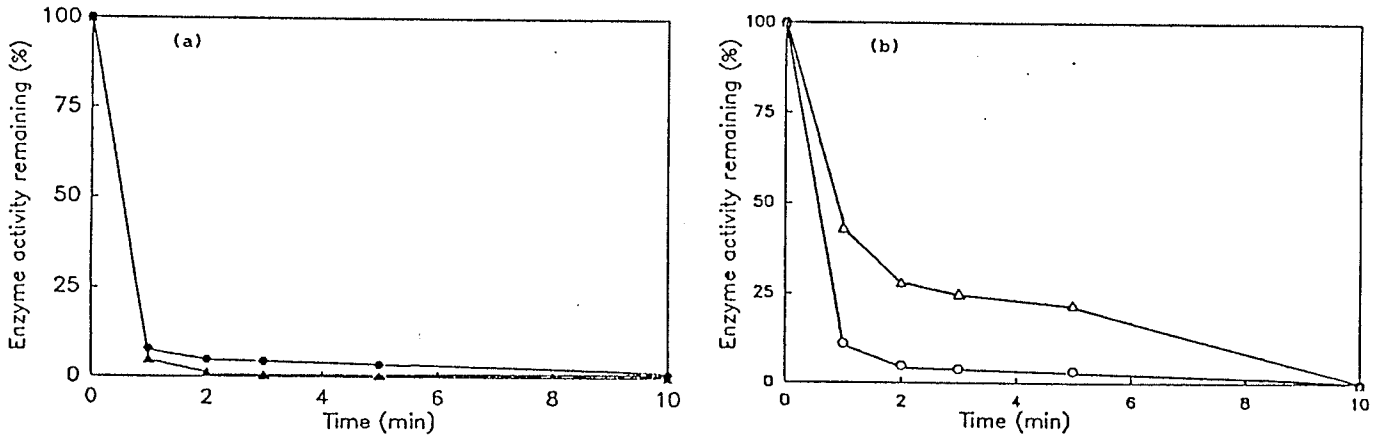
TABLE 3

Effect of phospholipids on microsomal cholinephosphotransferase and ethanolaminephosphotransferase

Lipid added	CPT activity (%)	EPT activity (%)
None	100	100
Phosphatidylcholine	108	109
Phosphatidylethanolamine	124	123
Lysophosphatidylcholine	79	92
Lysophosphatidylethanolamine	92	91

NOTE: Hamster liver microsomes (2 mg) were incubated with 0.2 mg phospholipid (in 0.05% Tween 20) at 4°C for 30 min. Subsequently, CPT and EPT activities were determined as described in Methods. The activities of CPT and EPT without the addition of phospholipid were 0.58 and 0.22 nmol/(min·mg protein), respectively, and are expressed as 100%. The results are the average of three separate experiments.

FIG. 18



The effect of heat treatment on cholinephosphotransferase and ethanolamine-phosphotransferase activities

The enzyme preparations were incubated at 55 °C for the indicated periods. Subsequently, the activities of microsomal cholinephospho-transferase (▲) and ethanolaminephosphotransferase (●) were determined and the results are depicted in *a*. the activities of partially purified cholinephosphotransferase (△) and ethanolaminephospho-transferase (○) were determined after incubation at 55 °C and the results are depicted in *b*. Each point is the mean of two separate sets of experiments, each determined in duplicate.

2. The Effect of Detergents on Cholinephosphotransferase and Ethanolaminephosphotransferase Activities in Microsomes

Detergents have been shown to affect cholinephosphotransferase and ethanolaminephosphotransferase activities in microsomal fractions (Kanoh and Ohno 1976; Coleman and Bell 1977; Arthur *et al.* 1984; Cornell and MacLennan 1985; Vechini *et al.* 1987). However, there was no comprehensive study on the differential effect of various detergents on these two enzyme activities. In this study, we tested the effects of cationic, anionic, and neutral detergents on cholinephosphotransferase and ethanolaminephosphotransferase activities in the hamster liver microsomes. As shown in Table 4, cholinephosphotransferase activity was inhibited by the presence of detergents, whereas ethanolaminephosphotransferase was activated by the addition of CHAPS, octyl glucoside, and taurocholate. The addition of 10 mM Triton X-100 to the reaction mixture completely inhibited cholinephosphotransferase activity, but did not affect ethanolaminephosphotransferase activity. To further elucidate the differential effect of detergents on both phosphotransferase activities, different amounts of detergents were added to the assay mixture. As depicted in Fig. 19, taurocholate and octyl glucoside inhibited cholinephosphotransferase activity in a dose-dependent manner. However, a biphasic response was obtained from ethanolaminephosphotransferase. Ethanolaminephosphotransferase activity was markedly enhanced at lower detergent levels, but the enzyme activity was inhibited

TABLE 4

Effect of detergents on cholinephosphotransferase and ethanolaminephosphotransferase activities of hamster liver microsomes

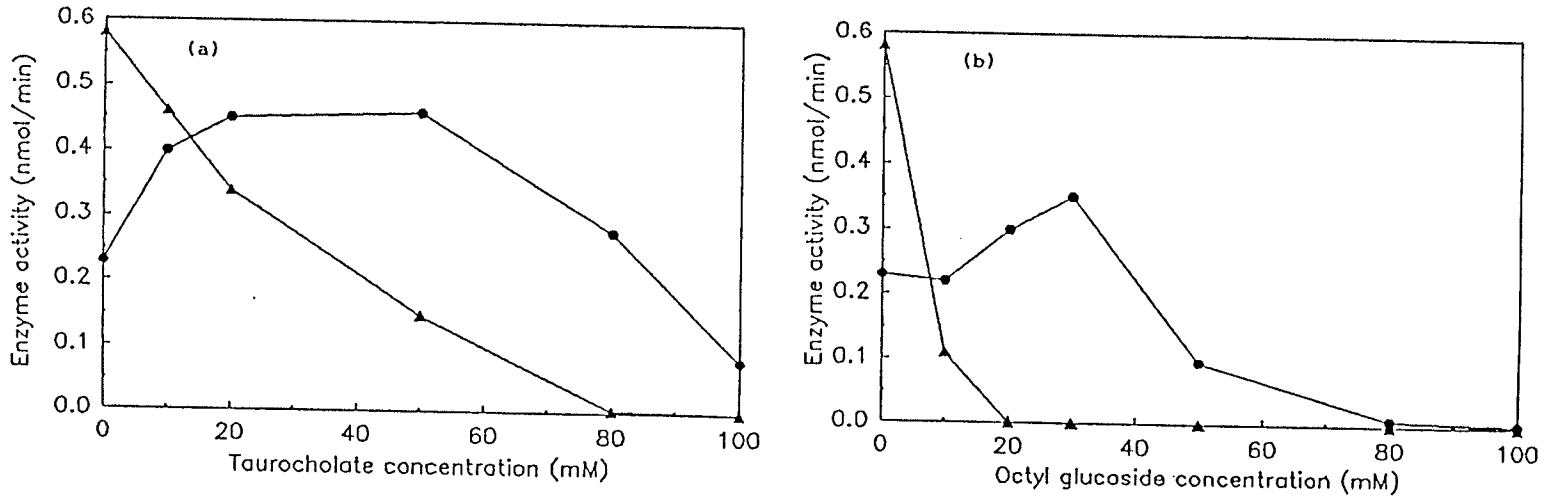
Detergent added	Detergent concn. (mM)	Enzyme activity (%)	
		CPT[a]	EPT[b]
None	—	100	100
CHAPS	1	97	113
	20	3	117
CHAPSO	1	90	90
	20	1	87
Octyl glucoside	10	19	96
	20	0	130
Taurocholate	10	79	174
	20	59	196
Triton X-200	10	0	100
	20	0	90
Tween 20	5	38	61
	20	15	96

NOTE: Detergent was added to the microsomal preparation (2 mg) and the mixture was incubated at 4°C for 15 min prior to the assay. The assay was initiated by the addition of labelled substrate. Detergent concentration was calculated from the total assay mixture. The activities of CPT and EPT were determined as described in Methods. The CPT and EPT activities without detergent were 0.58 and 0.22 nmol/(min·mg protein), respectively, and are expressed as 100%. The results are the mean of two separate experiments, each determined in duplicate.

[a]CPT, cholinephosphotransferase.

[b]EPT, ethanolaminephosphotransferase.

FIG. 19



The effect of detergent concentrations on microsomal cholinephosphotransferase and ethanolaminephosphotransferase activities

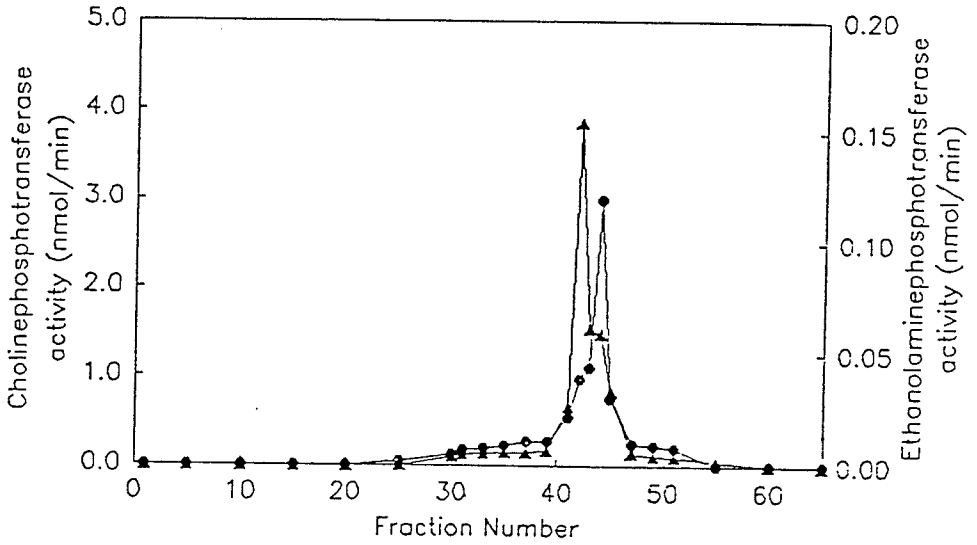
Different amounts of taurocholate (a) or octyl glucoside (b) were added to the microsomal preparation (2 mg protein) and the mixture was incubated at 4 °C for 15 min. Subsequently, cholinephospho-transferase (▲) and ethanolaminephosphotransferase (●) activities were determined. Detergent concentration was calculated from the total assay mixture. Each point is the mean of two separated experiments, each determined in duplicate.

at higher detergent concentrations.

3. Partial Purification of Cholinephosphotransferase and Ethanolamine-phosphotransferase from Hamster Liver Microsomes

Although differential effects were obtained on the microsomal choline-phosphotransferase and ethanolaminephosphotransferase activities in the presence of detergents, attempts to solubilize both phosphotransferase activities by these detergents (listed in Table 4) were not successful. However, both phosphotransferase activities were solubilized from the microsomes with 3% Triton QS-15 (with a detergent to protein ratio of 1:1.5, mg/mg). When the solubilized sample (in 3% Triton QS-15) was applied to a DEAE-Sepharose column, cholinephosphotransferase activity was eluted as a single peak in 0.3 - 1.0 M KCl gradient (Fig. 20). Ethanolaminephosphotransferase activity was also eluted from the column as a single peak. In four separate experiments, cholinephosphotransferase activity was always eluted ahead of ethanolaminephosphotransferase activity. The active fractions (fractions 41 - 45) were pooled and the specific activities of both phosphotransferases were determined. A two-fold purification of cholinephosphotransferase and three-fold purification of ethanolaminephosphotransferase were obtained. Dialysis of the pooled fractions did not cause any significant change in the specific activities of both phosphotransferases. In spite of changing the elution conditions, complete resolution

FIG. 20



Elution profiles of cholinephosphotransferase and ethanolaminephosphotransferase by DEAE-Sepharose chromatography

The solubilized hamster liver microsomes were applied to a DEAE-Sepharose column (1.5 x 15 cm) equilibrated with 10 mM phosphate buffer (pH 6.0) - 2 mM ß-mercaptoethanol. After the application of the sample, 150 ml of 10 mM phosphate buffer (pH 6.0) - 2 mM ß-mercaptoethanol was applied to the column, followed by a linear gradient of 0.3 - 1.0 M KCl (300 ml) in the same buffer. Fractions (5 ml) were collected. Cholinephosphotransferase (▲) and ethanolamine-phosphotransferase (●) activities were determined in each fraction.

between these two enzyme activities was not achieved. Further purification of these two enzymes by other chromatographic procedures was not successful.

4. Characterization of Partially Purified Cholinephosphotransferase and Ethanolaminephosphotransferase

Both partially purified phosphotransferases exhibited broad pH profiles. The pH optimum for cholinephosphotransferase was 8.0, whereas the pH optimum for ethanolaminephosphotransferase was 8.5. In general, the partially purified enzymes favoured a more alkaline condition than the microsomal enzymes (Fig. 16b).

The effects of divalent cations on both phosphotransferase activities were investigated (Fig. 17c and 17d). Similar to the results obtained with the microsomal preparation, both enzyme activities showed an absolute requirement for Mg^{2+} or Mn^{2+} and the activation of cholinephosphotransferase by Mg^{2+} was more prominent than ethanolaminephosphotransferase. The partially purified cholinephosphotransferase required 10 mM Mg^{2+} for maximal activity. Although ethanolaminephospho-transferase was also activated by Mg^{2+} or Mn^{2+}, it was less sensitive to the changes of Mg^{2+} concentrations. Both phosphotransferases in the partially purified form depicted a higher degree of sensitivity towards Mn^{2+} than that found in the microsomes.

The effect of exogenous phospholipids on the partially purified enzymes was also examined (Table 5). Phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylethanolamine caused the activation of the cholinephosphotransferase, but lysophosphatidylcholine inhibited the enzyme activity. Alternatively, phosphatidylcholine and lysophosphatidylethanolamine produced severe inhibitions on ethanolaminephosphotransferase activity.

The stability of the partially purified cholinephosphotransferase and ethanolaminephosphotransferase at high temperature was studied. Upon incubation of the enzyme preparation at 55 °C for various time periods, the two phosphotransferases were inactivated to different extent (Fig. 18b). Cholinephosphotransferase was found to be substantially less sensitive to heat treatment. After 1 min of incubation at 55 °C, over 40% of cholinephosphotransferase activity was recovered. Under identical treatment, only 10% ethanolaminephosphotransferase activity remained.

TABLE 5

Effect of phospholipids on partially purified cholinephosphotransferase and ethanolaminephosphotransferase

Lipid added	CPT activity (%)	EPT activity (%)
None	100	100
Phosphatidylcholine	125	67
Phosphatidylethanolamine	122	102
Lysophosphatidylcholine	10	5
Lysophosphatidylethanolamine	123	46

NOTE: After DEAE-Sepharose chromatography, the fractions containing both CPT and EPT activities were pooled. The partially purified protein sample (0.2 mg) was incubated with 0.2 mg phospholipid (in 0.05% Tween 20) at 4°C for 30 min. Subsequently, both phosphotransferase activities were determined as described in Methods. The activities for CPT and EPT without the addition of phospholipid were 1.25 and 0.6 nmol/(min·mg protein), respectively, and are expressed as 100%. The results are the average of three separate experiments.

III. Study of Metabolic Regulator - Argininosuccinate

1. The Isolation and Identification of a Cholinephosphotransferase Inhibitor in Hamster Cytosol

Subcellular fractions were prepared from the hamster liver and cholinephosphotransferase activities were determined in the cytosolic, crude microsomal and washed microsomal fractions (Table 6). The majority of enzyme activity was found in the microsomal fraction and a slight increase in total enzyme activity was detected after the wash. The observed small increase in total activity in the washed microsomes probably represents a greater increase in the actual total enzyme activity since the lost of enzyme activity during the wash (5-10%) was not taken into account. The increase in total enzyme activity after the wash is indicative of the removal of an inhibitor(s). In addition, the increase in the specific activity of the enzyme suggests the selective removal of non-enzyme protein during the wash.

A small amount of cytosol is normally associated with the crude microsomal preparation (Arthur *et al.* 1986). Hence, the effect of cytosol on the cholinephosphotransferase activity was investigated. Enzyme activity in the washed microsomes was assayed in the presence of of cytosol containing 0.05-1.0 mg protein. As depicted in Fig. 21, enzyme activity was inhibited by hamster liver cytosol in a

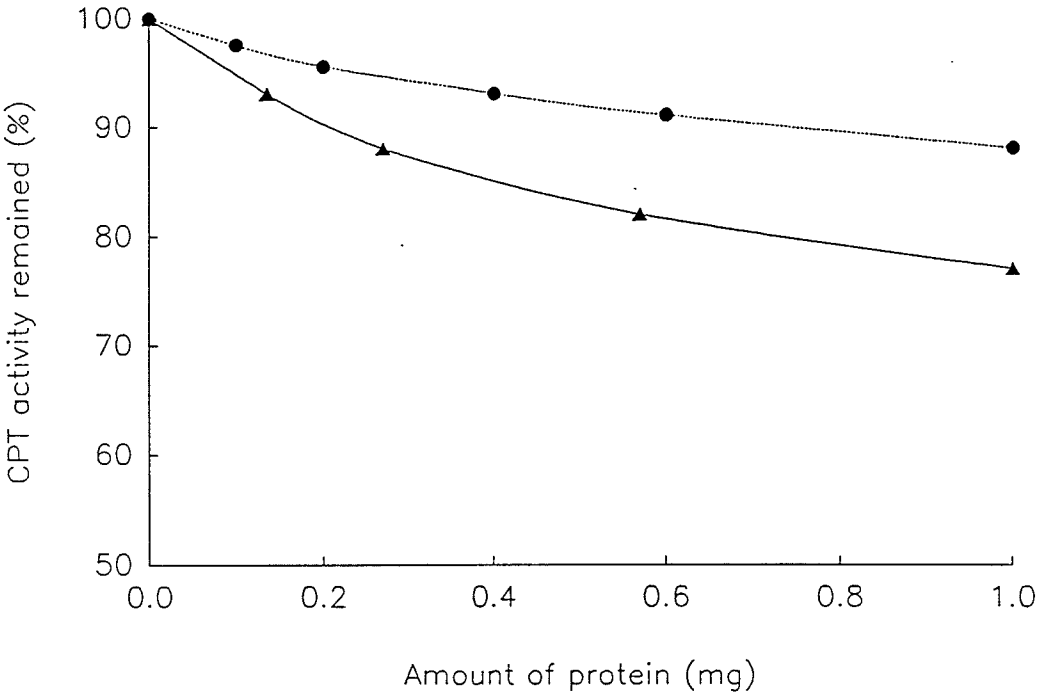
TABLE 6

Cholinephosphotransferase activity in hamster liver subcellular fractions

The microsomal pellet obtained from ultracentrifugation was suspended in 10 mM Tris-HCl (pH 7.4) and designed as the crude microsomal preparation. The crude microsomal preparation was centrifuged at 100,000 g for 60 min. The pellet was resuspended in the same buffer and designated as the washed microsomal preparation. The values shown are the mean of two determinations obtained from a typical set of experiments.

Subcellular fractions	Specific activity (nmol/min/mg protein)	Total activity (nmol/min)
Cytosol	0.06	26
Crude microsomes	5.60	1539
Washed microsomes	8.40	1546

FIG. 21



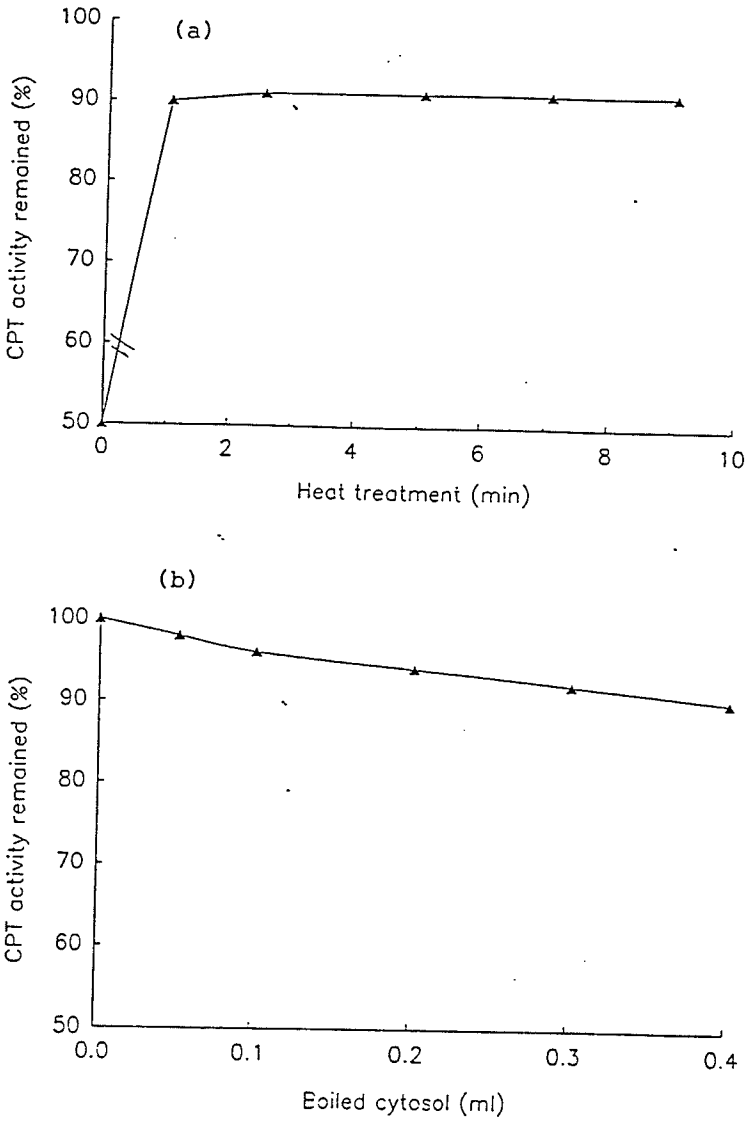
Inhibition of hamster liver microsomal cholinephosphotransferase by cytosolic fraction and albumin

Cholinephosphotransferase activity was determined in hamster liver microsomes in the presence of different amounts of cytosol (15 mg/ml protein) (▲) or bovine albumin (●). Enzyme activity in the absence of cytosol was 5.5 nmol/min/mg and expressed as 100%. Each point was the mean of three separate determinations.

progressive manner. Upon the addition of 0.07 ml of cytosol (containing 1 mg protein) to the assay mixture, a 22% inhibition of enzyme activity was observed. As a positive control, enzyme activity was assayed in the presence of albumin (Fig. 21). A 10% inhibition of enzyme activity was detected when an identical amount of albumin was present in the assay mixture. Other proteins included gamma globulin (1.0 mg) also displayed some inhibitory effect (3-5%) on the enzyme activity. It appears that enzyme activity was inhibited by high protein concentrations in a non-specific manner. However, the non-specific inhibitory effect of the proteins could not account for the inhibition of enzyme activity produced by the cytosol. In addition to the cytosolic proteins, the cytosol might contain other factors for the inhibition of enzyme activity.

In order to identify the nature of these factors, our initial approach was to remove the inhibitory effect of the non-specific proteins in the cytosol by heat treatment. These factors could then be studied in the heat-treated cytosol if they were not heat-sensitive. Hamster liver cytosol was incubated at 100°C for various time periods. The denatured proteins were removed by centrifugation and an aliquot (0.4 ml) of the supernatant was added to the cholinephosphotransferase assay mixture. Untreated cytosol (0.4 ml) was used as control (at time 0). As shown in Fig. 22a, a substantial portion of the inhibitory effect was eliminated by heat-treatment for only 1 min but further heat treatment had no effect on the remaining inhibitory activity. Analysis of

FIG. 22

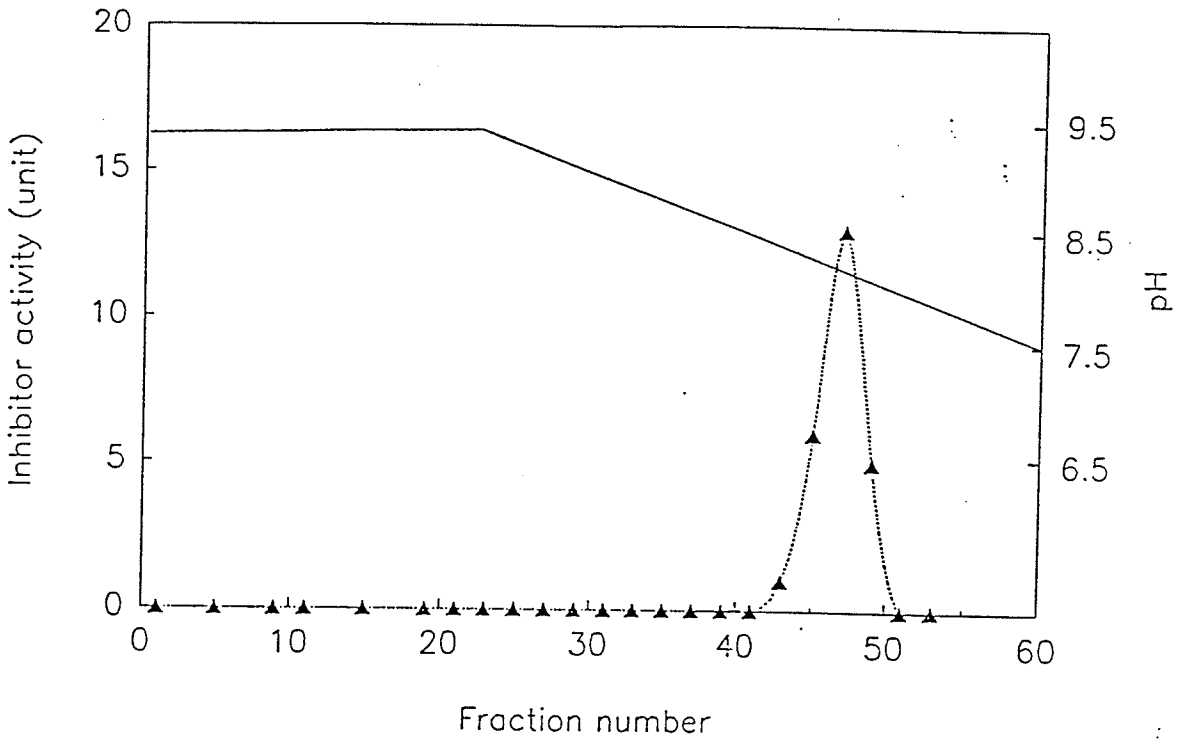


The effect of heat treatment on the inhibitory activity in hamster liver cytosol
Hamster liver cytosol was incubated at 100°C for 0-10 min followed by centrifugation at 5,000 g for 30 min. An aliquot of the supernatant (0.4 ml) from each sample was added to the cholinephosphotransferase assay and the results are depicted in 22 a. The supernatant (0-0.4 ml) obtained from the cytosol incubated for 10 min at 100°C was added to the cholinephosphotransferase assay and the results are shown in 22 b. The enzyme activity without any addition was 5.5 nmol/min/mg and expressed 100%. Each point represents the mean of three separate determinations.

the heat-treated cytosol revealed that enzyme activity was inhibited in a dose-dependent manner (Fig. 22b). It is clear that the cytosol contained two inhibitory components: a heat-sensitive component and a heat-stable component.

Our next objective was to isolate the inhibitor(s) in the heat-treated cytosol and to study its inhibitory effect on the enzyme. The cytosol (25ml) was incubated at 100°C for 10 min and centrifuged at 5,000 x g for 30 min. The supernatant was filtered through an Amicon centriflo cone with a molecular cut off at 25kDa. The filtered sample was applied to a FPLC Mono Q HR 5/5 column equilibrated with 10 mM Tris-HCl (pH 9.5). As depicted in Fig. 23, the fractions containing the inhibitory activity was eluted from the column when the pH was adjusted to 8.5. The fractions containing inhibitory activity were pooled and the volume reduced by lyophilization. The lyophilized sample was dissolved in 1 ml of water and 25 μl was applied to a cellulose thin-layer chromatogram. The plate was developed in a solvent system containing butanol:formic acid:water (75:15:10, v/v). After chromatography, the sample fractions and standards on the thin-layer plate were visualized by ninhydrin spray (Fig. 24). The sample was resolved into one major band and four minor bands. The major band had a Rf value of 0.08 which was identical to the Rf value of argininosuccinic acid. In a separate experiment, the chromatogram containing the sample was divided into 0.5 cm fractions ranging from the origin to the solvent front. The cellulose gel of each fraction was removed from the plate and the content of

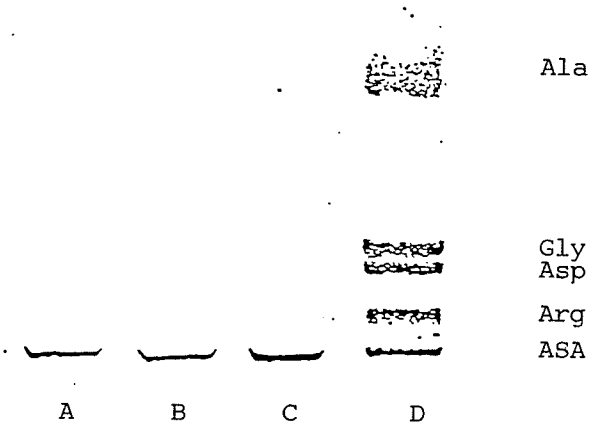
FIG. 23



Purification of cholinephosphotransferase inhibitor by Mono Q anion exchange chromatography

Heat-treated hamster cytosol containing 2.06 mg protein was applied to a Mono Q HR 5/5 column equilibrated with 10 mM Tris-HCl at pH 9.5. The column was washed with 10 ml of the equilibrating buffer followed by a linear pH gradient (pH 9.5 to pH 7.5) of 10 mM Tris-HCl (—). Fractions (1 ml) were collected and an aliquot (0.5 ml) was added in cholinephosphotransferase reaction mixture. The % inhibition is calculated from the difference in enzyme activity between the control assay and the assay in the presence of the the eluant (▲).

FIG. 24



Separation and identification of cholinephosphotransferase inhibitor by thin-layer chromatography

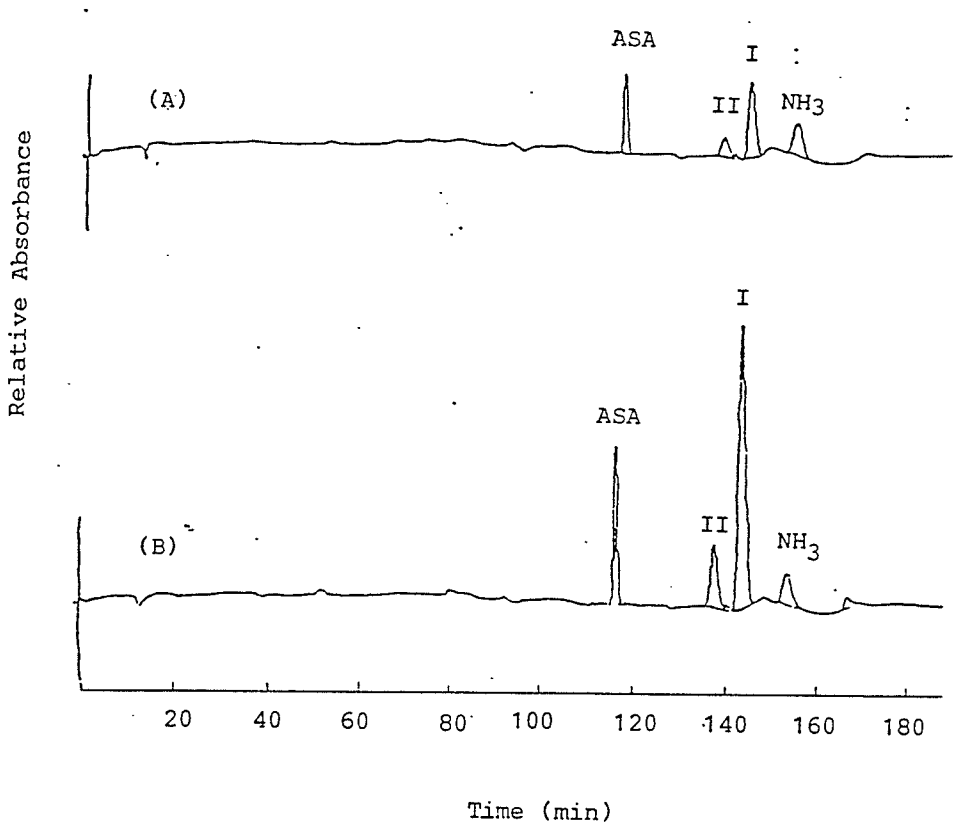
The active fractions from Mono Q column were pooled and an aliquot was applied to a cellulose thin-layer chromatographic plate. The plate was developed in the solvent containing butanol:formic acid:water (75:15:10, v/v). The locations of sample fractions and other standards were visualized with 0.2% ninhydrin spray. (A) 4 μg argininosuccinic acid ; (B) 25 μl sample; (C) 25 μl sample + 4 μg argininosuccinate acid; (D) a mixture of argininosuccinic acid, Arg, Asp, Gly, Ala (4 μg each).

each fraction was eluted by 0.5 ml water. An aliquot of the eluant (0.2 ml) from each fraction was analyzed for its ability to inhibit cholinephosphotransferase activity. Only the fractions corresponding to the major band were found to exhibit inhibitory activities.

2. The Characterization of the Cholinephosphotransferase Inhibitor

The purity and the chemical nature of the inhibitor isolated by thin-layer chromatography was evaluated by an amino acid analyzer equipped with a high resolution Ultrapac 8 resin cation exchange column. The column was calibrated with standard amino acids and argininosuccinate. Argininosuccinate has been shown to exist in free acid form and anhydride forms (Westall 1960, Portoles and Rubio 1986). The anhydrides are formed from the free form by heat treatment. The different forms of argininosuccinate cannot be separated by thin-layer chromatography but these forms are readily resolved by the cation exchange column. The sample was resolved into four separate fractions by the amino acid analyzer (Fig. 25) and the identity of the peaks were established by authentic standards: peak I (argininosuccinic acid), peak 2 (anhydride II), peak 3 (anhydride I) and peak 4 (ammonia). The analysis revealed that the sample contained only argininosuccinate and its anhydride forms.

FIG. 25



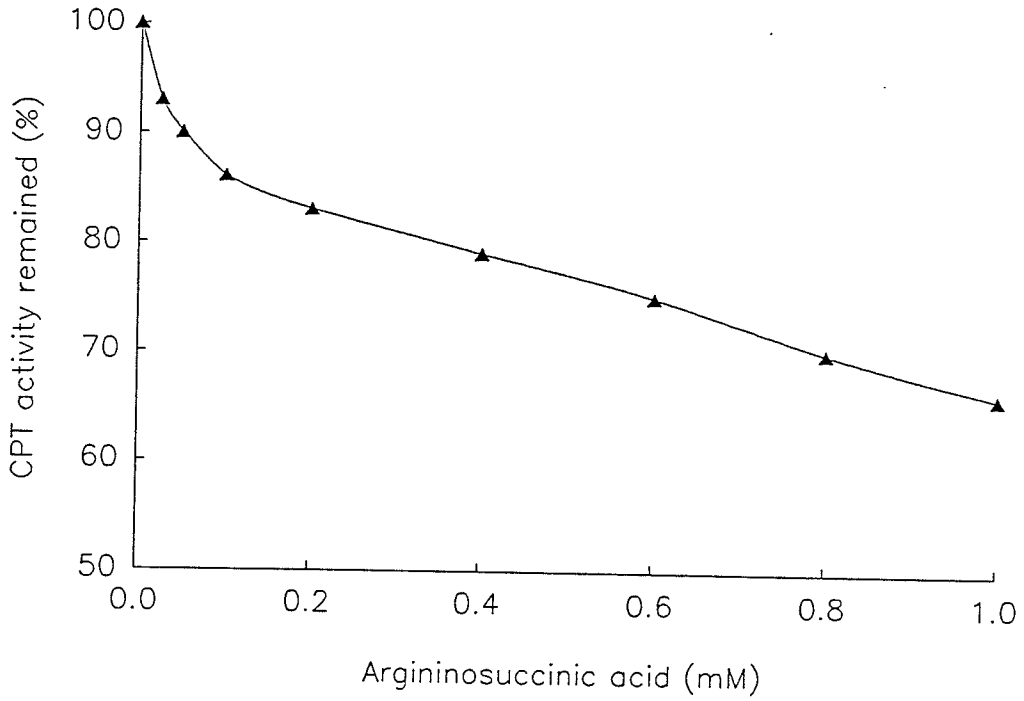
Amino acid analysis of isolated cholinephosphotransferase inhibitor from hamster liver cytosol and argininosuccinate

The sample isolated from thin-layer chromatography was analyzed by an automatic amino acid analyzer. (A) argininosuccinic acid; (B) sample isolated from hamster liver cytosol. A series of elution buffers were used in the following order: I. 0.2 M lithium citrate, pH 2.80 (0-17 min); II. 0.3 M lithium citrate, pH 3.0 (17-65 min); III. 0.6 M lithium citrate, pH 3.02 (65-93 min); IV. 1 M lithium citrate, pH 3.45 (93-141 min); V. 1.65 M lithium citrate, pH 3.55 (141-204 min).

3. The Effect of Argininosuccinate on the Activities of Phosphatidylcholine Biosynthetic Enzymes

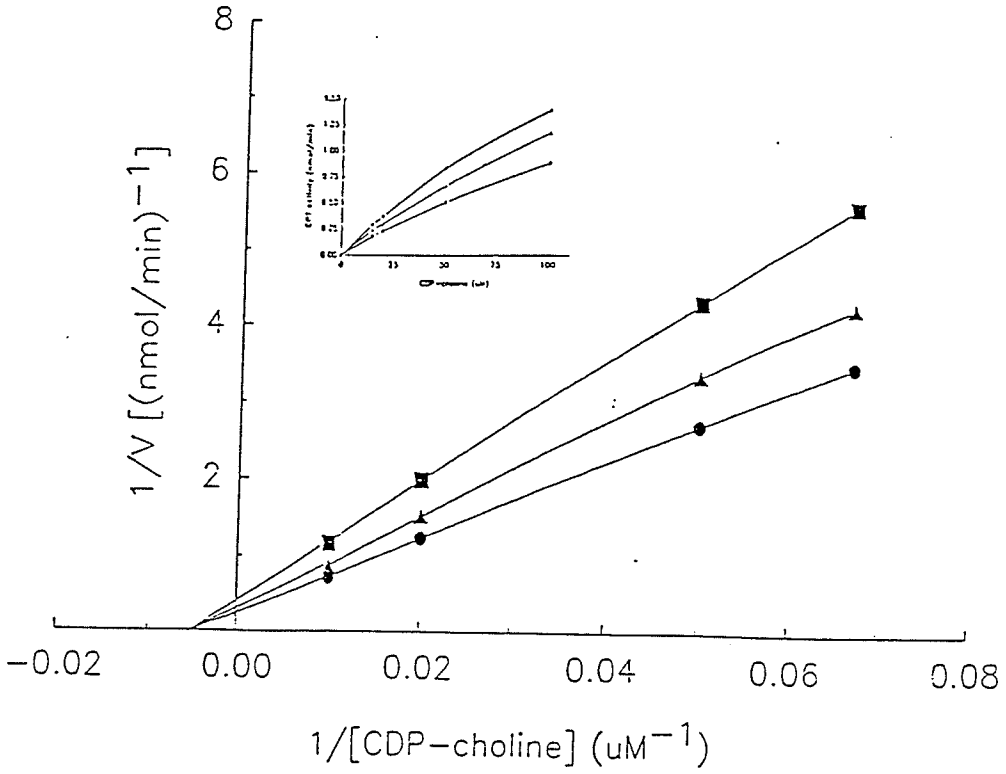
The direct effect of argininosuccinate on cholinephosphotransferase activity was investigated. Argininosuccinate (0.025-1.0 mM) was added to the enzyme assay mixture, and was found to cause inhibition of cholinephosphotransferase activity in a progressive manner (Fig. 26). The inhibition was more effective at low argininosuccinate concentrations (0.025-0.10 mM). A similar pattern of inhibition was obtained when heat-treated argininosuccinate (containing the free and anhydride forms) was used. The mechanism of inhibition was investigated by kinetic studies. Enzyme activities were assayed in the presence of argininosuccinate (0.6 and 1.5 mM) at different concentrations of CDP-choline or 1,2-diacylglycerol. As depicted in Fig. 27, the Lineweaver-Burk plot with varying concentrations of CDP-choline showed a classical non-competitive inhibition pattern in the presence of argininosuccinate. When enzyme activities were determined with different concentrations of diacylglycerol, a mixed type of inhibition was observed (Fig. 28). The effect of argininosuccinate on the activities of other enzymes in the CDP-choline pathway was also investigated (Table 7). No significant change in the activities of choline kinase or cytidylyltransferase was detected in the presence of 0.1-1.0 mM argininosuccinate.

FIG. 26



Effect of argininosuccinic acid on cholinephosphotransferase activity Cholinephosphotransferase activity in hamster liver microsomes was determined in the presence of 0-1.0 mM argininosuccinate. The enzyme activity in the absence of argininosuccinate was expressed as 100%. Each point was the mean of three separate determinations.

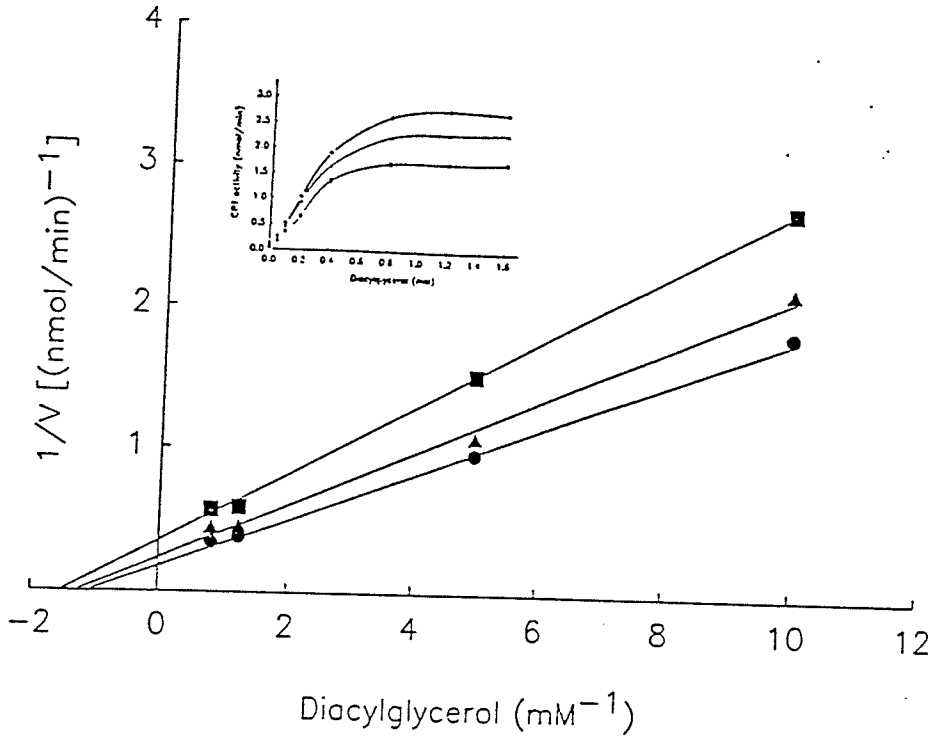
FIG. 27



Double-reciprocal plot of cholinephosphotransferase activity *vs* CDP-choline concentrations

Cholinephosphotransferase activity in hamster liver microsomes was determined in the absence (●) or presence of 0.6 mM (▲) and 1.0 mM (■) argininosuccinic acid. Enzyme activity as a function of CDP-choline concentration is depicted in the inset. Each point is the mean of three separate determinations.

FIG. 28



Double-reciprocal plot of cholinephosphotransferase activity *vs* diacylglycerol concentrations

Cholinephosphotransferase activity in hamster liver microsomes was determined in the absence (●) or presence of 0.6 mM (▲) and 1.0 mM (■) argininosuccinic acid. Enzyme activity as a function of diacylglycerol concentrations is depicted in the inset. Each point is the mean of three separate determinations.

TABLE 7

Effect of argininosuccinic acid on phosphatidylcholine biosynthetic enzymes in hamster liver

The activities of choline kinase and CTP:phosphocholine cytidylyltransferase were determined in the absence (control) or presence of argininosuccinate. The results are depicted as mean ± standard deviation from three separate experiments.

Enzymes	Specific activity (nmol/min per mg of protein)			
	Control	+argininosuccinic acid		
		0.1 mM	0.6 mM	1.0 mM
Choline kinase	0.68 ± 0.09	0.67 ± 0.02	0.67 ± 0.01	0.69 ± 0.02
Cytidylyltransferase				
(microsomes)	0.33 ± 0.05	0.33 ± 0.05	0.30 ± 0.04	0.26 ± 0.05
(cytosol)	1.14 ± 0.16	1.09 ± 0.07	1.13 ± 0.14	1.07 ± 0.01

4. The role of Argininosuccinate as a Physiological Fractor on Phosphatidylcholine Biosynthesis

The elevation of the enzyme activities of the urea cycle in the liver under fasting conditions has been well documented (Schimke 1962; Snodgrass 1981). Argininosuccinate is synthesized as a metabolite in the urea cycle (Snodgrass 1981) and the enrichment of this metabolite *in vivo* might affect phosphatidylcholine biosynthesis. In order to test this hypothesis, livers of fed (control) and 24h fasted hamsters were perfused with Krebs-Henseleit buffer containing 50 μM [Me-^{3}H]choline (1 μCi/ml) for 40 min. Subsequent to perfusion, phosphatidylcholine fractions were isolated from the livers of the control and the fasted animals and the radioactivities associated with these fractions were determined. A 25% decrease in radioactivity was detected in the phosphatidylcholine fraction from the fasted animal (Table 8). However, no significant difference in the uptake of labelled choline by the liver was observed between the two animal groups. The incorporation of [Me-^{3}H]choline into choline-containing metabolites was also determined. There was no significant difference in the labelling of phosphocholine but fasting caused a substantial increase (45%) in the labelling in CDP-choline. The decrease in the labelling of phosphatidylcholine, coupled with the increase in the labelling of CDP-choline clearly suggest that the conversion of CDP-choline to phosphatidylcholine was reduced in the liver of the fasted animal. The mechanism for such reduction was

TABLE 8

The incorporation of radioactivity into choline-containing metabolites and phosphatidylcholine in perfused hamster liver

Hamster liver was perfused with Krebs-Henseleit buffer containing 50 μM [Me-^{3}H]-choline. Subsequent to perfusion, the liver was homogenized in chloroform: methanol (1:1, v/v). The choline-containing metabolites in the aqueous phase and phosphatidylcholine in the organic phase were isolated by thin-layer chromatography. The results are depicted as mean ± standard deviation (number of experiments).

	Control	24 h Fasting
	dpm/g wet wt. (x 10^{-3})	
Phosphocholine	279 ± 121 (6)	227 ± 149 (4)
CDP-choline	3.7 ± 0.7 (6)	5.4 ± 1.5 (4)*
Phosphatidylcholine	48 ± 4 (5)	36 ± 10 (4)*

* $P < 0.05$

examined by determinating the specific activities of the enzymes of the CDP-choline pathway in the livers of the two animal groups (Table 9). No significant change in protein contents was detected in the cytosolic and microsomal fractions between the two animal groups. There was no difference in the specific activity of choline kinase was observed, but the specific activity of cytidylyltransferase in the microsomal fraction was substantially reduced in the fasted animal with a corresponding increase in the enzyme activity in the cytosolic fraction. Interestingly, there was no change in specific activity of cholinephosphotransferase between the fasted and control animals which indicates that fasting had no direct effect on the activity of the enzyme *per se*. When 0.02-0.2 ml cytosol from each animal group was added to the cholinephosphotransferase assay, the cytosol from the fasted animal caused a higher degree of inhibition (Fig. 29). Based on the results obtained from five sets of experiments, the difference in the inhibition of enzyme activity by 0.2 ml of liver cytosol between the fasted and control animals was 12±3%.

The ability of the heat-treated liver cytosol of the fasted animal to produce a higher degree of inhibition of enzyme activity was examined. Liver cytosols of two animal groups were incubated at 100°C for 10 min, and 0.05-0.20 ml of the heat-treated cytosols from each group was added to the enzyme assay (Fig. 30). The heat-treated cytosol from the fasted animal caused a higher degree of inhibition of enzyme activity than the control. Based on the results obtained from five sets of experiments, the

TABLE 9

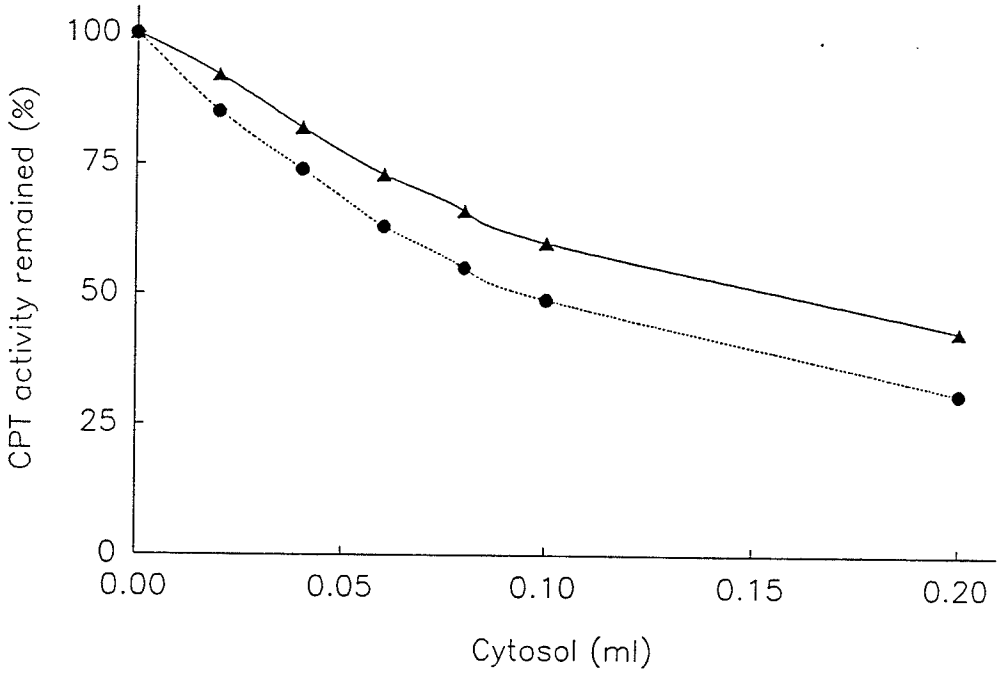
The activities of hepatic enzymes for phosphatidylcholine biosynthesis from fasted and control hamsters.

Subcellular fractions were prepared from the livers of the control and 24 h fasted hamsters. The activities of choline kinase, cytidylyltransferase and cholinephosphotransferase in the subcellular fractions were determined. The results are depicted as mean ± standard deviation from three experiments.

Enzymes	Specific activity (nmol/min per mg of protein)	
	Control	24 h Fasting
Choline kinase	0.69 ± 0.09	0.68 ± 0.08
Cytidylyltransferase		
(microsomes)	0.33 ± 0.05	0.22 ± 0.03*
(cytosol)	1.14 ± 0.16	1.67 ± 0.28*
Cholinephosphotransferase	5.88 ± 0.43	5.80 ± 0.43

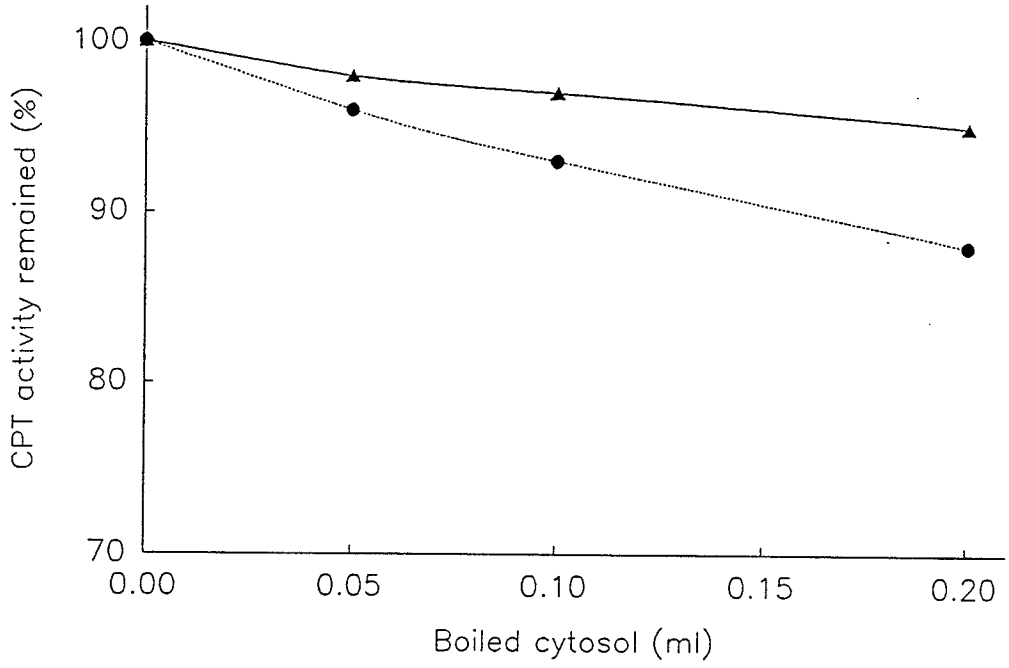
* $P < 0.05$

FIG. 29



Inhibition of cholinephosphotransferase activity by liver cytosol of the fasted hamster Cholinephosphotransferase activity in hamster liver was determined in the presence of 0-0.2 ml liver cytosol from the control (▲) or 24 h fasted (●) hamsters. Enzyme activity without the addition of cytosol was expressed as 100%. Each point was the mean of three separate determinations.

FIG. 30



Inhibition of cholinephosphotransferase activity by heat-treated liver cytosol of the fasted hamster

Liver cytosol of the control (▲) and 24 h fasting (●) hamsters were incubated at 100°C for 10 min. The heat-treated cytosol was centrifuged and an aliquot (0-0.2 ml) of the supernatant was added to the enzyme assay. Enzyme activity without any addition is expressed as 100%. Each point is the mean of three separate determinations.

difference in the inhibition of enzyme activity between these two animal groups was 7±2%.

Argininosuccinate was shown to be the sole factor for the inhibition of cholinephosphotransferase activity in the heat-stable component of the cytosol. If argininosuccine was indeed the factor responsible for the attenuation of enzyme activity, its level should be elevated in the liver of the fasted animal. Analysis of the pool size of argininosuccinate in the liver revealed that its level was significantly elevated during fasting (Table 10). A concomitant decrease in the levels of ATP and CTP were also detected. Our result confirms the previous findings that high energy nucleotide levels were reduced (Leelavathi and Guynn 1977) and argininosuccinate level was elevated in the liver of the fasted animal. Since other urea cycle intermediates were also increased during fasting, the effect of these compounds on choline kinase, cytidylyltransferase and cholinephosphotransferase activities was explored. None of the urea cycle intermediates had any effect on choline kinase or cytidylyltransferase, and only argininosuccinate displayed inhibitory effect on cholinephosphotransferase activity.

TABLE 10

The pool sizes of ATP, CTP and argininosuccinate in hamster livers

	Control	24h fasted
	(μmol/g wet weight)	
ATP	864 ± 97 (3)	630 ± 72 (3)*
CTP	70 ± 6.0 (3)	55 ± 7.8 (3)*
Argininosuccinate	0.50 ± 0.13 (5)	1.09 ± 0.18 (3)*

* $P < 0.05$

B. CONTROL OF PHOSPHATIDYLCHOLINE CATABOLISM

- Modulation by ethanol and vitamin E

Dietary treatment has been employed to unravel the regulation of phosphatidylcholine metabolism in mammalian tissues. Cardiac lysophosphatidylcholine level was shown to increase in rats fed with a vitamin E-deficient diet (Cao *et al.* 1987a). The alteration in cardiac lysophosphatidylcholine level by dietary vitamin E was attributed to a change in cardiac phospholipase A activity. It was therefore postulated that tissue vitamin E content may modulate the rate of phosphatidylcholine catabolism (Cao *et al.* 1987a). Since prolonged ethanol administration has been shown to affect phospholipid metabolism in the heart (Rubin 1982), perfusion of the isolated heart with ethanol could be a useful model to study the mechanisms which control phosphatidylcholine turnover in cardiac tissue. In this study, the combined effects of ethanol and vitamin E on phosphatidylcholine metabolism and on the ethanol-induced lysophosphatidylcholine accumulation was investigated in the isolated rat heart.

I. Effect of Ethanol Perfusion on Phosphatidylcholine Catabolism in Rat heart

1. Effect of Ethanol on Phospholipid Composition

The effect of ethanol perfusion on the rat heart phospholipid composition was investigated. After perfusion with 0.5 - 2.0% ethanol for 4 h, the lipids were extracted from the tissue and the phospholipid classes were separated by thin-layer chromatography. As depicted in Table 11, there was no significant difference in the major cardiac phospholipids between the control and the ethanol-perfused hearts, but lysophosphatidylcholine was significantly elevated in the hearts perfused with 0.5 and 1.0% ethanol. However, increasing ethanol to 2% in the perfusate did not cause the elevation of lysophosphatidylcholine level.

2. Effect of Ethanol on Phosphatidylcholine Catabolic Enzymes

(1) Phospholipase A

Since phospholipase A is the major enzyme responsible for the generation of lysophosphatidylcholine, its activity was determined in the subcellular fractions of the rat heart perfused with ethanol. As depicted in Table 12, phospholipase A activity was significantly elevated only in the cytosolic fractions of the hearts perfused with 0.5 or 1.0% ethanol. Although the enzyme activity in the microsomal fraction appeared to be elevated, analysis of the data revealed that the difference was not significant between the experimental and control groups. The mitochondrial phospholipase A activity was not significantly affected by ethanol perfusion. Perfusion

TABLE 11

Effect of ethanol on phospholipid composition in rat heart

Phospholipid class	No ethanol (control)	0.5% ethanol	1% ethanol	2% ethanol
Lysophosphatidylcholine	0.12 ± 0.01	0.17 ± 0.02 *	0.20 ± 0.03 *	0.14 ± 0.03
Sphingomyelin	0.64 ± 0.05	0.70 ± 0.07	0.67 ± 0.05	0.62 ± 0.07
Phosphatidylcholine	12.26 ± 0.70	11.71 ± 1.40	12.68 ± 0.93	12.11 ± 1.34
Phosphatidylserine and phosphatidylinositol	1.44 ± 0.37	1.53 ± 0.43	1.26 ± 0.48	1.30 ± 0.26
Phosphatidylethanolamine	10.03 ± 0.88	9.89 ± 0.32	10.54 ± 1.02	10.47 ± 1.11
Phosphatidic acid and cardiolipin	3.71 ± 0.40	3.38 ± 0.36	3.55 ± 0.43	3.21 ± 0.47

* $P < 0.05$ when compared to control.

TABLE 12

Activities of lysophosphatidylcholine metabolic enzymes in rat heart subsequent to perfusion with ethanol

Enzyme	nmol product formed per h per mg protein			
	no ethanol	0.5% ethanol	1% ethanol	2% ethanol
Phospholipase A				
cytosolic	4.45 ± 1.04	6.76 ± 0.82 *	7.84 ± 1.47 *	5.12 ± 1.55
microsomal	10.08 ± 1.17	11.72 ± 1.07	12.68 ± 1.88	10.86 ± 1.32
mitochondrial	8.40 ± 0.75	9.98 ± 0.84	9.45 ± 0.78	7.67 ± 1.26
Lysophospholipase				
cytosolic	48.1 ± 7.4	56.0 ± 8.6	54.3 ± 7.0	38.3 ± 9.8
microsomal	32.6 ± 4.6	30.7 ± 3.8	28.5 ± 5.1	27.7 ± 4.9
	nmol product formed per min per mg protein			
	no ethanol	0.5% ethanol	1% ethanol	2% ethanol
Lysophosphatidylcholine:				
acyl-CoA acyltransferase	12.8 ± 1.3	11.5 ± 1.6	11.2 ± 2.0	10.7 ± 2.6

* $P < 0.05$ when compared to no ethanol addition.

with 2% ethanol did not produce any significant change in phospholipase A activity in all subcellular fractions.

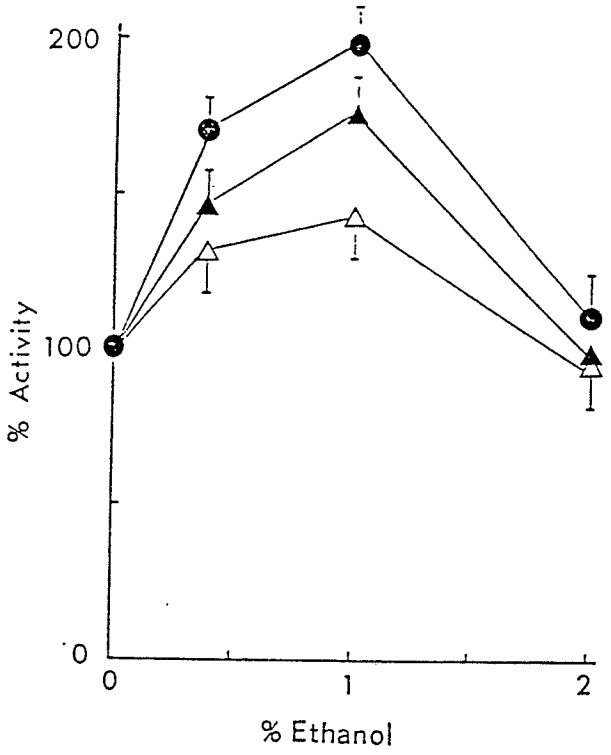
(2) Lysophospholipase and Lysophosphatidylcholine:acyl-CoA Acyltransferase

The increasing in lysophosphatidylcholine in the hearts perfused with 0.5 or 1.0% ethanol could also be caused by changes in lysophospholipase and/or lysophosphatidylcholine:acyl-CoA acyltransferase activities. Hence, the activities of these two enzymes were determined in the ethanol-perfused hearts. As shown in Table 12, the presence of 0-2% ethanol in the perfusate did not cause any significant changes in cardiac lysophospholipase or acyltransferase activities. These results suggested that both enzymes in the heart were not affected by ethanol perfusion.

II. In Vitro Effect of Ethanol on Phosphatidylcholine Catabolic Enzymes

The direct effect of ethanol on the heart phospholipase A activity was determined in all subcellular fractions. Cardiac subcellular fractions were preincubated with 0 - 2% ethanol at 37 °C for 10 min prior to the assay for phospholipase A activity. As shown in Fig. 31, preincubation with 0.5 or 1.0% ethanol caused a dose-dependent enhancement of phospholipase A activities in all subcellular fractions. The enhanced activity by ethanol was most prominent in the cytosolic enzyme and least prominent

FIG. 31



In vitro effect of ethanol on phospholipase A activity in the rat heart

Subcellular fractions of the rat heart were preincubated with 0.25 - 2.0% ethanol at 37 °C for 10 min prior to assay. The reaction was initiated by the addition of labelled substrate. Phospholipase A activities assayed in the absence of ethanol were used as controls (100%). The symbols represent the (△) mitochondrial, (▲) microsomal and (●) cytosolic fractions. The values depicted are the mean of three separate experiments, each assayed in duplicate. The vertical bars are standard deviations.

in the mitochondrial enzyme. At higher ethanol concentration (2%), there was a general decrease in enzyme activity over the maximum values observed in the 1% ethanol. These results demonstrated that ethanol elicited a direct but biphasic effect on phospholipase A activity. Preincubation with 1% ethanol had not effect on the cardiac lysophospholipase or acyltransferase activities.

III. Combined Effects of Ethanol and Vitamin E on Phosphatidylcholine Catabolism In Rat Heart

1. Effect of Vitamin E on Phosphatidylcholine Catabolism in Ethanol-Perfused Rat Heart

It has been demonstrated that both cardiac and platelet phospholipase A can be inhibited by dietary or *in vitro* enrichment of vitamin E (α-tocopherol) (Cao *et al.* 1987a; Douglas *et al.* 1986). In order to determine if the addition of vitamin E could alter the ethanol-induced stimulation of phospholipase A or the accumulation of lysophosphatidylcholine, rat hearts were perfused with different amounts of vitamin E (25-100 μM) in 1% ethanol. The presence of 50 or 100 μM of vitamin E in the perfusate produced a significant decrease of the cardiac phospholipase A activity in all the subcellular fractions (Table 13). At the concentration of 25 μM, vitamin E reduced the microsomal and mitochondrial phospholipase A activities below that of

TABLE 13

Effect of vitamin E on lysophosphatidylcholine metabolic enzymes in ethanol-perfused rat heart

Enzyme	nmol product formed per h per mg protein			
DL-α-tocopherol (μM):	0	25	50	100
Phospholipase A				
cytosolic	7.84 ± 1.47	6.38 ± 1.02	5.55 ± 0.98 *	5.59 ± 1.67
microsomal	12.68 ± 1.88	9.47 ± 1.19 *	7.85 ± 0.82 *	7.07 ± 1.46 *
mitochondrial	9.45 ± 0.78	7.70 ± 0.88 *	7.20 ± 1.00 *	7.34 ± 1.21 *
Lysophospholipase				
cytosolic	48.1 ± 7.4	–	41.2 ± 6.8	–
microsomal	32.6 ± 4.6	–	30.0 ± 5.1	–
	nmol product formed/min per mg protein			
DL-α-tocopherol (μM):	0		50	
Lysophosphatidylcholine:				
acyl-CoA acyltransferase	12.8 ± 1.3	–	11.5 ± 0.7	–

* $P < 0.05$ when compared to no addition of DL-α-tocopherol.

the controls. However, increase in vitamin E concentration beyond 50 μM did not produce any further reduction of the enzyme activities. The cardiac lysophospholipase and acyltransferase activities were unaffected by the presence of vitamin E in the perfusate (Table 13).

The combined effect of vitamin E and ethanol on phospholipid composition was also investigated. As shown in Table 14, the normalization of ethanol-induced phospholipase A activities by vitamin E was accompanied by a corresponding reduction in cardiac lysophosphatidylcholine. Other phospholipid classes in the heart were not affected by ethanol or vitamin E in the perfusate.

2. *In Vitro* Effect of Ethanol and Vitamin E on Phospholipase A

The *in vitro* effect of vitamin E on phospholipase A activity in the presence of ethanol was investigated. Subcellular fractions from rat heart were preincubated with 0 - 100 μM vitamin E in 1% ethanol at 37 °C for 10 min prior to the assay. As depicted in Fig. 32, exogenously added vitamin E caused an inhibition of phospholipase A in a dose-dependent manner. In the presence of 100 μM vitamin E the ethanol-induced increase of phospholipase A activities was completely abolished in all subcellular fractions, and enzyme activities were significantly lower than the controls without added ethanol.

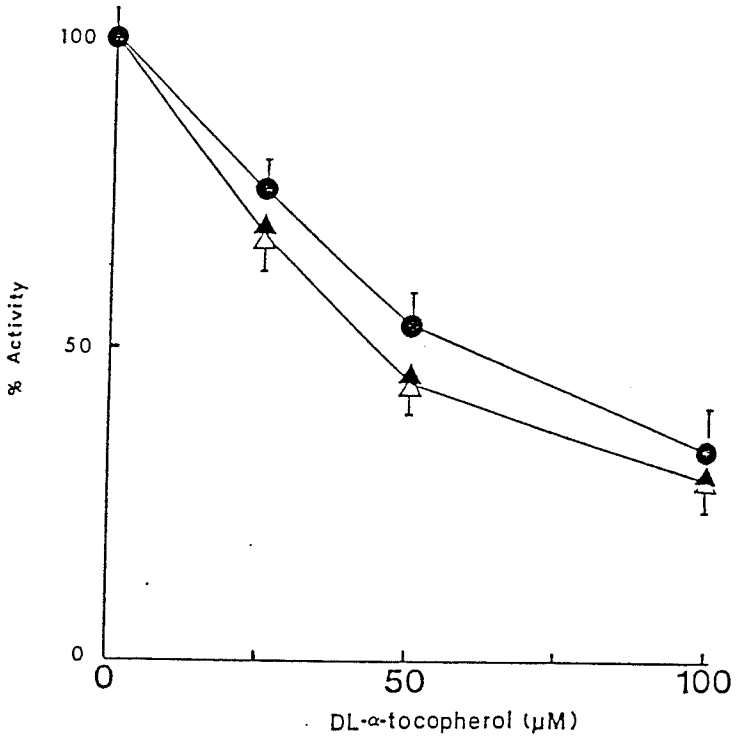
TABLE 14

Effect of vitamin E on phospholipid composition in ethanol-perfused rat heart

Phospholipid class	μmol of lipid-P per g heart wet weight			
DL-α-tocopherol (μM):	0	25	50	100
Lysophosphatidylcholine	0.20 ± 0.03	0.16 ± 0.02	0.11 ± 0.03 *	0.10 ± 0.03 *
Sphingomyelin	0.67 ± 0.05	0.57 ± 0.03	0.63 ± 0.06	0.77 ± 0.13
Phosphatidylcholine	12.68 ± 0.93	11.61 ± 1.65	12.68 ± 1.34	12.26 ± 0.48
Phosphatidylserine and phosphatidylinositol	1.26 ± 0.48	1.42 ± 0.27	1.52 ± 0.14	1.48 ± 0.15
Phosphatidylethanolamine	10.54 ± 1.02	9.73 ± 0.78	8.71 ± 1.05	9.35 ± 0.76
Phosphatidic acid and ardiolipin	3.55 ± 0.43	3.10 ± 0.51	3.64 ± 0.35	3.20 ± 1.00

* $P < 0.05$ when compared to no DL-α-tocopherol addition.

FIG. 32



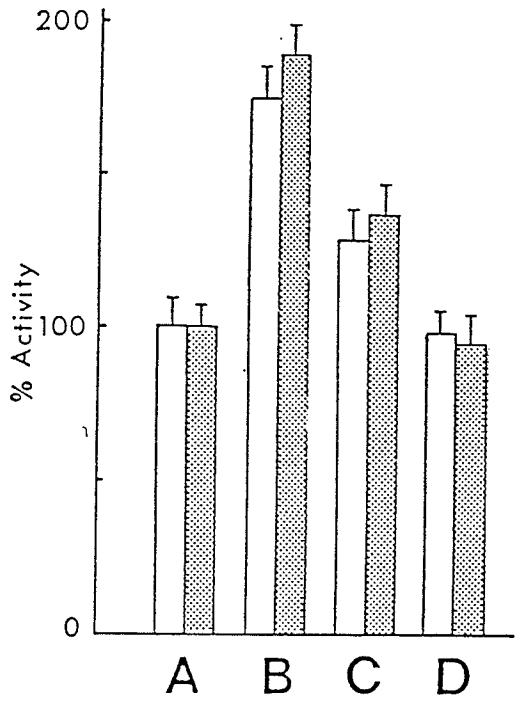
In vitro effects of ethanol and vitamin E on phospholipase A activity in the rat heart

Subcellular fractions of the rat heart were preincubated with 25 - 100 μM of DL-α-tocopherol in 1% ethanol for 10 min prior to the assay. The reaction was initiated by the addition of labelled substrate. Phospholipase A activities assayed in the absence of vitamin E (but in the presence of 1% ethanol) were used as controls (100%). The symbols used are the same as in Fig. 31.

In order to elucidate whether ethanol and vitamin E affect phospholipase A_1 or A_2 in the same manner, both enzyme activities in the cytosolic fractions were assayed with 1-palmitoyl-2-[^{14}C]arachidonoylglycerophosphocholine as substrate. Subsequent to the assay, phospholipase A_1 activity was estimated from the radioactivity present in the lysophosphatidylcholine fraction, whereas phospholipase A_2 activity was determined from the radioactivity associated with the released arachidonate. As shown in Fig. 33, both phospholipase A_1 and A_2 activities were decreased to the same degree when vitamin E was added to the incubating mixture. Similar results were obtained when phospholipase A_1 and A_2 activities were determined in the microsomal fraction.

In order to understand the modulatory mechanism of ethanol and vitamin E on phospholipase A activity, kinetic studies were undertaken. Phospholipase A was partially purified from rat heart cytosol by CM-Sepharose chromatography. The rat heart cytosol (50 mg protein) was applied to a CM-cellulose column (1 x 21 cm) equilibrated with 50 mM Hepes buffer containing 5 mM EDTA, pH 7.4. The column was washed with the same buffer (120 ml), followed by a linear gradient of 0 - 0.5 M NaCl (200 ml). Fractions (3 ml) were collected and assayed for phospholipase A activity. Most phospholipase A activity was eluted from the column at 0.1 M NaCl. The active fractions devoid of lysophospholipase activity were pooled and used in kinetic studies. The double-reciprocal plots of phospholipase A activity vs.

FIG. 33

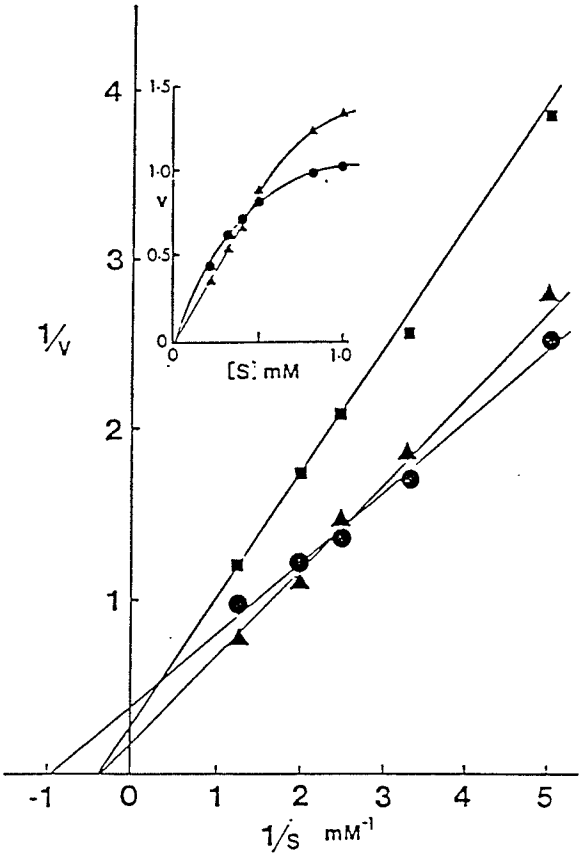


In vitro effects of ethanol and vitamin E on phospholipase A_1 and A_2 activities

Aliquots of the rat heart cytosol were preincubated with 1% ethanol or DL-α-tocopherol in 1% ethanol at 37 °C for 10 min. The reaction was initiated by the addition of 1-palmitoyl-2-[^{14}C]arachidonyl-glycerophosphocholine. Phospholipase A_1 activity was estimated from the radioactivity in the lysophosphatidylcholine fraction, whereas phospholipase A_2 activity was determined from the radioactivity in the free fatty acid fraction. Phospholipase A_1 activity (4.05 nmol/h per mg protein) and phospholipase A_2 activity (5.25 nmol/h per mg protein) assayed without ethanol and DL-α-tocopherol were used as control (100%) (A). Enzyme activities were assayed in the presence of 1% ethanol (B), 25 μM DL-α-tocopherol in 1% ethanol (C), and 50 μM DL-α-tocopherol in 1% ethanol (D) The open bars denote phospholipase A_1 activities and the shaded bars denote phospholipase A_2 activities. Each value is the mean of three assays. The vertical bars are standard deviations.

phosphatidyl[Me-^{3}H]choline concentrations in the presence of ethanol or a combination of ethanol and vitamin E are depicted in Fig. 34. At 1% ethanol, enzyme activities were reduced at low substrate concentrations and enhanced at high substrate concentrations when compared with the controls. Attempts to use higher ethanol concentration for the kinetic study were not successful because at 2% ethanol, a linear line could not be obtained from double-reciprocal plot. When the enzyme was assayed in the presence of 1% ethanol and 100 μM vitamin E, enzyme activities were inhibited. However, the presence of vitamin E in 1% ethanol did not alter the change in K_m induced by ethanol, but inhibited the reaction in a noncompetitive manner.

FIG. 34



Double-reciprocal plot of the activities of partially purified phospholipase A vs. phosphatidylcholine concentrations in the presence of added ethanol and vitamin E.

Partially purified rat heart cytosolic phospholipase A (40 μg) was incubated in the absence (●) or presence (▲) of 1% ethanol, 100 μM DL-α-tocopherol and 1% ethanol (■). The reaction was initiated by the addition of phosphatidyl-[Me-^{3}H]choline as substrate. Enzyme activity (nmol/min per mg protein) as a function of substrate concentration is depicted in the inset. Each point represents the mean of three separate assays.

DISCUSSION

I. Solubilization and Partial Purification of Cholinephosphotransferase

The importance of cholinephosphotransferase in phosphatidylcholine biosynthesis is well documented (Cornell 1989). However, only limited information was available on the properties of the enzyme. The objective of this study was to purify cholinephosphotransferase from hamster liver and heart, and to elucidate its regulatory mechanism without the interference of other enzymes presented in the microsomes. Since cholinephosphotransferase is an integral membrane protein, the solubilization of the enzyme by detergents is a prerequisite for its purification. A number of neutral and ionic detergents were employed for this study, including octyl glucoside, CHAPS, CHAPSO, taurocholate, Triton X-100 and Tween 20. These detergents were found to be ineffective for the solubilization of the enzyme from both sources, i.e. they either failed to solubilize the enzyme or caused irreversible inactivation of the enzyme. Alternatively, cholinephosphotransferase from hamster liver microsomal fraction was solubilized by 3% Triton QS-15 (with a detergent to protein ratio of 1:1.5). However, Triton QS-15 was not effective in the solubilization of the enzyme from hamster heart microsomes. The solubilized enzyme preparation from hamster liver was partially purified by DEAE-Sepharose chromatography and

Sepharose 6B chromatography. The seven-fold purification obtained was the highest purification of the enzyme from mammalian sources. The K_m values for CDP-choline were similar between microsomal and partially purified enzymes, whereas the K_m value for diacylglycerol was substantially lowered when the enzyme was partially purified.

When cholinephosphotransferase was partially purified, a differential effect of phospholipids on its activity was observed. Compared with the microsomal enzyme, the partially purified phosphotransferase was more sensitive to the modulation by exogenous phospholipids. The partially purified enzyme favoured a more alkaline condition than in the microsomal form. The increase in sensitivity to exogenous phospholipids and the shift in pH profile of the enzyme in partially purified form might be caused by changes in the hydrophobic environment during solubilization and purification. The ability to solubilize and partially purify cholinephosphotransferase from hamster liver will enable us and other investigators to closely examine the control mechanism of this enzyme and its role in the regulation of phosphatidylcholine biosynthesis.

II. Differentiation of Cholinephosphotransferase and Ethanolamine-phosphotransferase

Indirect evidence has indicated that cholinephosphotransferase and ethanolaminephosphotransferase are separate enzymes which catalyze the biosynthesis of phosphatidylcholine and phosphatidylethanolamine, respectively (Kanoh and Ohno 1976; Possmayer *et al.* 1977; Holub 1978; Morimoto and Kanoh 1978). The activities of both phosphotransferases are tightly associated with endoplasmic reticulum (Vance *et al.* 1977). Similar to cholinephosphotransferase, ethanolaminephosphotransferase has not been isolated from mammalian source and the regulation of its activity is largely unknown. In order to gain further insight into the origin and the regulation of two enzyme activities in the endoplasmic reticulum, the attempts were made to separate cholinephosphotransferase and ethanolaminephosphotransferase from hamster liver microsomes.

The solubilized phosphotransferases were partially purified and separated from each other by ion exchange chromatography. The partial resolution of the two activities suggests that cholinephosphotransferase and ethanolamine-phosphotransferase are separate proteins, but with very similar net charges. It is rather intriguing that cholinephosphotransferase became more heat stable subsequent to partial purification, and yet the stability of ethanolaminephosphotransferase upon heat treatment was not significantly changed before and after purification. One facile explanation is that both phosphotransferases are intimately associated with each other in the microsomes and heat denaturation of the microsomal ethanolamine-

phosphotransferase may cause the simultaneous inactivation of cholinephosphotransferase. However, the close association between these two enzymes was disrupted during solubilization, thus revealing the true stability of the individual protein upon heat treatment. Taken together, the results obtained from this study provide us with clear evidence for the separate identities and distinct properties of cholinephosphotransferase and ethanolaminephosphotransferase in hamster liver. However, the possibility that these two enzymes do not have absolute specificity towards CDP-choline and CDP-ethanolamine has not been precluded.

Differential effects of detergents on two phosphotransferase activities were observed. Cholinephosphotransferase activity was inhibited by the detergents tested, whereas with same detergent concentrations, ethanolaminephosphotransferase was activated by the addition of CHAPS, octyl glycoside, and taurocholate. It is possible that the detergents might disrupt the essential enzyme - membrane lipid interactions, which might be crucial for the activities of membrane-bound enzymes. The inactivation of cholinephosphotransferase by detergents might be due to changes in the hydrophobic environment, which in turn would impair the catalytic activity of the enzyme or the normal substrate binding process. The direct binding of detergents to the active site of cholinephosphotransferase might also contribute to the apparent inactivation of the enzyme. In the presence of detergents, ethanolamine-phosphotransferase might become more accessible to the substrates upon the disruption of its association with

membrane lipids.

Using mutants of *Saccharomyces cerevisiae* defective in cholinephosphotransferase or ethanolaminephosphotransferase, Hjelmstad and Bell demonstrated that the two phosphotransferases in yeast were encoded by separate genes (Hjelmstad and Bell 1988, 1990). The product of cholinephosphotransferase gene from yeast did not have ethanolaminephosphotransferase activity, whereas the product of ethanolaminephosphotransferase gene contained the activities of both ethanolaminephosphotransferase and cholinephosphotransferase. The presence of seven transmembrane helices of cholinephosphotransferase was predicted from the protein sequence which is in agreement with this enzyme being tightly associated with membrane (Hjelmstad and Bell 1990).

III. Regulation of Cholinephosphotransferase Activity by an Endogenous Metabolite - Argininosuccinate

In this study, we are the first to show that the activity of microsomal cholinephosphotransferase was inhibited by argininosuccinate. The specificity of the inhibition makes argininosuccinate an ideal metabolite for the regulation of cholinephosphotransferase activity. In addition, the inhibition was not affected by changes in CDP-choline levels. In view of this finding, our objective was to examine

the physiological role of argininosuccinate in the modulation of cholinephosphotransferase activity and consequently, its effect on phosphatidylcholine biosynthesis. The ability to regulate the biosynthesis of phosphatidylcholine by a metabolite in the urea cycle is an attractive model to demonstrate the occurance of coordination between phospholipid and protein metabolism.

The incorporation of labelled choline into phosphatidylcholine in the liver of the fasted hamster was employed as a model to study the regulatory role of argininosuccinate on phosphatidylcholine biosynthesis. When animals were fasted for 24h, the enzyme activities of the urea cycle were shown to be elevated (Schimke 1962, Snodgrass 1981). Fasting also caused a 27% reduction in ATP and 20% reduction in CTP levels, but such reductions were not severe enough to cause any significant change in phosphatidylcholine biosynthesis (Choy 1982, Hatch and Choy 1990). Although the enzyme activities of the urea cycle could be further increased by prolonged starvation, the levels of the high energy nucleotides would be reduced to an extend (>35%) where their levels would become rate-limiting in the CDP-choline pathway (Choy 1982, Hatch and Choy 1990). For practical purpose, hamsters fasted for 24h were used in the present study. To test the validity of the model, the pool size of argininosuccinate in the livers of both animal groups were determined. The pool size of argininosuccinate obtained from the control was very similar to that reported by other investigators (Coopes et al. 1986). A two-fold increase in the

argininosuccinate pool was detected in the liver of the fasted animal, and the increase in argininosuccinate level was reflected cytosolic fraction.

The conversion of labelled CDP-choline to phosphatidylcholine was reduced in the liver of the fasted animal, but no significant difference in the microsomal cholinephosphotransferase activity was detected between the two animal groups. It appears that the attenuation of enzyme activity for the conversion of CDP-choline to phosphatidylcholine was produced by a non-microsomal factor. This supposition was supported by the fact that cytosol from the fasted animal produced a higher degree of inhibition of enzyme activity than the control. When heat-treated cytosols were used in this study, the difference in the inhibition of enzyme activity between the two animal groups was not abolished. Taken together, our results indicate that an increase in argininosuccinate was the principal factor for attenuating the conversion of CDP-choline to phosphatidylcholine in the liver of the fasted hamster.

Quantitative analysis of the data revealed that the difference in attenuation of cholinephosphotransferase activity by 0.2 ml liver cytosol was 12% between fasted and the control animals. The difference in attenuation of enzyme activity by the same amount of heat-treated cytosol was reduced to 7% between the two animal groups. The difference in enzyme inhibition detected in heat-treated cytosols did not quantitatively account for the difference observed with untreated cytosols. Hence, it

is conceivable that some heat-sensitive factor(s) might also be involved in the inhibition of the enzyme activity in the cytosol of the fasted animal. The nature of these factors has not been defined.

It is generally accepted that the cytidylyltransferase catalyzes the rate-limiting step in the CDP-choline pathway (Vance 1990), and the translocation of the enzyme between the microsomal and cytosolic compartments is a principal mechanism for the control of the enzyme activity (Sleight and Kent 1983, Vance 1989). In the present study, the translocation of cytidylyltransferase activity from the microsomal to cytosolic compartment was observed during fasting. The translocation of cytidylyltransferase during fasting was documented in an earlier study (Pelech and Vance 1984). The redistribution of enzyme activity from the microsomal to the cytosolic compartment was attributed to the direct phosphorylation of the microsomal enzyme in the fasted animal. Since the microsomal form of the enzyme has been touted as the active form, the translocation of the enzyme to the cytosolic form in the liver of the fasted animal would have reduced the overall activity of the enzyme and caused an accumulation of labelled phosphocholine. However, no difference in the labelling of phosphocholine was detected between the two animal groups. It appears that the reduction of enzyme activity by translocation in the fasted animal had not reached the stage to produce a significant change in the conversion of phosphocholine to CDP-choline. Further deterioration in the energy status of the

tissue by prolonged starvation might expedit the translocation process and would cause a severe reduction in cytidylyltransferase activity and result in the accumulation of phosphocholine. We postulate that the attenuation of cholinephosphotransferase activity by a metabolite sensitive to the metabolic status of the tissue appears to be an additional mechanism for the immediate and direct control of phosphatidylcholine biosynthesis.

IV. Combined Effects of Ethanol and Vitamin E on Phosphatidylcholine Catabolism in Rat Heart

Chronic administration of ethanol has been found to cause cardiac dysrhythm in rat (Gimeno *et al.* 1962; Lochner *et al.* 1969), whereas rapid ethanol consumption has been reported to cause fatal cardiac disturbances in human subjects (Koskenvuo *et al.* 1986). Analysis of the membrane phospholipids upon prolonged ethanol administration in mammalian organs revealed that there were significant changes in the composition and acyl contents of the phospholipid classes (Pachinger *et al.* 1973). These observations suggest that ethanol-induced alterations in cell membrane properties and impaired cardiac function may be related to derange or defects in phospholipid metabolism. However, little is known regarding the short-term effect of ethanol on phospholipid metabolism. The initial goal of this study was to examine the effect of ethanol on cardiac phospholipid metabolism. Ethanol perfusion of the

rat heart was found to cause an increase in lysophosphatidylcholine level. The accumulation of lysophosphatidylcholine was probably caused by the enhancement of cardiac phospholipase A_1 and A_2 activities during ethanol perfusion. Although prolonged ethanol administration has been shown to affect the phospholipid contents and the acyltransferase activity (Pachinger *et al.* 1973; Reitz *et al.* 1973), the results obtained from this study clearly indicate that such changes were not produced by short-term ethanol perfusion.

There were some discrepancies in phospholipase A activities in response to different ethanol treatments. When the heart was perfused with 1% ethanol, the microsomal enzyme activity was marginally enhanced (25%), but when the assay mixture was preincubated with 1% ethanol, the enzyme activity was significantly increased (75%). Similar changes were also observed with the mitochondrial enzyme. The difference between the two experimental designs can be explained as follows. While the presence of ethanol in the perfusate quickly equilibrated with the perfused heart (Erickson 1979), the majority of the ethanol in the heart remained in the cytosol. It is therefore logical to surmise that the effect of ethanol on phospholipase A activity would be most prominent in the cytosolic compartment. This would explain the lack of ethanol effect on the mitochondrial and microsomal enzyme observed when the heart was perfused with ethanol. Indeed, the majority of the ethanol that was associated with the membrane fraction arising from ethanol perfusion would be lost

during the preparation of the subcellular fraction. Hence, determination of phospholipase A activities in different subcellular fractions after perfusion might not truly reflect the degree of enzyme activity enhancement when ethanol was present in the intact perfused heart. We postulate that a higher degree of enhancement of phospholipase A would occur in the intact organ.

In this study, phosphatidylcholine isolated from the heart was used as substrate for the assay of phospholipase A activity. It has been shown previously that cardiac phospholipase A (both A_1 and A_2) displays acyl-group specificity towards certain phosphatidylcholine species (Tam *et al.* 1984; Cao *et al.* 1987b). The advantages of employing a natural substrate (with mixed acyl groups) for the determination of phospholipase A activity has been documented (Cao *et al.* 1987a, 1987b). The use of phosphatidyl[Me-^{3}H]choline as a natural substrate allowed us to assay the sum of phospholipase A_1 and A_2 activities. In order to discriminate the effects of ethanol and vitamin E on phospholipase A_1 and A_2, it was necessary to use a phospholipid substrate containing a defined labelled acyl group. The use of 1-palmitoyl-2-[^{14}C]arachidonoylglycerophosphocholine as substrate permitted us to determine both phospholipase A_1 and A_2 activities simultaneously by monitoring the formation of labelled lysophosphatidylcholine and labelled fatty acid, respectively (Cao *et al.* 1987a, 1987b). However, it should be noted that the sum of phospholipase A_1 and A_2 obtained from this assay was not the same as that obtained with the natural

substrate. Such a discrepancy has been reported in previous studies (Cao *et al.* 1987a, 1987b; Tam *et al.* 1984) and was probable due to the specificity of the enzyme towards phosphatidylcholine with defined acyl group.

The enhancement of phospholipase A activity by ethanol appears to occur only at high substrate concentrations. At lower substrate concentrations, 1% ethanol was found to be inhibitory to the reaction. One explanation for the dual effect of ethanol is that ethanol is essentially a mild inhibitor to the reaction, but it may also increase phospholipid hydrolysis by facilitating a better substrate dispersion (Arthur and Choy 1989). The change in K_m value suggests that ethanol has the ability to reduce the affinity between the substrate and the enzyme. We postulate that at low substrate concentrations, the phospholipid was adequately dispersed, so that the inhibitory effect of ethanol became apparent. At higher substrate concentrations, the inhibitory effect of ethanol was still maintained but the beneficial effect of substrate dispersion by ethanol became more prominent. The attenuation of enzyme activity by vitamin E appears to occur by an entirely different mechanism. Vitamin E did not alter the shift of K_m produced by ethanol, but inhibited the enzyme activity in a non-competitive manner.

This study clearly demonstrates that short-term administration of ethanol caused the accumulation of lysophosphatidylcholine in the heart. We also confirm that vitamin

E has the ability to reduce the accumulation of lysophosphatidylcholine by attenuating the cardiac phospholipase A activity (Cao *et al.* 1987a). Phospholipase A is the major enzyme responsible for the degradation of phosphatidylcholine into its lyso-form, and the lysophosphatidylcholine is further metabolized by the action of lysophospholipase or reacylated back to the parent phospholipid by lysophosphatidylcholine:acyl-CoA acyltransferase. Due to its detergent property, lysophosphatidylcholine is cytolytic at high concentrations (Choy and Arthur 1989) and its level in mammalian tissue is under rigid control. At present, the exact mechanism for the control of cardiac lysophosphatidylcholine level is not entirely clear. Since the alterations of cardiac lysophosphatidylcholine levels by ethanol and the combination of ethanol and vitamin E were not accompanied by changes in lysophospholipase or acyltransferase activities, the modulation of phospholipase A activity appears to be a key factor for the control of lysophosphatidylcholine metabolism in the heart.

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