REGULATION OF PHOSPHATIDYLCHOLINE METABOLISM IN MAMMALIAN TISSUES

by

© Kar-min O

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Faculty of Graduate Studies
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To My Parents and My Brother

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ABSTRACT

Phosphatidylcholine is the major phospholipid in mammalian tissues. The majority of phosphatidylcholine is synthesized via the CDP-choline pathway, and the remainder is formed from the progressive methylation of phosphatidylethanolamine or from the base-exchange reaction with an existing phospholipid. In the CDP-choline pathway, choline is phosphorylated into phosphocholine which is converted into CDP-choline. CDP-choline is condensed with diacylglycerol for the formation of phosphatidtylcholine. The conversion of phosphocholine to CDP-choline is regarded as the rate-limiting step of this pathway and is catalyzed by CTP:phosphocholine cytidylyltransferase. The principal catabolic route for phosphatidylcholine is through the hydrolytic action of phospholipase A_1 or A_2, resulting in the formation of lysophosphatidylcholine. Lysophosphatidylcholine can be further deacylated by lysophospholipase or can be reacylated back to the parent phospholipid through the acyltransferase reaction. In this study, the control mechanisms of phosphatidylcholine biosynthesis and catabolism in mammalian tissues including the heart were investigated. Administration of diethyl ether and dietary treatment with different levels of vitamin E were used to establish model systems for this study.

Diethyl ether is an anesthetic for small laboratory

animals. The effect of ether anesthesia on phosphatidylcholine biosynthesis in hamster organs was investigated. Ether administration did not affect the incorporation of radioactive choline into phosphatidylcholine in the liver, heart, lung, brain and spleen. A significant (29%) decrease in the labelling of phosphatidylcholine was detected in the kidney of ether-treated hamsters. Reduction in phosphatidylcholine labelling was not due to a diminished level of choline uptake but a decrease in the conversion of phosphocholine to CDP-choline. The resultant accumulation of labelled phosphocholine was caused by a deminished microsomal cytidylyltransferase activity in the ether-treated kidney. It appears that ether treatment caused the translocation of CTP:phosphocholine cytidylyltransferase from microsomal (more active) form to cytosolic (less active) form. Since the other hamster organs were not affected by ether treatment, the cytidylyltransferase in hamster kidney may be modulated differently than those in other hamster organs.

The role of vitamin E in the regulation of phosphatidylcholine catabolism was also studied in the rat heart. The level of lysophosphatidylcholine was increased in animals fed with a vitamin E-deficient diet but was decreased when animals was fed with a high vitamin E diet. The alterations in cardiac lysophosphatidylcholine levels by dietary vitamin E were attributed to the changes in the activity of cardiac phospholipase A. Dietary vitamin E affected both phos-

pholipase A_1 and A_2 in the same manner, but had no effect on the other major enzymes which are responsible for the metabolism of lysophosphatidylcholine. Kinetic studies revealed that the inhibition of enzyme activity by vitamin E was essentially non-competitive. It is clear from this study that the vitamin E status in the animal is a biochemical factor for the maintenance of lysophosphatidylcholine levels in the heart. We postulate that vitamin E may have an important role in the regulation of phosphatidylcholine catabolism.

INTRODUCTION

I. BIOLOGICAL MEMBRANES AND PHOSPHOLIPIDS

The plasma membrane and the internal membranes of eucaryotic cells are collectively known as biological membranes. The development of the plasma membrane was a key step in the generation of the earliest forms of life. Biological membranes are involved in a number of cellular functions. For example, they not only define the geographical limit of the cell, but also form closed boundaries resulting in different compartments within the cell. In addition, the plasma membrane provides a selective barrier to the outside environment. It is also a selective filter that maintains the concentration gradient of ions on either side and allows nutrients to enter and waste products to leave the cell [1].

All biological membranes can be interpreted by the 'fluid mosaic model' first proposed by Singer and Nicholson [2]. Fig. 1 shows the topography of membrane protein, lipid and carbohydrate in the fluid mosaic model of a typical 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 of the biological membrane are soluble in organic solvents. They constitute about 50% of

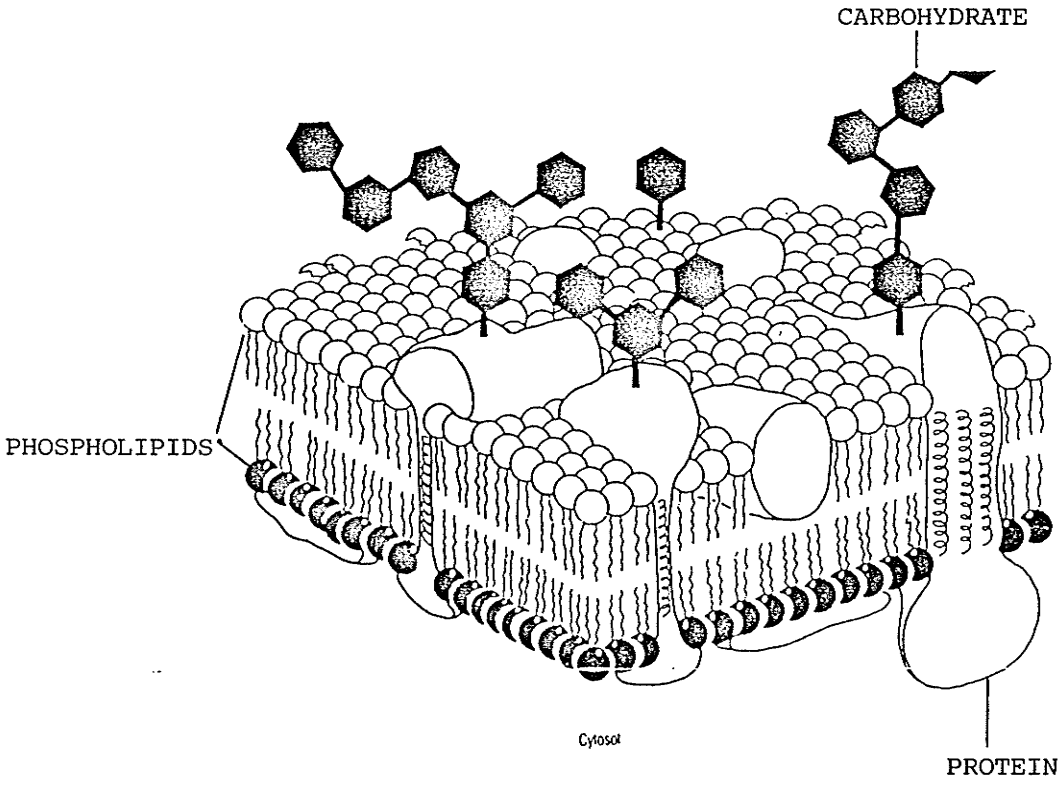


Fig. 1

A model of plasma membrane structure (14)

the mass of mammalian cell plasma membranes [1]. 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 nonpolar (hydrophobic) region. As shown in Fig. 2, the typical phospholipid molecule has a polar head group and two hydrophobic acyl groups. The length of the acyl groups (tails) normally varies from 14 to 24 carbon atoms and the acyl group at C-2 usually contains one or more double bonds. The differences in chain length and degree of unsaturation are important in the fluidity of the membrane. There are three major types of lipids in cell membranes: phospholipid, cholesterol and glycolipids. Glycerol-based phospholipids are the most abundant lipids in membrane [1].

Fig. 3 depicts the different classes of phospholipids including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and cardiolipin. Phosphatidylcholine is the major phospholipid in mammalian tissues. Beyond its structural role in membrane formation, it is a donor of fatty acyl group for cholesterol ester and serves as a source of arachidonic acid for prostaglandin biosynthesis [3]. It is also involved in the modulation of membrane bound enzymes [4]. A portion of phosphatidylcholine synthesized in the liver is incorporated into lipoprotein and the dipalmitoyl-phosphatidylcholine synthesized in the lung is secreted as surfactant [5]. Cholesterol is also a

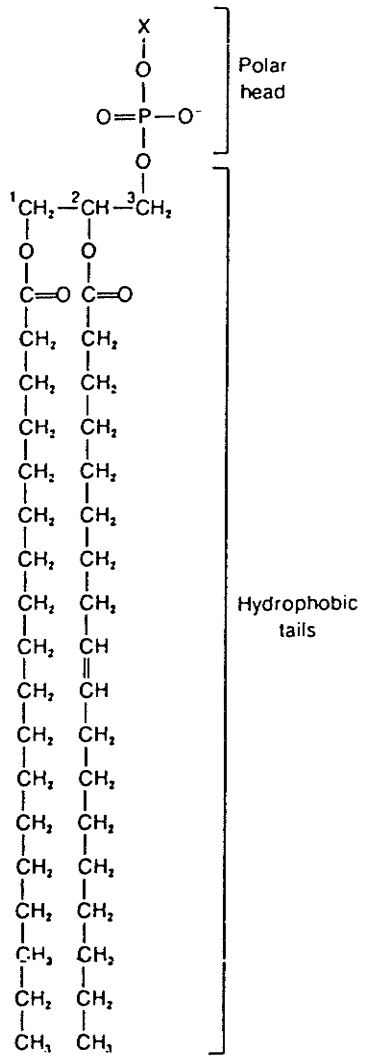
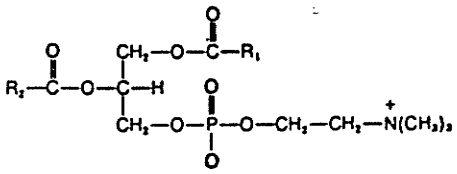
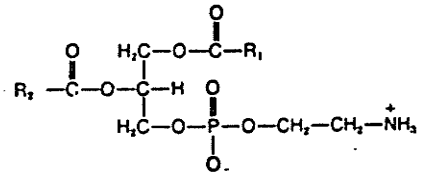


Fig. 2

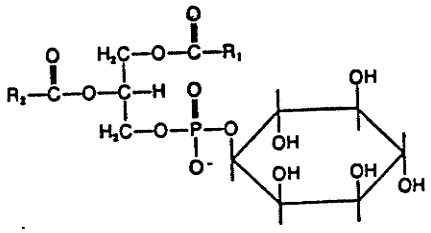
General structure of phosphoglycerides (98)



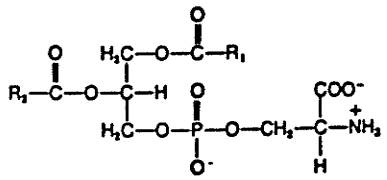
Phosphatidylcholine



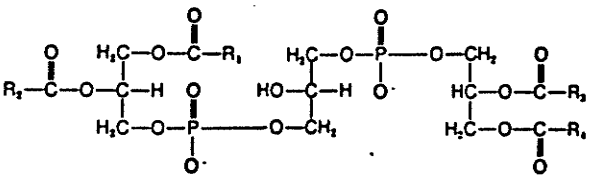
Phosphatidylethanolamine



Phosphatidylinositol



Phosphatidylserine



Cardiolipin

Fig. 3

Classification of phospholipids

major component in some membranes, accounting for as much as one molecule for every phospholipid molecule [1]. The major functions of cholesterol are for 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 [1].

II. Phosphatidylcholine Biosynthesis in Mammalian Tissues

Phosphatidylcholine was discovered by Gobley in 1847 [6]. He demonstrated the presence of the phospholipid which was later named lecithin in egg yolk. In 1862 Diakonow and Strecker identified that lecithin contained two fatty acid groups linked to a glycerol backbone with choline attached to the third hydroxyl group of glycerol by a phosphodiester linkage [7]. The structure of phosphatidylcholine was confirmed by Grün and Linpächer using chemical synthesis in 1927 [8].

The pathways for phosphatidylcholine biosynthesis were elucidated in 1960's [9]. 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 base exchange of choline with other phospholipids. The contribution of each pathway to total phosphatidylcholine biosyn-

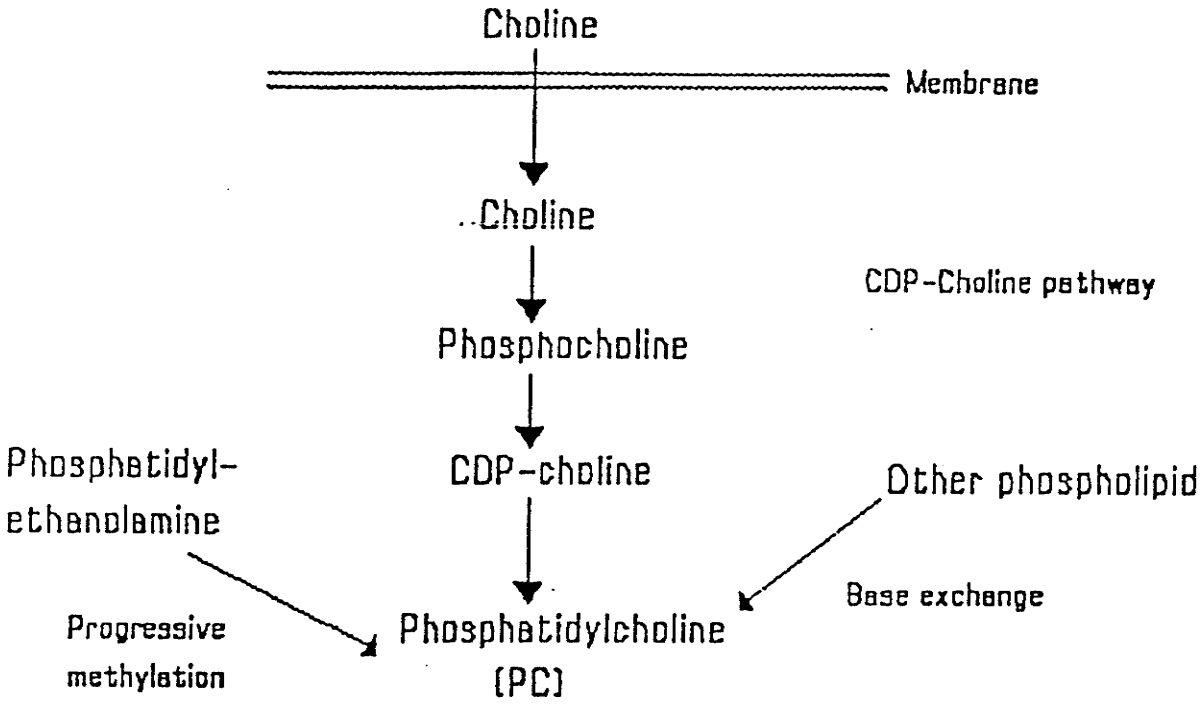


Fig. 4
Pathways for the biosynthesis of phosphatidylcholine

thesis appears to be tissue specific. However, the major pathway for phosphatidylcholine biosynthesis occurs via the CDP-choline pathway [10].

a) CDP-Choline Pathway

This major pathway for the biosynthesis of phosphatidylcholine was elucidated by Kennedy and co-workers [10]. This pathway is therefore known as the Kennedy pathway or the *de novo* synthesis pathway. In this pathway (Fig 5), choline taken up by the cell is first phosphorylated into phosphocholine. This step is catalyzed by choline kinase. Phosphocholine is then converted to CDP-choline by the action of CTP:phosphocholine cytidylyltransferase. The final step in this pathway is the formation of phosphatidylcholine from CDP-choline and diacylglycerol, catalyzed by CDP-choline: 1,2-diacylglycerol choline phosphotransferase.

i) Choline Kinase

Choline kinase catalyzes the phosphorylation of choline and appears to be present in the cytosol of various tissues [11-13]. This enzyme has been highly purified from the liver, lung and kidney. Its molecular weight is in the range of 75,000-160,000 [14]. It is generally accepted that choline kinase and ethanolamine kinase are separate enzymes, which catalyze the phosphorylation of choline and ethanolamine, respectively [14].

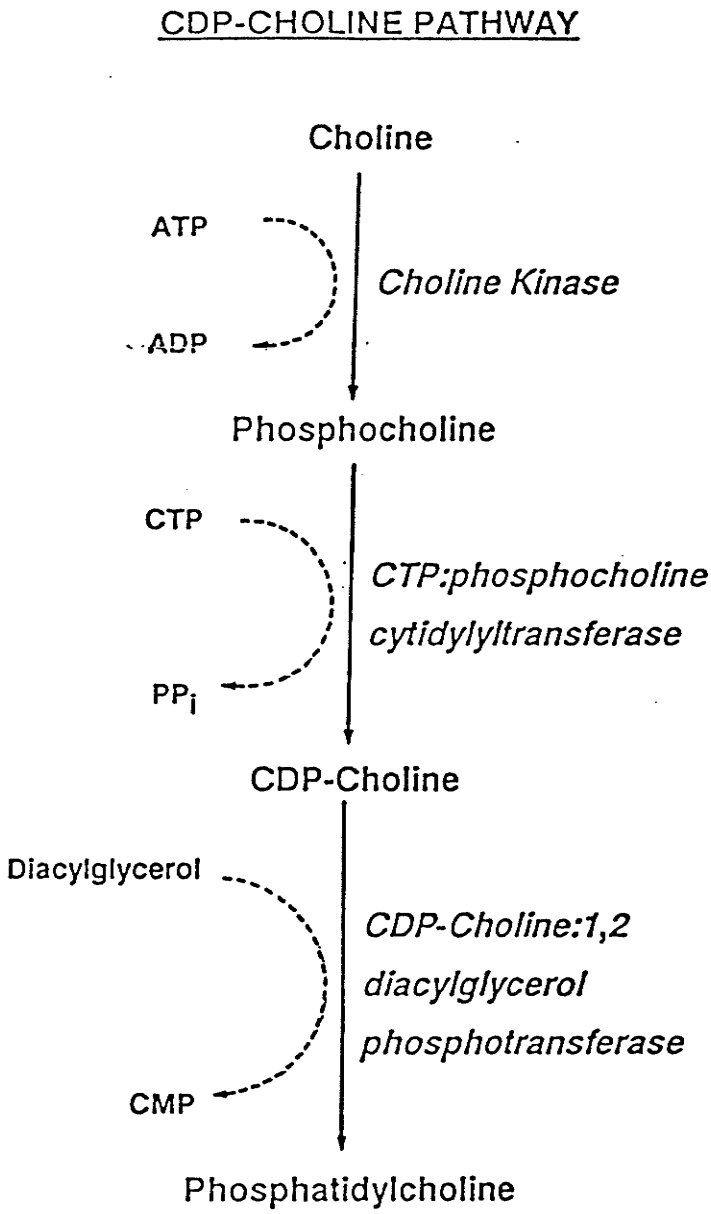


Fig. 5

Phosphatidylcholine biosynthesis via the CDP-choline pathway

ii) CTP:Phosphocholine Cytidylyltransferase

Cytidylyltransferase is located in both cytosolic and microsomal fractions [15]. The step catalyzed by cytidylyl-transferase has been shown to be the rate-limiting step in CDP-choline pathway. The evidence for the rate-limiting role of this reaction was obtained by a number of approaches including pulse-chase studies in HeLa cells [16]. In this study, the radioactivity of the exogenously added [Me-^{3}H]-choline was quickly converted to phosphocholine (97 % at the end of 1 hr). Accumulation of labelled phosphocholine was observed, and the labelled material was transiently associated with CDP-choline and was subsequently converted to phosphatidylcholine.

The rate-limiting step of the CDP-choline pathway was also determined in the isolated heart by pulse-chase studies [17]. The isolated heart was perfused with labelled choline for 5 min followed by non-labelled choline perfusion for 1-60 min. The [Me-^{3}H]-choline taken up by the heart was converted immediately into phosphocholine. The radioactivities associated with choline, phosphocholine, CDP-choline and phosphatidylcholine were determined. The labellings of choline and CDP-choline were lower than that of phosphocholine during the chase period. As the radioactivity in phosphocholine decreased during the chase,

a concomitant increase in phosphatidylcholine labelling was observed. These results clearly demonstrate that the rate-limiting step for phosphatidylcholine biosynthesis is at the conversion of phosphocholine to CDP-choline. This rate-limiting step has also been studied in liver, lung, intestine, kidney and other tissues [18].

Cytidylyltransferase has been purified from rat liver cytosol in 1986 [19]. The purified enzyme appears to contain equal amounts of two nonidentical proteins, with molecular weights of 38,000 and 45,000 [20].

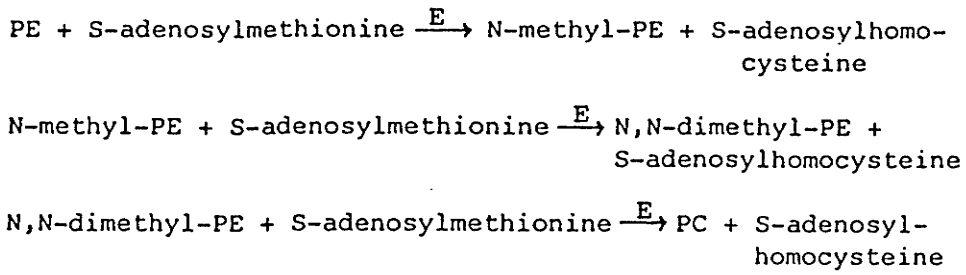
iii) CDP choline : 1,2-diacylglycerol choline phosphotransferase

The final step in the CDP-choline pathway is the formation of phosphatidylcholine from diacylglycerol and CDP-choline by the action of CDP-choline:1,2-diacylglycerol choline phosphotransferase. This enzyme is tightly bound to the microsomal fraction in most tissues [21]. The exception to this is the report of enzyme activity in the lung mitochondria [21-22]. The enzyme has been solubilized and purified 4-5 fold from rat liver microsomes [23]. Some investigators have reported that choline phosphotransferase and ethanolamine phosphotransferase are separate enzymes [18]. At present, there is no evidence to support the separate identities of these two enzymes. Both enzymes have not been purified from any mammalian sources.

b) Progessive Methylation of Phosphatidylcholine

An alternative pathway for phosphatidylcholine biosynthesis is the conversion of phosphatidylethanolamine to phosphatidylcholine via the methylation pathway [24-25]. This pathway was described by Bremer and Greenberg in 1961 [25]. In this pathway (Fig. 6), phosphatidylethanolamine is methylated to phosphatidylcholine by successive transfer of methyl groups from S-adenosyl-methionine. This methylation results in the synthesis of phosphatidyl-monomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME), and finally phosphatidylcholine. The methylation pathway contributes significantly to phosphatidylcholine biosynthesis in the liver where it accounts for the formation of 20 % of phosphatidylcholine [26]. Only 2.5 % of total phosphatidylcholine in hamster heart is formed by methylation of phosphatidylethanolamine [17].

The methylation of phosphatidylethanolamine is catalyzed by phosphatidylethanolamine N-methyltransferase [25,27-28]. This enzyme is localized in the microsomal fraction [18]. Recently, this enzyme has been purified to apparent homogeneity from rat liver microsome [29]. The purified enzyme is composed of a single subunit with a molecular mass of 183,000 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This single membrane-



E: Phosphatidylethanolamine-N-methyltransferase

Fig. 6

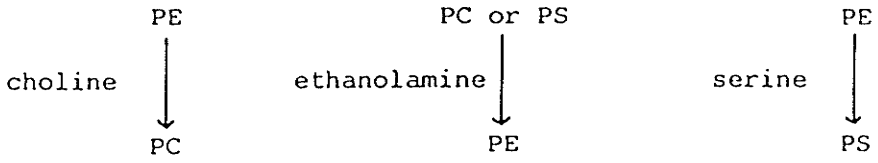
Progressive methylation of phosphatidylethanolamine

bound enzyme catalyzes all three methylation steps for the conversion of phosphatidylethanolamine to phosphatidylcholine. The regulation of this enzyme has not been examined in detail. However, it has been suggested that phosphatidylethanolamine N-methyltransferase is regulated by phosphorylation [30-31]. Glucagon and cAMP analogues cause a 2-fold activation of this enzyme in hepatocytes [18]. The exact regulatory mechanisms on this methyltransferase *in vivo* and *in vitro* still remains to be investigated.

c) Base Exchange Pathway

The biosynthesis of phosphatidylcholine can occur via a third pathway which is the Ca^{2+}-mediated exchange of choline for another phospholipid head group (Fig. 7). The reaction occurs in the microsomal fraction. Dils and Hübscher [32] demonstrated that the exchange of choline with the headgroup of a pre-existing phospholipid to yield phosphatidylcholine is energy-independent and but calcium-dependent [18]. The significance for the base exchange of choline is unknown. However, this pathway appears to be active principally in brain [33-36]. It has also been found that this pathway contributes in a minor way to the biosynthesis of phosphatidylcholine in hamster heart [17].

III. Control of Phosphatidylcholine Biosynthesis in Mammalian Tissues



PE: Phosphatidylethanolamine

PC: Phosphatidylcholine

PS: Phosphatidylserine

Fig. 7

Base exchange reaction

The control of phosphatidylcholine biosynthesis has been extensively studied in the liver and heart as well as other tissues. Phosphatidylcholine biosynthesis in mammalian tissues is highly regulated by a number of control machanisms [18]. For example, phosphatidylcholine biosynthesis is regulated at the levels of choline uptake, the energy status of the cells, modulation of rate-limiting step and the selection of diacylglycerol moieties.

a) Choline Uptake

Choline is taken up by a low-affinity Na^+-independent transport system [37]. Ethanolamine inhibits choline uptake in a competitive manner [38]. On the other hand, neutral amino acids such as glycine, L-alanine, L-serine or L-phenylalanine cause an increase in choline uptake in isolated hamster heart [39-40]. Although short term inhibition of choline uptake by ethanolamine or enhancement by neutral amino acids has no immediate effect on phosphatidylcholine biosynthesis, it has been postulated that prolonged inhibition or enhancement of choline uptake may ultimately affect phosphatidylcholine biosynthesis in the heart.

b) Energy Status of the Organs

ATP is the substrate of choline kinase whereas CTP is

the substrate of cytidylyltransferase. It was reported that the intracellular level of ATP and CTP were reduced in the isolated myopathic hamster heart [41]. The reduction in ATP level in the myopathic heart had no effect on the phosphorylation of choline to phosphocholine. However, the diminished CTP level caused a reduction in CDP-choline formation. In order to maintain the normal rate of phosphatidylcholine biosynthesis, cytidylyltransferase activity in the myopathic heart was activated. These results suggest that the level of CTP and modulation of cytidylyltransferase activity may be two important mechanisms for the regulation of phosphatidylcholine biosynthesis *in vivo* [41].

c) Modulation of the Rate-limiting Enzyme

CTP:phosphocholine cytidylyltransferase is located in both cytosolic and microsomal fractions. Since cytidylyltransferase is the rate-limiting enzyme in the CDP-choline pathway, the regulatory mechanisms of this enzyme have been studied in different tissues [42-44].

One regulatory mechanism is direct activation of cytidylyltransferase by fatty acids [18]. Pelech *et al.* reported that phosphatidylcholine biosynthesis in isolated hepatocytes was stimulated by exogenous fatty acids such as oleic acid and stearic acid [45-46]. Phosphatidylcholine biosynthesis in hamster heart was stimulated only by stearic acid [47]. The stimulation of phosphatidylcholine bio-

synthesis was mediated by the activation of cytidylyl-transferase in the microsoaml fraction [47].

Another regulatory mechanism is proposed in the trans-location theory. The microsomal enzyme is regarded as the active form of the enzyme whereas the cytosolic enzyme is less active [18]. The conversion of the cytosolic enzyme to the microsomal form will activate the conversion of phospho-choline to CDP-choline [18]. Alternatively, the translocation of the microsomal enzyme to the cytosolic form will slow down this reaction [18]. Two different modes of translocation of cytidylyltransferase have been suggested [18]. One involves reversible phosphorylation of the cytidylyltransferase. Indirect evidence shows that trans-location of cytidylyltransferase from one subcellular compartment to another is promoted by phosphorylation-dephosphorylation of the enzyme [48]. Phosphorylation causes a decrease in the cytidylyltransferase activity associated with microsomes [49], whereas dephosphorylation promotes the binding of the enzyme to microsomes where it is activated [49]. A second mechanism involves the association of fatty acids with cytidylyltransferase [45-46]. Fatty acids promote the affinity of cytidylyltransferase for membranes where the enzyme is activated [46].

d) Selection of Molecular Species of Diacylglycerol

Another control factor is acyl specificity of CDP-choline: 1,2-diacylglycerol choline phosphotransferase for diacylglycerol This enzyme is located in the microsomal fraction and catalyzes the final step of the CDP-choline pathway. In the isolated hamster heart, enzyme activity was determined with 1,2-diacylglycerols of known acyl content [50]. Maximum activity was obtained with diacylglycerol containing a monoenoic acyl group. It has been postulated that in the hamster heart, choline phosphotransferase has only limited ability to select the appropriate acyl groups for phosphatidylcholine biosynthesis. It appears that the majority of the newly formed phosphatidylcholine in the heart via the CDP-choline pathway is subsequently re-synthesized by the deacylation-reacylation process [51].

e) Phosphatidylcholine Biosynthesis in Model System
- the Use of Anesthetic as a Probe

The effect of aromatic anesthetics on phosphatidyl-choline biosynthesis is well documented [52-53]. Administration of polychlorinated biphenyls reduced the incorporation of the labelled phosphate into the choline containing phospholipids in rat liver whereas phenobarbital enchanced such incorporation [52]. In a subsequent study [53], it was demonstrated that the administration of phenobarbital result-ed in substantial decreases in choline kinase and microsomal phosphocholine cytidylyltransferase activities.

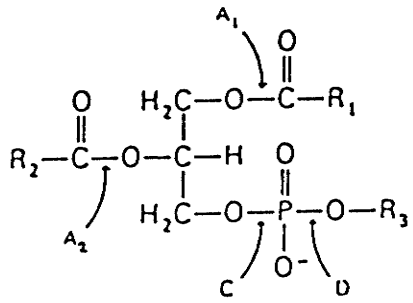
Choline phosphotransferase and cytosolic phosphocholine cytidylyltransferase activities were not significantly affected by such treatment.

Diethyl ether is a commonly administered anesthetic for small laboratory animals. The reduction in the incorporation of labelled ethanolamine into phosphatidylethanolamine in the kidney of diethyl ether-anesthetized hamsters was reported [54]. In the present study, the effect of diethyl ether on phosphatidylcholine biosynthesis in hamster organs was examined.

IV. Phosphatidylcholine Catabolism in Mammalian Heart

Phospholipases are a group of enzymes which catalyze the hydrolysis of ester bonds in glycerophospholipids [55]. Each phospholipase has its own specificity. Fig. 8 shows the classification of the phospholipases based on the site of hydrolysis. These phospholipases include phospholipases A_1, A_2, C and D. The phospholipases A are acyl hydrolases which hydrolyze 1-acyl ester (phospholipase A_1) or the 2-acyl ester (phospholipase A_2). Phospholipase C catalyzes the cleavage of the glycerol phosphate bond and phospholipase D catalyzes the removal of the base group. Both phospholipase C and phospholipase D are phosphodiesterases.

The principal catabolic pathway for phosphatidylcholine is through the hydrolytic action of phospholipase A_1 or A_2,



A_1: Phospholipase A_1

A_2: Phospholipase A_2

C: Phospholipase C

D: Phospholipase D

Fig. 8

Sites of action of phospholipases

which releases an acyl group and results in the formation of lysophosphatidylcholine [55]. Lysophospholipases catalyze the hydrolysis of the remaining acyl group in the lysophospholipids.

Phospholipid metabolism in mammalian cells is in a dynamic state. The content and composition of the membrane phospholipids are closely regulated by the rates of biosynthesis and catabolism. It has been postulated that lipid catabolism may be connected with the maintenance of cellular viability, and may involve in the 'repair' and maintenance of the membranes of the living cell [56].

V. Regulation of Phosphatidylcholine Catabolism in Mammalian Heart

Phosphatidylcholine is the major phospholipid (35-45% total phospholipids) in mammalian heart [57]. Its content and composition in the cardiac membrane are under rigid control. It has been postulated that, regardless of etiological factors, cardiac failure is always associated with changes in the membrane phospholipid composition [58]. Such changes may result from abnormalities in the catabolism of the phospholipid.

a) Phospholipases A

It has been reported that phospholipase A activity is ubiquitously distributed in all subcellular fractions in

hamster heart, but high specific activity has been found in the microsomal fraction [59]. Recently our laboratory has reported the purification of a cytosolic protein in the hamster heart which exhibits both phospholipase A_1 and A_2 activities [60]. The purified phospholipase A is active towards the acyl groups of phosphatidylcholine and phosphatidylethanolamine, but not lysophosphatidylcholine. With purified enzyme, our laboratory has demonstrated for the first time that the activity of phospholipase A_1 or A_2 is dependent on both the C-1 and C-2 acyl groups of phosphatidyl-choline. For example, both phospholipases favor a highly unsaturated acyl group at C-2. The phospholipase A_1 activity prefers a C-1 steroyl group, whereas phospholipase A_2 activity exhibits a higher specificity toward a C-1 palmitoyl group.

b) Remodelling of the Acyl Group in Phospholipids

Lysophosphatidylcholine generated by the action of phospholipase A can be further deacylated by lysophospholipase or , alternatively, it can be reacylated to the parent phospholipid through the reacylation reaction (Fig. 9). Reacylation of lysophospholipids, catalyzed by the action of acyltransferase which is located in both mitochondria and microsomes, serves the part of the mechanism for the remodelling of the fatty acyl groups of cellular phospholipids [61]. It has been demonstrated that the

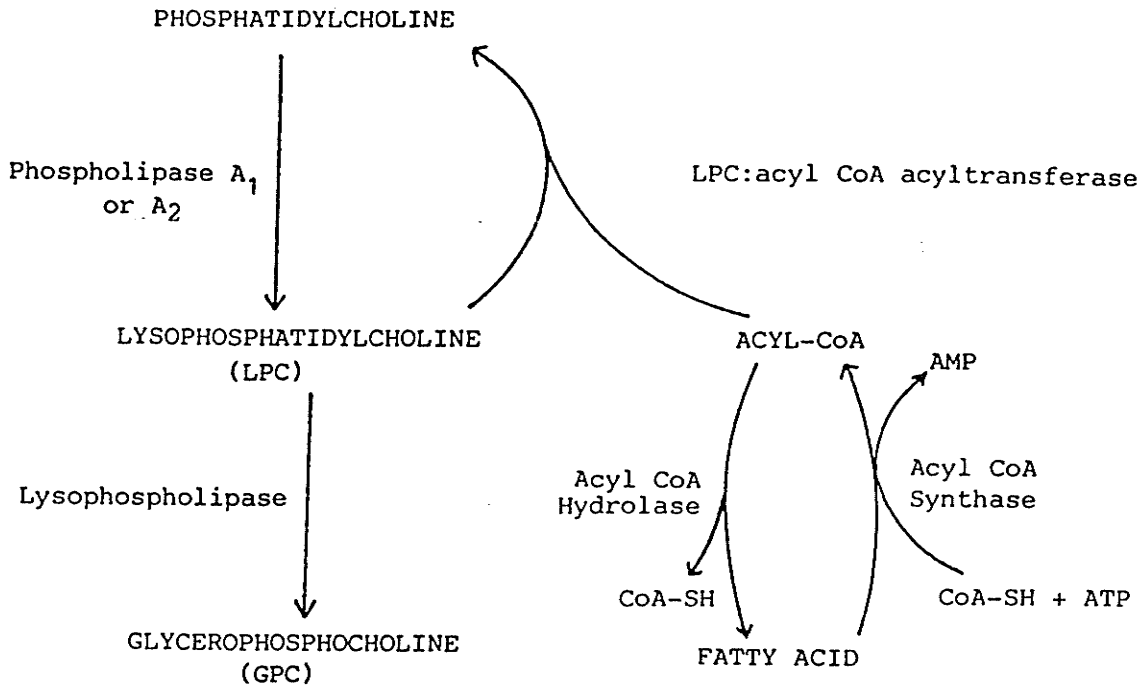


Fig. 9

The deacylation-reacylation pathway of phosphatidylcholine

deacylation-reacylation of intracellular phosphatidylcholine is a facile mechanism for the cell to obtain phosphatidylcholine with the required acyl groups [9,61]. Reacylation of exogenous lysophosphatidylcholine may also be an important pathway for formation of phosphatidylcholine. It has been reported that lysophosphatidylcholine is transported actively into the heart and then reacylated exclusively by the action of lysophosphatidylcholine:acyl CoA acyltransferase [62]. In the same study, it has been found that phosphatidylcholine is not formed from transacylation of two lysophosphatidylcholine molecules but by a reacylation process. From the pool size of total lysphosphatidylcholine, the amount of phosphatidylcholine formed via the reacylation process was estimated to be 6.6 nmol/min/g heart. It has been postulated that the reacylation of lysophosphatidylcholine and acyl-CoA represents a major pathway for the resynthesis of phosphatidylcholine and 14 % of the total phosphatidylcholine in the heart may be formed via this pathway [62].

c) Control of the Level of Lysophosphatidylcholine in the Mammalian Heart

It is obvious that the reacylation process is an important mechanism for the remodelling of the acyl groups in phosphatidylcholine in the heart. Together with lysophospholipase, acyltransferase serves as one of the principal

enzyme for the eliminiation of lysophospholipids in the cell.

Since all lysophospholipids are cytolytic at high concentrations, the maintenance of normal cellular function necessitates a rigid control of their transient presence [63]. The accumulation of lysophospholipids in the ischemic myocardium has been suggested as one of the biochemical factors in the generation of arrhythmias [64-65]. The development of cardiac arrhymias following the onset of cardiac ishchemia has been well established [66]. However, the exact biochemical factors involved in the production of cardiac arrhythmias *in vivo* remain obscure.

The direct action of lysophosphatidylcholine and other lysophospholipids on cardiac arrhythmias has been observed in isolated perfused hamster hearts [67]. During short intervals of experimentally produced ischemia, elevated levels of lysophosphatidylcholine in the ischemic myocardium have been reported [64-65]. Recently, Man *et al* investigated the relationship between the production of arrythmias and the elevation of tissue lysophosphatidylcholine level [68]. In these studies, a temporal relationship between the accumulation of lysophosphatidylcholine and occurrence of arrhythmias was established after five hours of ischemia. A significant elevation of lysophosphatidylcholine was detected at three hours of ischemia without the occurrence

of arrhythmias. The results indicate that the cardiac arrhythmias do not cause the elevation of lysophosphatidylcholine. Alternatively, if lysophospholipids are causually related to the production of arrhythmias, a critical level of the lysophospholipid must accumulate in order to elicit electrophysiological alterations.

A significant increase in lysophosphatidylcholine was observed in liver of vitamin E-deficient rats [69]. Such an increase was attributed to an increase in mitochondrial phospholipase A_2 activity [69]. More recently, the direct inhibitory effect of vitamin E on platelet phospholipase A_2 has been demonstrated [70]. These studies clearly show that vitamin E may act as a modulator of phospholipid metabolism in mammalian tissues. Although the effect of vitamin E on cardiac phospholipid metabolism was not assessed electrocardiac abnormalities and cardiac necrosis as a direct result of vitamin E deficiency in the rabbit heart have been well documented [71]. Abnormalities in membrane conductivity and subsequent necrosis appeared to originate from the membrane defects that might result from altered phospholipid metabolism. In the present study we provide evidence that cardiac phospholipase A activity and, consequently, the level of lysophosphatidylcholine in the heart, can be regulated by dietary vitamin E level.

MATERIALS AND METHODS

A. MATERIALS

I. Animals

Male Syrian golden hamsters, 120 ± 10 g, were used for the study of phosphatidylcholine biosynthesis. These animals were maintained on Purina hamster chow and tap water, *ad libitum* in a light and temperature-controlled room. Weanling male Sprague-Dawley rats were used for the study of phosphatidylcholine catabolism.

II. Chemicals

Choline iodine, phosphorylcholine, CDP-choline, synthetic vitamin E (-DL-tocopherol) and natural vitamin E (-D-tocopherol acetate) were obtained from Sigma Chemical Company, St. Louis, MO. [Me-^{3}H]-choline and CDP-[Me-^{14}C]-choline were purchased from NEN division, DuPont Company, Dorval, Quebec. 1-[1-^{14}C] Palmitoyllysophosphatidylcholine (58.5 mci/mmol), 1-stearoyl-2-[^{14}C] arachidonyl-glycerophosphocholine (60.1 mci/mmol), and aqueous counting scintillant were obtained from Amersham International. Phosphatidylcholine (from pig liver), lysophosphatidylcholine (from pig liver) and other lipid standards were purchased from Serdary Research Laboratory (London, Ontario, Canada). Thin-layer chromatographic plates (sil-G25) were the product of Brinkmann, Rexdale, Ontario. CM-cellulose (CM-11) was the product

of Whatmann. All other chemicals and TLC plates (Redi-plate silica-gel G) were obtained from the Fisher Scientific Co. Phospho-[Me-^{3}H]-choline was synthesized enzymatically from [Me-^{3}H]-choline by the action of yeast choline kinase [72]. Labelled phosphatidyl-[Me-^{3}H]-choline was isolated from the rat heart after perfusion with [Me-^{3}H] choline [59].

B. METHODS

I. Phosphatidylcholine Biosynthesis Studies

a) Animal Anesthesia

Diethyl ether was administered to the hamsters by inhalation [54]. The animal was placed in 2 L sealed container and a cotton ball containing 2 ml of dithyl ether was introduced into the container. Subsequent to the loss of the right reflex (2-4 min), the animal was removed from the container and labelled choline (0.5 ml of a 20 mM choline chloride solution) containing 20 uCi was injected intraperitoneally. Light anesthesia was admistered by the inhalation of a diethyl ether / air mixture for the next 60 min. A minimum respiratory rate of 40 per min was maintained throughout the experiment. Identical doses of labelled choline were injected into the non-anesthetized animals and these animals were used as controls. At the prescribed time interval, the control and the anesthetized animals were sacrificed by decapitation, and the organs were rapidly removed and placed

in ice-cold saline (0.9 % NaCl).

b) Uptake of Labelled Choline by the Hamster Organs

The heart, liver, kidney, lung, brain and spleen, taken from anethetized or contol hamsters, were washed with ice-cold saline and their wet weights were determined. The organs were cut into small pieces and then homogenized in chloroform/methanol (2:1; v/v) with a Polytron homgenizer (Brinkman PT 10/35) to yield a 10% (w/v) homogenate. The homogenate was centrifuged and the pellet was re-extracted twice with chloroform/methanol (2:1; v/v). The extracts were pooled and an aliquot was taken for the determination of total uptake of radioactivity by organs.

c) Analysis of Phospholipids

Phase separation of the pooled extract was achieved by adding water to the extract until chloroform/methanol/water ratio of 4:2:3 (v/v) was obtained. The upper phase (aqueous) was removed by Pasteur pipette and placed in a round bottom flask and the solvent was removed by evaporation *in vacuo*, at 37 °C. The content was resuspended in 1 ml of water, transferred to a tube and stored at -20 °C. The lower phase (organic) was dried by evaporation *in vacuo*, at 30 °C. The content was resuspended in 3 ml of chloroform/methanol (2;1; v/v) and the solution was filtered (Whatman 4 qualitative filter paper) into a screw cap tube. The solvent was then

totally removed by evaporation under nitrogen. 1 ml of chloroform was added to the tube and stored at -20 °C. Radioactivity in the organic phase was determined by liquid scintillation counting . Phosphatidyicholine in the organic phase was analyzed by thin-layer chromatographic plate (Redi-plate, Silica gel G, Fisher Scientific) with a solvent containing chloroform/methanol/acetic acid/water (70:30:2:4; v/v) [73]. Phosphatidylcholine on the thin-layer chromatogram was visualized by exposure of the plate to iodine vapor in a closed chamber.

d) Analysis of Choline-containing Metabolites

Choline-containing metabolites in aqueous phase were analyzed by thin-layer chromatography (TLC plates, sil-G25, Brinkman) with a solvent containing methanol/0.6% sodium chloride/ammonium hydroxide (50:50:5; v/v) [17]. The location of CDP-choline fraction was visualized under UV light. The locations of choline and phosphocholine fractions were visualized by exposure of the plate to iodine vapor. Since a substantial amount of choline was oxidized into betaine in the liver [18], the labelling of betaine was also determined. Betaine was separated from CDP-choline after thin-layer chromatography by a Norit A charcoal column (0.5 x 3 cm) equilibrated with 2 % ethanol whereas CDP-choline was eluted by subsequent addition of 10 ml of 40 % ethanol containing 1% ammonium hydroxide [17].

e) Preparation of Subcellular Fractions

Hamsters were anesthetized by diethyl ether as described previously [54]. Non-anesthesized hamsters were used as controls. These hamsters were sacrificed by decapitation and the heart, liver, kidney, lung, brain and spleen were rapidly removed and placed in ice-cold saline. The organs were weighed and homogenized in 0.145 M NaCl-5 mM Tris-HCl (pH 7.4) to yield a 10% (w/v) homogenate. The homogenate was centrifuged at 12,000 x g for 15 min and the supernatant was subsequently centrifuged at 100,000 x g for 60 min. The resulting supernatant was designated as cytosolic fraction. The pellet obtained from high speed centrifugation was resuspended in 0.145 M NaCl-5 mM Tris-HCl (pH 7.4) which is defined as microsomal fraction. Choline kinase activity was determined in the cytosol whereas the CTP:phosphocholine cytidylyltransferase activities were determined in the cytosolic and microsomal fractions. Choline phosphotransferase activity was determined in the microsomal fraction.

f) Assay of Enzyme Activities for Phosphatidylcholine Biosynthesis in Hamster Organs

i) Choline Kinase

Choline kinase in the cytosolic fraction was assayed with labelled choline [41]. The reaction mixture contained 80 ul cytosol, 20 ul 1.0 M Tris-HCl (pH 8.0), 20 ul 0.1 M

$MgCl_2$, 20 ul 0.1 M ATP, 50 ul water and 10 ul 10 mM [Me-^{3}H]-choline in a final volume of 200 ul. The mixture was incubated at 37 C for 20 min. The reaction was stopped by boiling for 3 min. The mixture was then centrifuged at 2,000 rpm for 10 min and 50 ul of supernatant was applied onto a thin-layer chromatographic plate with unlabelled phosphocholine as carrier. The thin-layer chromatographic plate was developed in a solvent containing methanol/0.6% NaCl/ ammonium hydroxide (50:50:5; v/v). Phosphocholine fraction on the plate was visualized by exposure of the plate to iodine vapor. The fraction was removed and the radioactivity associated with phosphocholine was determined by liquid scintillation counting.

ii) CTP:Phosphocholine Cytidylyltransferase

CTP:phosphocholine cytidylyltransferase in the cytosolic and microsomal fractions was assayed with labelled phosphocholine at exactly 4 hours after the tissues were homogenized [74]. A typical reaction mixture contained 10 ul 0.12 M Magnesium acetate, 10 ul 1 M Tris-succinate (pH 8.0), 10 ul 25 mM CTP, 10 ul mM phosph-[Me-^{3}H]-choline and 60 ul either cytosol or microsomal preparation. In addition, cytosolic cytidylyltransferase activity was determined in the presence of total liver phospholipid extract (2 mg / ml) and 0.1 mM oleate. Maximal stimulation of the cytosolic cytidylyltransferase was obtained in the presence of these

lipids [75]. The mixture was incubated at 37 °C for 30 min and the reaction was stopped by boiling for 3 min. An aliquot was applied onto a thin-layer chromatographic plate with unlabelled CDP-choline as carrier. The thin-layer chromatographic plate was developed in a solvent containing methanol/0.6 % NaCl/ammonium hydroxide (50:50:5; v/v). The CDP-choline fraction was visualized under UV light. The fraction was removed and radioactivity determined by liquid scintillation counting.

iii) CDP-choline: 1,2-diacylglycerol choline phospho transferase

Microsomal CDP-choline:1,2-diacylglycerol choline phosphotransferase was assayed with labelled CDP-choline in the presence of Tween 20 and exogenous diacylglycerol [50]. The reaction mixture contained 100 ul microsome, 100 ul 1.0 M Tris-HCl (pH 8.5), 100 ul 0.1 M $MgCl_2$, 100 ul 50 mM EDTA, 100 ul 2 uM diacylglycerol (in 1 % Tween 20), 400 ul water and 4 mM [Me-^{14}C] CDP-choline (specific radioactivity=0.1 uCi/umol). The mixture was incubated at 37 °C for 15 min and the reaction was stopped by adding 6 ml chloroform/methanol (2:1; v/v). Two milliliter of 0.9% NaCl was added to cause phase separation. The mixture was centriguged at 2,000 rpm for 10 min and the organic phase was washed two additional times with 4 ml 40% methanol. The organic phase was then evaporated and the radioactivity determined.

g) Extraction and Preparation of Total Hamster Liver Phospholipids

Hamsters were sacrificed by decapitation and the livers were removed and placed on ice-cold saline. The livers were weighed and cut into small pieces. The tissue was homogenized in chloroform/methanol (2:1; v/v) to yield 33% homogenate (w/v). The homogenate was centrifuged at 2,000 rpm for 10 min and the supernatant removed and placed in a separatory funnel. The pellet was extracted twice with 10 ml of chloroform/methanol/water (1:2:0.8; v/v). The extracts were pooled and the phase separation was achieved by the addition of 10 ml chloroform and 10 ml water. The lower phase was removed and the upper phase was washed with 20 ml chloroform/methanol/water (86:14:1; v/v). The lower phase from this wash was removed and combined with the extract from the first separation. The solvent was removed *in vacuo* and the lipid was resuspended in chloroform/methanol (2:1; v/v) [76]. Quantitation of lipid phosphorus was determined by the method of Bartlett [77].

h) Preparation of phospho-[Me-^{3}H]-choline

Phospho-[Me-^{3}H]-choline was synthesized from [Me-^{3}H]-choline [72]. The incubation mixture contained 0.1 M Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP, 0.1 mM choline chloride, 0.1 mM [Me-^{3}H]-choline (10 uCi/nmol) and 1 unit of

choline kinase in a final volume of 1.0 ml. The reaction was started by the addition of enzyme and incubated overnight at 37 °C. The reaction was stopped by boilling for 3 min and the mixture was centrifuged at 2,000 rpm for 15 min. The supernatant was applied to a thin-layer chromatographic plate (100 ul/4 cm lane) which was developed in methanol/0.6% NaCl/ammonium hydroxide (50:50:5; v/v). The fraction corresponding to phosphocholine was scraped from the plate and placed into a glass-fibre-plugged Pasteur pipet. The phospho-[Me-^{3}H]-choline was eluted with 20 ml of distilled water. The eluents was lyophilized and the phospho-[Me-^{3}H]-choline was redissolved in water at approximately 100 uCi/ml and stored at -20 °C in small aliquots. The yield was about 70%.

II. Phosphatidylylcholine Catabolism Studies

a) Animals and Diet

Weanling male Sprague-Dawley rats were fed a purified diet consisting of (% by weight): vitamin-free casein (20), DL-methionine (0.3), corn starch (10.2), dextrose (50), Alphacel fibre (5), salt mix 4164 (3.5), AIN (American Institute of Nutrition) vitamin mix without tocopherol (1.0) and stripped corn oil (10.0) [78]. A batch of 20 rats was separated into three groups and fed on this diet containing (a) no added vitamin E, (b) 50 p.p.m.of vitamin E or (c)

5000 p.p.m. of vitamin E as α-D-tocopherol acetate. After 4 months of feeding, vitamin E deficiency or sufficiency was verified by plasma tocopherol values and pyruvate kinase activity [78-79]. The animal was put under light diethyl ether anesthesia and a sample of blood was obtained by aortic puncture [70]. The heart was rapidly removed and stored at -70 °C until used.

b) Pyruvate Kinase and Vitamin E Analysis

The activity of pyruvate kinase which is an indicator of myopathy and the level of plasma vitamin E were determined by Dr. A.C. Chan. An aliquot of the plasma was used for the determination of pyruvate kinase activity [79]. Plasma vitamin E content was analyzed by high-pressure liquid chromatography (HPLC) as described by Bieri [80] with all-rac-α-tocopherol acetate as internal standard.

c) Preparation of Subcellular Fractions from Rat Heart

Each rat heart was cut into small pieces and homogenized in 10 ml of 20 mM Tris-HCl buffer (pH 7.5), containing 0.25 M sucrose and 1 mM EDTA at 4 °C. The subcellular fractions were prepared by differential centrifugation as described previously [50].

d) Preparation of Labelled Phosphatidylcholine

Labelled phosphatidyl-[Me-^{3}H]-choline was prepared by

perfusion of [Me-^{3}H] choline into the isolated rat heart [59]. The isolated rat heart was perfused in the Langendorff mode with 10 mM Krebs-Henseleit buffer containing 5 uM of [Me-^{3}H] choline (6-8 uCi/ml). After 90 min of perfusion, the heart was homogenized in chloroform/methanol (2:1; v/v) and the precipitable materials were sedimented by centrifugation. Chloroform and water were added to the supernatant to a final ratio of 4:2:3 (v/v) of chloroform/methanol/water. After phase separation, the solvent in the lower phase was removed by evaporation and the lipid residue was reconstituted in chloroform/methanol (2:1; v/v). Phosphatidylcholine was separated from the other lipids in the sample by thin-layer chromatography with a solvent containing chloroform/methanol/water/acetic acid (70:30:4:2; by volume). The labelled phosphatidyl-[Me-^{3}H] choline was eluted from the silica gel and the specific radioactivity was 8,000-10,000 dpm/noml.

e) Phospholipase A assay

Phospholipase A activity was assayed with phosphatidyl-[Me-^{3}H]-choline as substrate [59]. The reaction mixture (0.5 ml) contained 20 mM Tris/HCl (pH 8.5), 5 mM $CaCl_2$ and 200-500 nmol of labelled phosphatidylcholine (3,000-4,000 dpm/nmol) dispersed in water by sonication. Owing to the presence of lysophospholipase activity in some subcellular fractions, 200 nmol of unlabelled lysophosphatidylcholine

was also included in the reaction mixture. As demonstrated previously, the presence of lysophosphatidylcholine effectively inhibited the further hydrolysis of lysophosphatidyl-[Me-^{3}H]-choline formed, but did not significantly inhibit the hydrolysis of phosphatidylcholine [59]. The assay was initiated by the addition of enzyme (0.3-0.5 mg of subcellular fractions) to the mixture containing the buffer and substrate, and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 1.5 ml of chloroform/methanol (2:1; v/v). Water was added to cause phase separation. The lysophosphatidylcholine in the lower phase was isolated by thin-layer chromatography with a chloroform/methanol/water/acetic acid (70:30:4:2; v/v) solvent system. When the enzyme activity was assayed in the presence of vitamin E, the vitamin was first dissolved in 6% (v/v) dimethyl sulphoxide [70] and 0.1 ml (containing the appropriate amount of vitamin E) was added to the assay mixture and preincubated for 10 min at 37 °C before the addition of the labelled substrate. The reaction was then initiated by the addition of the labelled substrate. Enzyme activity in the presence of 0.1 ml of 6% dimethyl suphoxide was used as control.

f) Determination of Phospholipase A_1 and A_2 Activities

To assay for phospholipase A_1 and phospholipase A_2 activities, 1-stearoyl-2-[^{14}C]-arachidonylglycerophospho-

choline was used as substrate [59]. The conditions of the assay were identical with those described in the preceding sub-section. After the reaction, radioactivities in the fatty acid and lysophosphatidylcholine fractions were determined. Phospholipase A_1 activity was calculated from the radioactivity in the lysophosphatidylcholine fraction, whereas phospholipase A_2 activity was estimated in the same manner from the fatty acid fraction.

g) Lysophospholipase Assay

Lysophospholipase activities in microsomal and cytosolic fractions of rat hearts were assayed with 1-[1-^{14}C]-palmitoyl-sn-glycerol-3-phosphocholine [62]. The reaction mixture (0.5 ml) contained 20 mM Tris/HCl (pH 7.0), 0.2 mM labelled lysophosphatidylcholine (1,200 dpm/nmol) and 0.3-0.5 mg of enzyme protein. The reaction was initiated by the addition of microsomal or cytosol preparation. After incubation at 37 ℃ for 30 min, the reaction was terminated by the addition of chloroform/methanol (2:1; v/v). The lipids were separated by thin-layer chromatography with a solvent system of chloroform/methanol/water/acetic acid (70:30:4:2; v/v). Enzyme activity was calculated from the radioactivity associated with the fatty acid fraction.

h) Partial purification of Phospholipase A from Rat Cytosol

Rat heart cytosol was applied to a column (1 X 21 cm) of CM-cellulose equilibrated with 50 mM Hepes buffer / 5 mM-EDTA, pH 7.4. The column was washed with 120 ml of the same buffer, followed by the application of a linear gradient of 0-0.5 M NaCl in 200 ml of the same buffer. Each fraction was assayed for phospholipase A activity. The majority of phospholipase A (containing both phospholipase A_1 and A_2 activities) was eluted from the column at 0.1 M NaCl. The fractions containing high enzyme activities were pooled and used for kinetic studies.

III. Other Procedures

a) Protein Assay

Protein concentrations of subcellular fractions were determined by the method of Lowry *et al* [81]. Subcellular fractions of 5 ul or 10 ul were incubated in 1.5 ml of 0.66 N NaOH at 37 C overnight. Afterwards, 1.5 ml of Reagent A (containing 1 ml of 2% $CuSO_4$, 1ml of 4% sodium potassium tartrate and 33 ml of 13% Na_2CO_3) was added to each tube and mixed thoroughly. The mixture was allowed to sit at room temperature for 10 min and 0.5 ml 2N phenol reagent was then added. The reaction mixture was incubated at room temperature for 30 min. Absorbance was measured at 625 nm with bovine serium albumin as standard (5-120ug/tube).

b) Separation of Phospholipids

An aliquot of tissue lipid extract or phospholipid standards were applied to a thin-layer chromatogaraphic plate. The plate was developed in chloroform/methanol/water/ ammonium hydroxide (70:30:4:2; v/v). The fraction corresponding to a phospholipid standard was scraped and placed in a test tube. Subsequently, 4 ml of chloroform/methanol/water/ acetic acid (50:39:10:1; v/v) was added into the tube and the mixture was centrifuged. The supernatant was removed and the pellet was washed twice with same solution. The supernatants were combined and 4 ml of 4 M ammonium hydroxide was added to cause phase separation. The upper phase (aqueous) was discarded and the solvent in the lower phase (organic) was evaporated under nitrogen. The content was redissolved in chloroform and an aliquot was taken for lipid phosphorus determination.

c) Determination of Lipid Phosphorus by Acid Digestion

The lipid phosphorus was determined by the method of Bartlett [77]. An aliquot of the sample was taken and the solvent was evaporated under nitrogen. Inorganic phosphorus was used as standards (0-20.00 ug). Perchloric acid (1.1 ml) was added to each sample and the mixture was incubated at 160 $^{\circ}$C for 2 hours. The mixture was then allowed to cool to room temperature. Subsequently, 8 ml water and 0.8 ml of 5% ammonium molybdate were added and mixed thoroughly. Freshly prepared 1-amino, 2-naphol, 4-sulphonic acid reagent (0.2

ml) was added to each tube. These tubes were placed in a boiling water bath for 10 min. After cooling to room temperature, the absorbance was measured at 830 nm.

d) Statistical Analysis

All results are expressed as mean + standard deviation. The statistical significance was determined by Student's t-test. The level of signifance was $p<0.05$.

e) Radioactivity Determination

Radioactivity was determined by liquid scintillation counting using channels' ratio calibration method.

EXPERIMENTAL RESULTS

A. REGULATION OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS - Effect of diethyl ether on hamster organs

I. Incorporation of Radioactivity into Phosphatidylcholine in Hamster Organs.

The effect of diethyl ether on the labelling of phosphatidylcholine in hamster organs was investigated in a time couse study. Choline chloride solution (0.5 ml of a 20 mM, 20 uCi) was injected into ether-anesthetized hamsters intraperitoneally. Identical doses of labelled choline were injected into unanesthetized hamsters in same manner and these hamsters were used as controls. The control and ether-treated hamdsters were sacrificed at prescribed time intervals and the phosphatidylcholine content and labelling of phosphatidylcholine in the organs were determined. Ether anesthesia had no effect on the total phosphatidylcholine content (umol of Pi/g wet weight) in hamster organs at 30 and 60 min of treatment. However, at 30 min of ether anesthetic treatment, a 12% decrease ($0.05<P<0.1$) in phosphatidylcholine labelling was observed in the kidney. At 60 min, the decrease in phosphatidylcholine labelling became more prominent (29%). No significant change in labelling of phosphatidylcholine was detected in other organs (Table 1). There was no change in the labelling of lysophosphatidylcholine in the tested organs during ether anesthesia (Table

Table 1

Incorporation of Radioactivity into Phosphatidylcholine in Hamster Organs during Diethyl Ether Anesthesia

[Methyl-^{3}H]choline was injected intraperitoneally into control and diethyl ether anesthetized hamsters as described in Methods. After 60 minutes, hamsters were decapitated and the organs were removed and homogenized in chloroform:methanol (2:1, v/v). The homogenates were separated into aqueous and organic phases by the addition of chloroform and water. Phosphatidylcholine in the organic phase was isolated by thin-layer chromatography. The results are depicted as mean±standard deviation (number of experiments).

Tissue	Control	Diethyl ether-treated
	dpm/g wet weight ($x10^{-5}$)	*dpm/g wet weight ($x10^{-5}$)*
Heart	1.25±0.45 (8)	0.95±0.37 (8)
Liver	8.27±1.36 (10)	9.58±1.80 (10)
Kidney	8.19±2.20 (8)	5.84±1.36* (8)
Lung	7.58±1.74 (5)	6.22±0.50 (5)
Brain	0.31±0.04 (5)	0.32±0.04 (5)
Spleen	2.77±0.44 (4)	2.37±0.65 (3)

*$P<0.05$

2).

II. Total Uptake of [Me-^{3}H] Choline by Hamster Organs

One factor which might cause the reduction of phosphatidylcholine labelling in the kidney was a decrease in the uptake of labelled choline. Hence, total labelled choline uptake in hamster organs at 60 min of treatment was determined (Table 3). The total radioactiveity taken up by the kidney was similar between the control and anesthetized hamsters. Although the total choline uptake in the liver might be slightly increased during anesthesia, such differences were not statistically significant.

III. Radioactivity Incorporated into Choline-containing Metabolites

a) Hamster Kidney

The labelling of choline-containing metabolites in the CDP-choline pathway in the kidney at 60 min of ether treatment was also determined. As depicted in Table 4, labelling of phosphocholine was substantially increased (41%). Such increase suggests that the ability to convert phosphocholine into CDP-choline was impaired in the kidney during ether anesthesia.

b) Hamster Liver

Table 2

Incorporation of Radioactivity into Lysophosphatidylcholine in Hamster Organs during Diethyl Ether Anesthesia

[Methyl-^{3}H]choline was injected intraperitoneally into control and diethyl ether anesthetized hamsters as described in Methods. After 60 minutes, hamsters were decapitated and the organs were removed and homogenized in chloroform:methanol (2:1, v/v). The homogenates were separated into aqueous and organic phases by the addition of chloroform and water. Lysophosphatidylcholine in the organic phase was isolated by thin-layer chromatography. The results are depicted as mean±standard deviation (number of experiments).

Tissue	Control	Diethyl ether-treated
	dpm/g wet weight ($x10^{-3}$)	*dpm/g wet weight ($x10^{-3}$)*
Heart	1.55±0.17 (8)	1.51±0.30 (8)
Liver	7.26±5.61 (8)	7.12±3.32 (8)
Kidney	6.65±1.05 (8)	7.95±5.48 (8)
Lung	8.35±1.25 (4)	9.94±2.76 (4)
Brain	2.10±1.75 (4)	1.70±1.18 (4)
Spleen	9.00±3.84 (4)	9.32±0.47 (4)

Table 3

Total Uptake of [Methyl-^{3}H]Choline by Hamster Organs

[Methyl-^{3}H]choline was injected intraperitoneally into control and diethyl ether anesthetized hamsters as described in Methods. After 60 minutes, hamsters were decapitated and the organs were removed and homogenized in chloroform:methanol (2:1, v/v). An aliquot was taken for radioactivity determination. The results are depicted as mean±standard deviation (number of experiments).

Tissue	Control	Diethyl ether-treated
	dpm/g wet weight (x10^{-5})	*dpm/g wet weight (x10^{-5})*
Heart	4.64±1.71 (8)	4.14±1.01 (8)
Liver	24.34±4.17 (10)	27.64±2.92 (10)
Kidney	18.75±4.64 (8)	18.56±4.52 (8)
Lung	11.68±2.47 (5)	12.01±4.30 (5)
Brain	1.87±0.55 (5)	1.40±0.15 (5)
Spleen	9.07±2.53 (4)	8.58±1.14 (4)

Table 4

Radioactivity Incorporated into Choline-containing Metabolites in Hamster Kidney

Tissue homogenates were separated into aqueous and organic phase by the addition of chloroform and water as described in Methods. The aqueous phase was analyzed for labelled metabolites of the CDP-choline pathway. The results are depicted as mean±standard deviation (number of experiments).

Metabolites	Control	Diethyl ether-treated
	dpm/g wet weight ($x10^{-5}$)	
Choline	0.34±0.23 (8)	0.30±0.18 (8)
Phosphocholine	1.45±0.56 (8)	2.05±0.17* (8)
CDP-Choline	0.32±0.25 (8)	0.56±0.30 (8)

*$P<0.05$

Since the uptake of labelled choline in the liver might also be perturbed by ether anesthesia (Table 3), the labelling of the hepatic choline-containing metabolites was also determined. No significant defference was detected between the two animal groups (Table 5). In addition, labelling of betaine was not significantly different between the control (6.53×10^5 dpm/g wet weight) and experimental animals (5.78×10^5 dpm/g wet weight). These results confirm that total choline uptake in the liver was not changed between the control and ether treated animals.

IV. The Activities of Phosphatidylcholine Biosynthetic Enzymes from Hamster Organs

a) Choline Kinase and Choline Phosphotransferase

The accumulation of radioactivity in the phosphocholine fraction in the kidney of the anesthetized hamster might be caused by a change in the activity of the enzymes in the CDP-choline pathway. Thus the activities of these enzymes in the hamster organs were determined after 60 min of ether administration. In the kidney, no significant change in specific activities of both choline kinase and choline phosphotransferase was detected between the two animal groups (Table 6).

b) CTP:Phosphocholine Cytidylyltransferase

CTP:Phosphocholine cytidylyltransferase activity was

Table 5

Radioactivity Incorporated into Choline-containing Metabolites in Hamster Liver

Tissue homogenates were separated into aqueous and organic phase by the addition of chloroform and water as described in Methods. The aqueous phase was analyzed for labelled metabolites of the CDP-choline pathway. The results are depicted as mean±standard deviation (number of experiments).

Metabolites	Control	Diethyl ether-treated
	dpm/g wet weight ($x10^{-5}$)	
Choline	0.15±0.02 (4)	0.17±0.03 (3)
Phosphocholine	1.51±0.46 (4)	1.46±0.24 (3)
CDP-Choline	0.02±0.01 (4)	0.02±0.01 (3)

Table 6

The Activities of Phosphatidylcholine Biosynthetic Enzymes from Hamster Kidney

Kidneys were obtained from control and diethyl ether-anesthetized hamsters. Subcellular fractions from kidney were prepared as described in Methods. These fractions were assayed for phosphatidylcholine-synthesizing enzyme activities. The results are depicted as mean±standard deviation (number of experiments).

Enzymes	Specific Activity	
	Control	Diethyl ether-treated
	nmol/min/mg protein	*nmol/min/mg protein*
Choline kinase		
(cytosolic)	1.05±0.05 (4)	1.04±0.05 (4)
Phosphocholine cytidylyltransferase		
(microsomal)	3.37±0.78 (4)	1.71±0.57* (3)
(cytosolic)	0.66±0.29 (4)	0.62±0.24 (4)
(cytosolic+lipids)	2.07±0.22 (4)	2.65±0.27* (4)
Choline phosphotransferase		
(microsomal)	8.11±0.66 (4)	8.33±1.10 (4)

*P<0.05

also determined. The specific activity of cytidylyltransferase in the microsomal fraction was substantially reduced in the anesthetized animal, whereas the specific activity in the cytosol was not changed (Table 6).

Total cytidylyltransferase activity (umol/min/g tissue wet weight) was calculated from the specific activity and the amount of protein in each subcellular fraction (Table 7). The total enzyme activity in the microsomes was significantly decreased in the kidney of the anesthetized animal. No change was observed in the cytosolic enzyme between these two groups in the absence of exogenous lipid activators. In the presence of lipid activators (2 mg/ml total liver phospholipid extract, 0.1 mM oleate), the cytosolic enzyme activities in the control and ether anesthetized animals were both enhanced. Such enhancement was more prominent in anesthetized kidney than the control (Table 7). Although the sum of the cytosolic and microsomal enzyme activities was lower in the kidney of the anesthetized animal than the control in the absence of exogenous lipid activators in the cytosol, such difference was abolished when the cytosolic enzyme was fully activated.

B. REGULATION OF PHOSPHATIDYLCHOLINE CATABOLISM IN THE HEART- Effect of dietary vitamin E

I. Effect of Diet on the Vitamin E Status of Animals

TABLE 7

Total Phosphocholine Cytidylyltransferase Activity from Hamster Kidney

Kidneys were obtained from control and diethyl ether-anesthetized hamsters. Subcellular fractions from kidney were prepared as described in Methods. These fractions were assayed for phosphocholine cytidylyltransferase activities. The results are depicted as mean±standard deviation (number of experiments).

Subcellular Fraction	Total Activity (*nmol/min/g tissue wet weight*)	
	Control	Diethyl ether-treated
Microsome	15.70±2.18 (4)	6.49±0.82* (3)
Cytosol	15.75±1.78 (4)	15.45±3.27 (4)
Cytosol + Lipids	68.18±5.85 (4)	80.95±4.78* (4)
Microsome + Cytosol	31.45±2.87 (4)	23.51±3.73* (3)
Microsome + (Cytosol + Lipids)	82.21±6.01 (4)	88.33±2.43 (3)

*P<0.05

The plasma pyruvate kinase activity and plasma tocopherol levels were used to verify the vitamin E status of the rats. Pyruvate kinase activities in the vitamin E-deficient rats were markedly elevated in comparison with rats fed with 50 or 5000 p.p.m. dietary vitamin E. Plasma tocopherol levels, quantified by high pressure liquid chromatography, were found to be significantly different in all three groups of rats. The differences were in accordance with the level of vitamin E present in the diets (Table 8). There were no significant differences in body weight or heart weight among these three groups of rats.

II. Effect of Vitamin E on Phospholipid Composition

Rat hearts were homogenized in chloroform/methanol (2:1; v/v). Lipids were extracted from a portion of the homogenate by the procedure of Folch *et al.*[76]. The phospholipid classes were separated by thin-layer chromatography, and the amount of lipid phosphorus in each fraction was determined. As depicted in Table 9, no significant difference in major cardiac phospholipids was detected among the animal groups. However, the levels of lysophosphatidylcholine were significantly elevated in the heart of the rats fed with the vitamin E-deficient diet. The levels of lysophosphatidyl-choline were decreased in the rats fed with a high vitamin E diet.

Table 8

Effect of dietary vitamin E on plasma pyruvate kinase activity and serum tocopherol levels

Plasma pyruvate kinase activity and serum tocopherol content were determined as described in Methods. The results are expressed as mean±standard deviation (number of animals). Means not sharing a common superscript letter are significantly different ($p<0.05$).

	Dietary Vitamin E (*ppm*)		
	0	50	5,000
Pyruvate Kinase (*Units/ml*)	2.27 ± 0.41^{a} (7)	0.35 ± 0.12^{b} (7)	0.10 ± 0.02^{b} (6)
Tocopherol (*mg/dl*)	0.10 ± 0.01^{a} (7)	1.24 ± 0.25^{b} (7)	2.65 ± 0.19^{c} (6)

Table 9

Phospholipid Composition in the Hearts of Rats fed different levels of Vitamin E

Lipids were extracted from the hearts of the rats and phospholipid classes were separated from the lipid extracts by thin-layer chromatography. Only the phospholipid classes containing more than 0.1 umol of phospholipid/g wet weight are given. The results are expressed as mean±standard deviation calculated from four separate experiments. Means not sharing a common superscript letter are significantly different ($p<0.05$).

Phospholipids	Dietary Vitamin E (*ppm*)		
	0	50	5,000
	(umol/g heart)		
Phosphatidylethanolamine	9.76±0.35	10.08±0.24	9.86±0.58
Phosphatidylcholine	12.04±1.00	12.66±0.88	12.35±0.93
Phosphatidylserine & Phosphatidylinositol	1.29±0.23	1.23±0.12	1.32±0.09
Phosphatidic acid & Cardiolipin	3.56±1.21	3.87±1.74	3.07±0.89
Sphingomyelin	0.69±0.05	0.65±0.03	0.71±0.02
Lysophosphatidylcholine	0.26±0.06[a]	0.12±0.02[b]	0.07±0.02[c]

III. Effect of Vitamin E on Cardiac Phospholipase A

Since phospholipase A (both phospholipase A_1 and A_2) is the major enzyme responsible for the generation of lysophosphatidylcholine. Its activity in the subcellular fractions of the rat heart among the three animal groups was measured. Phospholipase A activity was determined with phosphatidyl-[Me-^{3}H]-choline as substrate, enzyme activity being estimated by the amount of radioactivity from the lysophosphatidylcholine fraction after the reaction (Table 10) Enzyme activities were significantly elevated ($P<0.05$) in all subcellular fractions of vitamin E-deficient rat hearts when compared with those of vitamin E-supplemented (50 or 5000 p.p.m.) rat hearts. In addition, enzyme activities in the hearts of the 5000 p.p.m vitamin E-supplemented groups appeared to be generally lower than those supplemented with 50 p.p.m. of vitamin E. Statistical analysis revealed that differences between the phospholipase A activities in the 50 p.p.m. and the 5000 p.p.m. vitamin E -supplemented groups were significant ($P<0.05$). only in mitochondrial and microsomal fractions. It appears that the hydrolysis of acyl groups of phosphatidylcholine in the rat heart was inhibited by increasing dietary vitamin E. Whether such inhibition affects both phospholipase A_1 and A_2 in the same manner was unknown. In order to assess each phospholipase A activity independently in the same assay, we used 1-stearoyl-2-[^{14}C]-

Table 10

Effect of dietary Vitamin E on Phospholipase A activity in Subcellular Fractions of the Rat Heart

Phospholipase A (phospholipase A_1 and A_2) activity in the rat heart subcellular fractions was assayed using rat heart phosphatidyl-[Me-^{3}H]-choline as substrate. Enzyme activity was calculated from the radioactivity associated with lysophosphatidylcholine after the reaction. The results are expressed as mean±standard deviation (number of animals). Means not sharing a common superscript letter are significantly different ($p<0.05$).

Subcellular Fraction	Dietary Vitamin E (*ppm*)		
	0	50	5,000
	Enzyme Activity *(nmol/h/mg protein)*		
Mitochondrial	8.63 ± 1.27^a (7)	6.99 ± 0.73^b (7)	4.80 ± 1.51^c (6)
Microsomal	9.07 ± 1.11^a (7)	7.93 ± 0.81^b (7)	6.39 ± 1.29^c (6)
Cytosolic	5.79 ± 0.94^a (7)	4.44 ± 1.17^b (7)	3.62 ± 1.51^b (6)

arachidonylglycerophosphocholine as substrate. 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 free arachidonate. As shown in Fig.10, both phospholipase A_1 and A_2 activities in the microsomal fractions were found to be higher in the vitamin E-deficient group than those in the vitamin E-fed groups. The magnitude of inhibition by dietary vitamin E was similar for both phospholipases. Enzyme activities were inversely related to the amount of vitamin E present in the diets.

IV. Effect of exogenous Vitamin E on the Activity of Phospholipase A

The direct effect of vitamin E on phospholipase A activity in the rat heart was investigated. Different amounts of α-DL-tocopherol were added to the subcellular fractions before enzyme assays. Addition of vitamin E markedlly lowered phospholipase A activities in all rat heart subcellular fractions (Fig. 11). Over the range of vitamin E tested (0.025-1.0 mM), the magnitude of inhibition was similar among various cardiac subcellular fractions. In order to understand better the mechanism of inhibition by vitamin E, rat heart cytosolic phospholipase A was partially purified by column choromatography, and the fractions that contained high phospholipase A activities devoid of lyso-

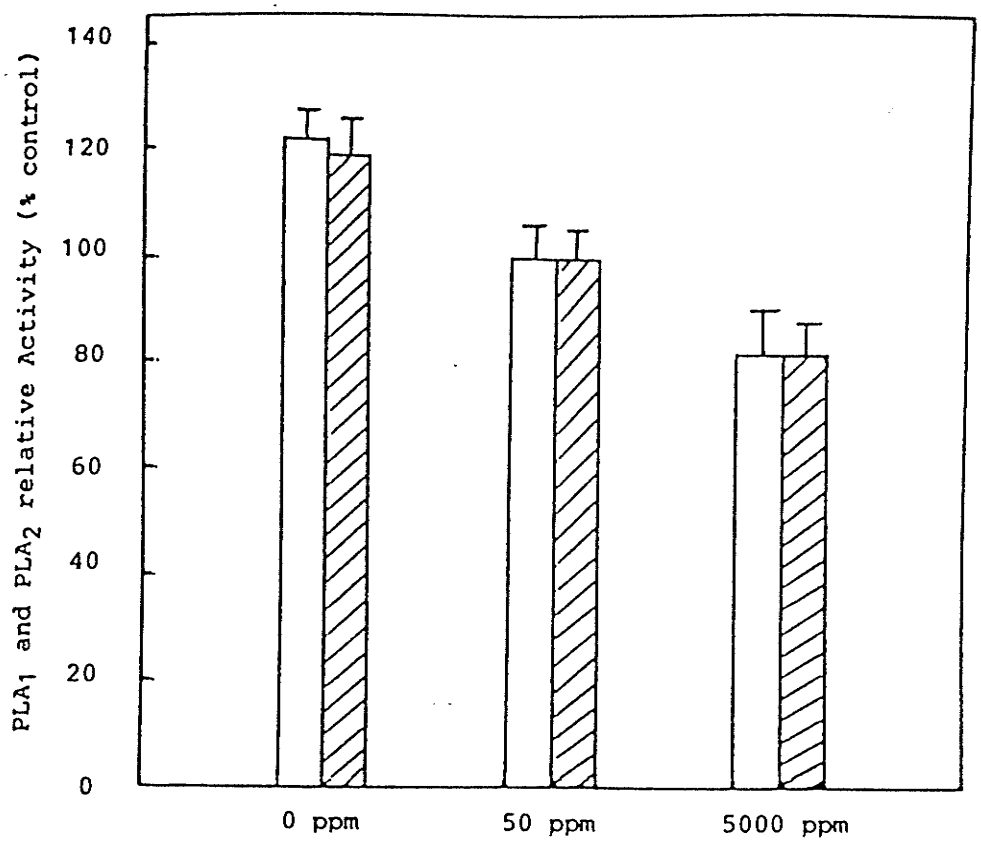


Fig. 10

Phospholipase A_1 and A_2 activities in the microsome fractions from the heart of rats fed 3 levels of dietary vitamin E.

Enzyme activities were assayed with 1-stearoyl-2-[^{14}C]-arachidonyl-glycerophosphocholine as substrate. Subsequent to incubation, phospholipase A_1 activity (unshaded bars) as estimated from the radioactivity present in the lysoglycerophosphocholine fraction whereas phospholipase A_2 (shaded bars) activity was determined from the radioactivity associated with the released free arachidonate. Values are mean ±standard deviation from three separate samples in each group, and each sample was determined in duplicate. Enzyme activity in the 50 ppm vitamin E-supplemented group is expressed as 100%. The activities of phospholipase A_1 or A_2 are significantly different between all groups ($p<0.05$).

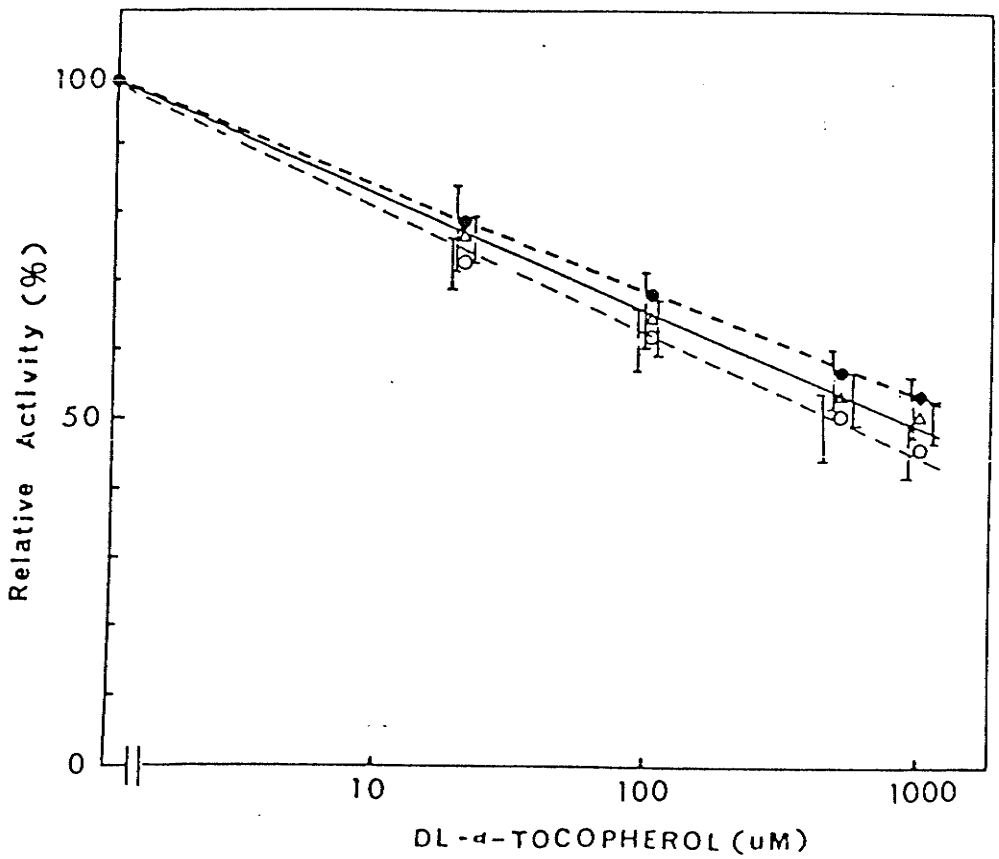


Fig. 11

In vitro inhibition of phospholipase A activity by DL-alpha-tocopherol.

The subcellular fractions from normal rat hearts were preincubated with DL-alpha-tocopherol for 10 min at 37° prior to assay. The reaction was initiated by the addition of phosphatidyl-[Me-^{3}H]-choline as substrate. Enzyme activity was calculated from the amount of radioactivity associated with lysophosphatidylcholine fraction after the reaction. Symbols used are (●) cytosol, (O) mitochondria, (Δ) microsomes . Values depicted are mean ± standard deviation of three separate samples, each determined in duplicate.

phospholipase activity were pooled and used for kinetic studies. Fig. 12 shows that partially purified phospholipase A activities were also inhibited by the addition of exogenous vitamin E. The inhibition of phospholipase A activity by vitamin E was in a dose-dependent manner. The double-reciprocal plot (Fig. 12) revealed that the nature of inhibition was essentially non-competitive.

V. Effect of Vitamin E on Lysophospholipase and Lysophosphatidylcholine Acyl-CoA Acyltransferase Activities

The significant increase in lysophosphatidylcholine from the vitamin E-deficient rat hearts depicted in Table 9 could be due to an increase in endogenous phospholipase A activities, or conversely, decreases in lysophospholipase or lysophosphatidylcholine: acyl-CoA acyltransferasee activities. Lysophospholipase catalyzes the further deacylation of lysophosphatidylcholine to form glycerophosphocholine and fatty acid. Lysophosphatidylcholine: acyl-CoA acyltransferase acylates lysophosphatidylcholine to phosphatidylcholine. Hence the activities of those two major enzymes responsible for the metabolism of lysophosphatidylcholine in the cardiac subcellular fractions of the three animal groups were determined. As depicted in Table 11, there is no significant difference in the activities of cardiac lysophospholipase or acyltransferase among the three animal groups.

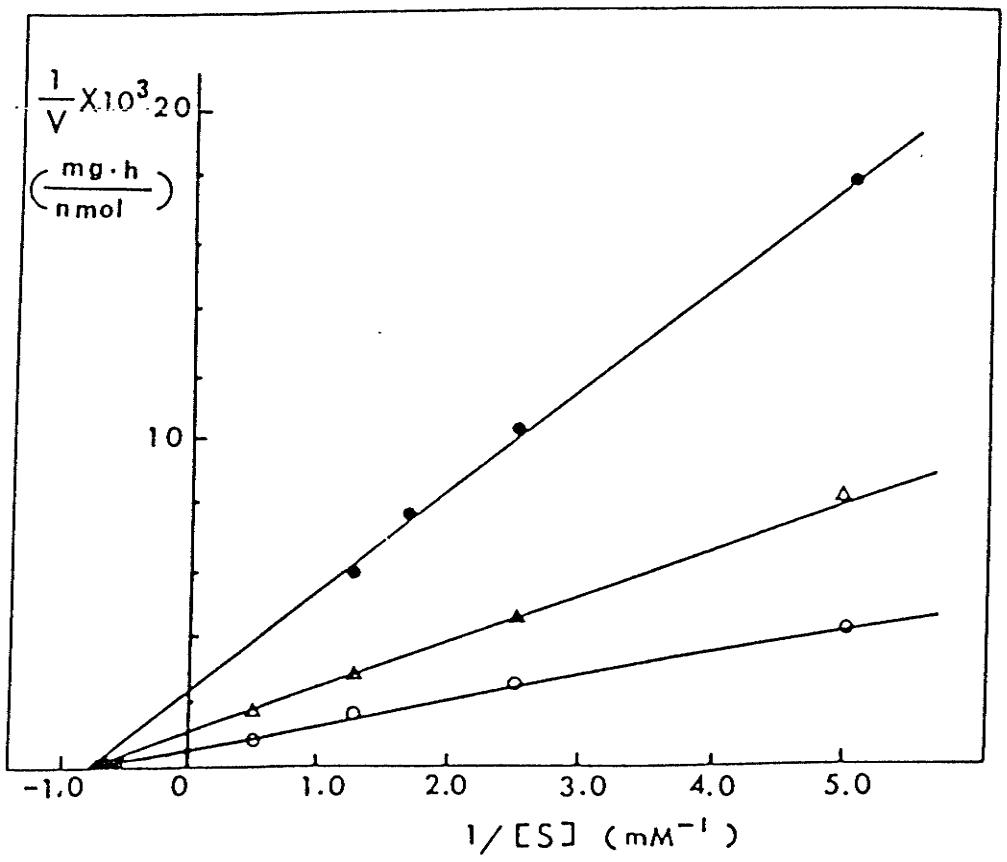


Fig. 12

Double reciprocal plot of partially purified rat heart phospholipase A in the presence and absence of DL-alpha-tocopherol.

Partially purified rat heart cytosolic phospholipase A (40 ug) was incubated in the absence (○) or in the presence of 50 uM (△) or 500 uM (●) DL-alpha-tocopherol for 10 min at 37°. The reaction was initiated by the addition of phosphatidyl-[Me-^{3}H]-choline as substrate. Enzyme activity was calculated from the amount of radioactivity associated with the lysophosphatidylcholine fraction after the reaction. Each point was the mean of three separate determinations.

Table 11

Effect of dietary Vitamin E on Rat Heart Lysophospholipase and Lysophosphatidylcholine (LPC): Acyl CoA acyltransferase Activities

Lysophospholipase activity was assayed with 1-[1-^{14}C]-palmitoyl-glycerolphosphocholine as substrate. LPC:acyl CoA acyltransferase activity was assayed with 1-[^{14}C]-oleoyl CoA as the labelled substrate. The results are expressed as mean±standard deviation from six separate experiments.

	Dietary Vitamin E (*ppm*)		
	0	50	5,000
	Lysophopholipase Activity *(nmol/h/mg protein)*		
Microsomal	30.00±5.04	31.50±3.40	35.27±6.38
Cytosolic	51.54±3.13	50.82±2.55	49.83±3.30
	LPC: Acyl CoA acyltransferase Activity *(nmol/h/mg protein)*		
Microsomal	10.1±2.9	13.4±2.3	12.1±3.9

DISCUSSION

I. Effect of Diethyl Ether Anesthesia on Phosphatidylcholine Biosynthesis

Phosphatidylcholine is the major phospholipid in mammalian tissues. The majority of phosphatidylcholine in the tissues is synthesized via the CDP-choline pathway and CTP:phosphocholine cytidylyltransferase is the rate-limiting enzyme in this pathway. The main objective of this part of the research was to study the regulatory mechanisms of phosphatidylcholine biosynthesis in mammalian heart as well as other tissues.

Anesthetics such as phenobarbital, trifluoperazine, chlorpromazine and tetracaine were found to affect rat liver cytidylyltransferase activity, thus influencing the rate of phosphatidylcholine biosynthesis [18]. Phosphatidylethanolamine synthesis was shown to be reduced in the kidney of diethyl ether anesthetized hamsters [54]. However, the effect of ether on phosphatidylcholine biosynthesis was not known. In this study, we have used diethyl ether as a probe to study the regulatory mechanism of phosphatidylcholine biosynthesis via the CDP-choline pathway in hamster organs [82].

The results from this study clearly demonstrated that diethyl ether treatment did not produce any effect on phos-

phatidylcholine biosynthesis on hamster organs other than the kidney. The reduction of phosphatidylcholine biosynthesis in hamster kidney under ether anesthesia (29%) was quantitatively similar to that reported for phosphatidylethanolamine biosynthesis (30% reduction) [54]. There might be at least three possible explanations for the reduction of phosphatidylcholine biosynthesis seen in hamster kidney under ether anesthesia. One of the possible explanations was that the rate of choline uptake by the hamster kidney might be affected under ether treatment. Exogenous choline is actively taken up by mammalian tissues and diethyl ether treatment might affect the choline transport system in the kidney which might lead to a decrease in phosphatidylcholine formation. Another explanation was that phosphatidylcholine biosynthesis via the CDP-choline pathway might be perturbed. The third possibility was that degradation of phosphatidylcholine might be affected.

In this study, we have tested these possibilities for the change of phosphatidylcholine biosynthesis in the anesthetized kidney. Our results clearly indicate that ether treatment had no effect on the rate of choline uptake by the kidney or by other hamster organs.

Phosphatidylcholine is hydrolyzed to lysophosphatidylcholine by the action of phospholipases. The labelling of lysophosphatidylcholine was determined after ether treat-

ment. There was no difference in the labelling of lyso-phosphatidylcholine between control and anesthetized hamster kidneys. It is obvious that the rate of phosphatidylcholine degradation was not changed during ether treatment.

The effect of ether treatment on phosphatidylcholine biosynthesis via CDP-choline pathway was extensively studied in the hamster kidney. The labelling of phosphocholine was significantly increased in the anesthetized kidney, but no change in the labelling of choline and CDP-choline was observed. Such increase in the labelling of phosphocholine, combined with decrease in the labelling of phosphatidyl-choline, suggest that the reduced labelling of phosphatidyl-choline in the anesthetized kidney probably resulted from a reduction in the conversion of phosphocholine to CDP-choline. Such conversion, catalyzed by CTP:phosphocholine cytidylyltransferase, has been regarded as the rate-limiting step in the CDP-choline pathway [16-17, 83-87]. Cytidylyl-transferase activity is located in both the microsomal and cytosolic fractions [88], and the cytosolic enzyme has been shown to increase in activity upon storage at 4 °C or at room temperature [17]. In order to obtain valid comparisons, all cytidylyltransferase assays in the cytosol were performed at exactly four hours after tissue homogenization [17]. Since the cytosolic enzyme has a lipid requirement [18, 74-75], lipids were added to the assay mixture in order to obtain maximum enzyme activity.

Current evidence suggests that the microsomal form of the enzyme is the active form responsible for the conversion of phosphocholine to CDP-choline *in vivo* [43,89]. The translocation of the cytidylyltransferase from one subcellular fraction to another is thought to be an important mechanism for the overall regulation of phosphatidylcholine biosynthesis [18-19]. The conversion of cytosolic enzyme to microsomal form will enhance the conversion of phosphocholine to CDP-choline. Alternatively, translocation of the microsomal enzyme to cytosolic form will diminish this conversion. The present study revealed that ether treatment caused a decrease in the specific activity and total activity of the cytidylyltransferase in the microsomal fraction of the kidney, with a corresponding increase in the cytosolic enzyme in the presence of lipid activators. In addition, the combined enzyme activities in these two forms were not changed. Since the cytosolic enzyme is subjected to lipid activation, assay of enzyme activity in the absence of lipids may not provide a realistic estimation of maximal total enzyme activity. We feel that a more valid comparison can be made when the cytosolic enzyme is fully activated. Based on the results obtained, we postulate that ether treatment promotes the translocation of the kidney cytidylyltrtansferase from microsomal form (more active) to cytosolic form (less active) by an undefined mechanism. Such

translocation would cause a reduction in the conversion of phosphocholine to CDP-choline which was confirmed by the analysis of choline-containing metabolites. The transfer of the microsomal cytidylyltransferase to the cytosolic fraction during ether anesthesia would provide the kidney with a facile mechanism to attenuate the rate of phosphatidylcholine biosynthesis.

One intriguing aspect of this study is the differential effect of diethyl ether on the translocation of the cytidylyltransferase in the kidney but not in other hamster organs. At present, only the cytidylyltransferase from the rat liver has been purified and studied in detail [19-20]. Our results suggest that the enzyme in hamster kidney may be modulated differently than those found in other hamster tissues.

II. Regulation of Phosphatidylcholine Catabolism by Vitamin E

Phospholipase A is mainly responsible for the catabolism of phosphatidylcholine in mammalian heart. Although the inhibition of phospholipase A activity by vitamin E has been reported in the liver and platelets [69-70], this is the first study to show that this enzyme in the heart is also inhibited by vitamin E. There were several reasons for using vitamin E deficient rat for this study. First and the foremost, this is a widely accepted model and there is a wealth of information on the biochemical and physiological changes during vitamin E deficient which may allow us to assess our

model easily. Secondly, the organs from this model were made available to us through the generosity of Dr. A.C. Chan. It is clear from the present study that the vitamin E status of the animal is an important biochemical factor for the maintenance of lysophosphatidylcholine levels in the heart. Since the vitamin E content in the cardiac tissue has recently been shown to be directly proportional to dietary vitamin E intake [91], our results indicate that there is a reverse relationship between phospholipase A activity in the rat heart and the tocopherol content of the tissue [92]. The other lysophosphatidylcholine metabolic enzymes in the heart are not affected by the vitamin E status of the animal.

It is noteworthy that phosphatidylcholine isolated from the heart was used as substrate for the assay of phospholipase A activity. Cardiac phospholipase A was found to display acyl specificity towards certain phosphatidylcholine species [59]. The problems and limitations of using synthetic substrates (with defined acyl groups) for studies of phospholipase A in mammalian tissues have been well-documented [59, 93]. We consider that the use of a natural substrate (with mixed acyl groups) would provide us with a better assessment of enzyme activity. Owing to the difficulties in obtaining uniform labelling of the different acyl groups in the natural substrate, the radioactive label was placed at the base group. The use of phosphatidyl-[Me-^{3}H]-

choline as substrate allowed us to assay for the sum of phospholipase A_1 and A_2 activities simultaneously. The sum of phospholipase A_1 and A_2 activities was determined by monitoring the formation of lysophosphatidyl[Me-^{3}H]-choline. In order to discriminate the effect of vitamin E between phospholipase A_1 and phospholipase A_2, it was necessary to use a phospholipid substrate containing a labelled acyl group [53,59]. The utilization of 1-stearoyl-2-[^{14}C]-arachidonyl-glycerophosphocholine as substrate permitted us to assay for phospholipase A_1 and A_2 activities simultaneously by monitoring the formation of labelled lysophosphatidylcholine and labelled fatty acyl group, respectively. The sum of phospholipase A_1 and A_2 activities obtained in this manner (10.02 nmol/h/mg, see Fig. 10) was not the same as that obtained with phosphatidyl-[Me-^{3}H]-choline (7.93 nmol/h/mg, see Table 10). Such a discrepancy was also observed in other studies [59], and probably resulted from the specificity of the enzyme towards phosphatidylcholine with an arachidonyl group [59], as well as the possible difference in the dispersion of substrates. Since lysophospholipase activity is present in the cytosolic and microsomal fractions [94], the addition of unlabelled lysophosphatidylcholine in the assay mixture was shown to be effective in the inhibition of the hydrolysis of the labelled lysophosphatidylcholine formed in the reaction under normal assay conditions [59]. However, the presence of high

concentrations of lysophosphatidylcholine was found to inhibit phospholipase A hydrolysis at very low substrate concentrations [59]. This was circumvented by the removal of lysophospholipase activity from the enzyme preparation used in kinetic studies. Lysophosopholipase activity in the cytosol fraction was readily separated from the phospholipase activity by CM-cellulose chromatography [92].

The mechanism of inhibition of phospholipase A activity by vitamin E was examined. Although the inhibition might occur at the substrate level, kinetic studies revealed that vitamin E is a non-competitive inhibitor of phospholipase A activity. Vitamin E inhibits both phospholipase A_1 and A_2 to similar extents, and the inhibition appears to be dose-dependent. The inhibition of the cardiac enzyme by vitamin E is similar to the other finding that vitamin E inhibits phospholipase A_2 activity [70]. Apart from the release of lysophospholipids, phospholipase A_2 is the principal enzyme responsible for the liberation of arachidonic acid, a rate-limiting step in eicosanoid biosynthesis. Evidence presented in the present and in other studies clearly demonstrated that vitamin E inhibits phospholipase A_2 in various mammalian tissues and thus, may contribute to the regulation of eicosanoid biosynthesis at the level of substrate release. Indeed, endogenous thromboxane synthesis in platelets was demonstrated to be inversely related to the amount of vitamin E received by normal and diabetic rats [95]. The

potential role of vitamin E as a nutrient regulator in decreased eicosanoid production merits further exploration.

From the results obtained in these studies we postulate that, under normal physiological conditions, phospholipase A activity in the heart is partially inhibited by vitamin E. The degree of inhibition is dependent on the vitamin E status of the animal. Cardiac phospholipase A is responsible for the production of lysophosphatidylcholine, which is further metabolized by lysophospholipase or reacylated back to the parent phospholipid by lysophosphatidylcholine: acyl-CoA acyltransferase. Since the activities of lysophospholipase and acyltransferase in the rat heart were changed, the elevated lysophosphatidylcholine level during vitamin E deficiency might result from the increase in phospholipase A activity. If this was the case, then the regulation of phospholipase A activity in this manner could constitute an important control mechanism for the maintenance of the levels of lysophosphatidylcholine and other lysophospholipids in the cardiac tissue. In the heart and other mammalian tissue the levels of lysophosphatidylcholine and other lysophospholipids must be rigidly regulated because of their detergent nature. High concentrations of lysophosphatidylcholine have been shown to be cytolytic [63]. Over the last few years the accumulation of lysophosphatidylcholine in the heart during ischaemia has been suggested as

a biochemical cause of the development of arrhythmias [67,96]. It is therefore plausible that the accumulation of lysophosphatidylcholine in the heart of the vitamin E-deficient animal may be one of the biochemical factors preceding the cardiac dysfunctions observed in previous studies [71].

REFERENCES

1) Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1983) in Molecular Biology of the Cell, Garland Publishing Inc., New York

2) Singer, S.J. and Nicholson, G.L. (1972) Science 175, 720-731

3) Blackwell, G.J. (1978) Adv. Prostaglandin Thromboxane Res. 3, 137-142

4) Coleman, R. (1973) in Form and Function of Phospholipids (Ansell, G.B., Haethorne, J.N. and Dawson, R.M.C., eds.) Elsevier Scientific Publishing Company, Amsterdam, pp 1-30

5) King, R.J. and Clements, J.A. (1972) Am. J. Physiol., 223, 715-726

6) Gobley, M. (1850) J. Pharm. Chim. (Paris) 17, 401-417

7) Diakanow, C. (1868) Zbl. Med. Wiss. 2, 434-435

8) Deuel, H.J. (1951) in The Lipids - Their Chemistry and Biochemistry, Vol. 1 (Deuel, H.J., ed.) Interscience Publishers, New York, pp 405-418

9) Van Golde, L.M.G. and Van den Bergh, S.G. (1977) in Lipid Metabolism in Mammals, Vol. 1 (Snyder, F., ed.) Plenum Press, New York, pp 1-33

10) Kennedy, E.P. (1962) Harvey Lect. 57, 143-171

11) Weinhold, P.A. and Rethy, V.B. (1974) Biochemistry 13, 5135-5141

12) Infante, J.P. and Kinsella, J.E. (1976) Lipids 11, 727-

735

13) Brophy, P.J., Choy, P.C., Toone, J.R. and Vance, D.E. (1977) Eur. J. Biochem. 78, 491-495

14) Vance, D.E. (1985) in Biochemistry of Lipids and Membranes, (Vance, D.E. and Vance, J.E., eds.), Benjamin Cummings Publishing Company, Menlo Park, pp 242-270

15) Schneider, W.C. (1963) J. Biol. Chem. 238, 3572-3578

16) Vance, D.E., Trip, E.M. and Paddon, H.B. (1980) J. Biol. Chem. 255, 1064-1069

17) Zelinski, T.A., Savard, J.D., Man, R.Y.K. and Choy, P.C. (1980) J. Biol. Chem. 255, 11423-11428

18) Pelech, S.L. and Vance, D.E. (1984) Biochim. Biophys. Acta 779, 217-251

19) Weinhold, P.A., Pounsifer, M.E. and Feldman, D.A. (1986) J. Biol. Chem. 261, 5104-5110

20) Feldman, D.A. and Weinhold, P.A. (1987) J. Biol. Chem. 262, 9075-9081

21) Jelsema, C.L. and Morre, D.J. (1987) J. Biol. Chem. 253, 7960-7971

22) Sikpi, M.O. and Das, S.K. (1987) Biochim. Biophys. Acta 899, 35-43

23) Kanoh, H. and Ohno, K. (1976) Eur. J. Biochem. 66, 201-210

24) Stetten, D. (1941) J. Biol. Chem. 138, 437-438

25) Bremer, J. and Greenberg, D.M. (1961) Biochim. Biophys. Acta 46, 205-216

26) Sundler, R. and Akesson, B. (1975) J. Biol. Chem. 250, 3359-3367

27) Bremer, J. and Greenberg, D.M. (1960) Biochim. Biophys. Acta 37, 173-175

28) Gibson, K.D., Wilson, J.D. and Udenfried, S. (1961) J. Biol. Chem. 236, 673-678

29) Ridgway, N.D. and Vance, D.E. (1987) J. Biol. Chem. 262, 17231-17239

30) Castano, J.G., Alemany, S., Nieto, A. and Mato, J.M. (1980) J. Biol. Chem. 255, 9041-9043

31) Pritchard, P.H., Pelech, S.L. and Vance, D.E. (1981) Biochim. Biophys. Acta 666, 301-301

32) Dils, R.R. and Hübscher, G. (1961) Biochim. Biophys. Acta 46, 505-513

33) Porcellati, G., Arienti, G., Pirotta, M.G. and Giorgini, D. (1971) Neurochem. 18, 1395-1417

34) Bjerve, K.S. (1971) Biochim. Biophys. Acta 296, 549-562

35) Kanfer, J.N. (1980) Can. J. Biochem. 58, 1370-1380

36) Filler, D.A. and Weinhold, P.A. (1980) Biochim. Biophys. Acta 618, 223-230

37) Jenden, D.J. (1979) in Nutrition and the Brain, (Barbeau, A., Growden, J.H. and Wurtman, R.J., eds) Raven Press, New York, pp 13-24

38) Zelinski, T.A. and Choy, P.C. (1984) Biochim. Biophys. Acta 617, 205-217

39) Hatch, G.M. and Choy, P.C. (1986) Biochim. Biophys. Acta

884, 259-264

40) Hatch, G.M. and Choy, P.C. (1988) Biochem. Cell Biol. 66, 418-424

41) Choy, P.C. (1982) J. Biol. Chem. 257, 10928-10933

42) Sleight, R. And Kent, C. (1983) J. Biol. Chem. 258, 824-830

43) Sleight, R. and Kent, C. (1983) J. Biol. Chem. 258, 831-835

44) Sleight, R. and Kent, C (1983) J. Biol. Chem. 258, 836-839

45) Pelech, S.L., Pritchard, P.H., Brindley, D.N. and Vance, D.E. (1983) J. Biol. Chem. 258, 6782-6788

46) Pelech, S.L., Pritchard, P.H., Brindley, D.N. and Vance, D.E. (1983) Biochem. J. 216, 129-136

47) Mock, T., Slater, T.L., Arthur, G., Chan, A.C. and Choy, P.C. (1986) Biochem. Cell Biol. 64, 413-417

48) Pelech, S.L. and Vance, D.E. (1982) J. Biol. Chem. 257, 14198-14202

49) Pelech, S.L., Pritchard, P.H. and Vance, D.E. (1981) J. Biol. Chem. 256, 8283-8286

50) Arthur, G. and Choy, P.C. (1984) Biochim. Biophys. Acta, 795, 221-229

51) Arthur, G. and Choy, P.C. (1986) Biochem. J. 236, 481-487

52) Ishidate, K. and Nakazawa, Y. (1976) Biochem. Pharmacol. 25, 1255-1260

53) Ishidate, K. and Tsuruoka, M. and Nakazawa, Y. (1980) Biochim. Biophys. Acta 620, 49-58

54) Tam, S.W. and Choy, P.C. (1983) Biochim. Biophys. Acta 488, 181-189

55) Waite, M. (1985) in Biochemistry of Lipids and Membranes (Vance, D.E. and Vance, J.E., eds), Benjamin Cummings Publishing Co., Menlo Park, pp 299-324

56) Dawidowicz, E.A. (1987) Ann. Rev. Biochem. 56, 43-61

57) White, D.A. (1973) in Form and Function of Phospholipids (Ansell, G.B., Hawthorne, J.N. and Dawson, R.M.C., eds.), Elsevier Scientific Publishing Co., Amsterdam, pp 441-482

58) Dhalla, N.S., Ziegelhoffer, A. and Harrow, J.A.C. (1977) Can. J. Physiol. Pharmacol. 55, 1211-1234

59) Tam, S.W., Man, R.Y.K. and Choy, P.C. (1984) Can. Biochem. Cell Biol. 62, 1269-1274

60) Cao, Y.Z., Tam, S.W., Arthur, G., Chen, H.L. and Choy, P.C. (1987) J. Biol. Chem. 262, 16927-16935

61) Lands, W.E.M. (1960) J. Biol. Chem. 235, 2233-2237

62) Savard, J.D. and Choy, P.C. (1982) Biochim. Biophys. Acta 711, 40-48

63) Weltzien, H.U. (1979) Biochim. Biophys. Acta 559, 259-287

64) Katz, A.M. and Messineo, F.C. (1981) Circ. Res. 48, 1-16

65) Corr, P.B., Gross, R.W. and Sobel, B.E. (1984) Circ. Res. 55, 135-154

66) Elharrar, V. and Zipes, D.P. (1977) Am. J. Physiol. 233, H329-H345

67) Man, R.Y.K. and Choy, P.C. (1982) J. Mol. Cell. Cardiol. 14, 173-175

68) Kinnaird, A.A., Choy, P.C. and Man, R.Y.K. (1988) Lipids 23, 32-35

69) Pappu, A.S., Fatterpaker, P. and Sreenivasan, A. (1978) Biochim. Biophys. Acta 172, 349-352

70) Douglas, C.E., Chan, A.C. and Choy, P.C. (1986) Biochim. Biophys. Acta 876, 639-645

71) Gatz, A.J. and Houchin, O.B. (1951) Anat. Rev. 110, 249-260

72) Paddon, H.B. and Vance, D.E. (1977) Biochim. Biophys. Acta 488, 181-189

73) Choy, P.C. and Vance, D.E. (1978) J. Biol. Chem. 253, 5163-5167

74) Vance, D.E., Pelech, S.D. and Choy, P.C. (1981) Methods in Enzymol. 68, 576-581

75) Cornell, R. and Vance, D.E. (1987) Biochim. Biophys. Acta 919, 26-36

76) Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 495-509

77) Bartlett, G.R. (1958) J. Biol. Chem. 234, 466-468

78) Hamelin, St-J.S. and Chan, A.C. (1983) Lipids 18, 267-269

79) Chan, A.C. and Leith, M.K. (1981) Am. J. Clin. Nutr. 34,

2341-2347

80) Bieri, J.G., Tolliver, T.J. and Catignani, G.L. (1979) Am. J. Clin. Nutr. 32, 2143-2149

81) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

82) O, K., Hatch, G.M. and Choy, P.C. (1988) Lipids 23, 656-659

83) Pritchard, P.H. and Vance, D.E. (1981) Biochem. J. 196, 261-267

84) Post. M., Batenburg, E., Schuurmans, A.J.M. and van Golde, L.M.G. (1982) Biochim. Biophys. Acta 712, 390-394

85) Whitehead, F.W., Trip, E. and Vance, D.E. (1981) Can. J. Biochem. 59, 38-47

86) Cornell, R.B. and Goldfine, H. (1983) Biochim. Biophys. Acta 750, 504-520

87) O'Doherty, P.J.A. (1980) Can. J. Biochem. 58, 527-533

88) Wilson, J.E. (1978) Trends Biochem. Sci. 3, 124-125

89) Sleight, R. and Kent, C. (1980) J. Biol. Chem. 255, 10644-10650

90) Vance, D.E. and Pelech, S.L. (1984) Trends Biochem. Sci. 9, 17-20

91) Whitacre, M.E., Combs, G.F., Jr. and Parker, R.S. (1987) J. Nutr. 117, 460-467

92) Cao, Y.Z., O, K., Choy, P.C. and Chan, A.C. (1987) Biochem. J. 247, 135-140

93) Nalbone, G. and Hostetler, K.Y. (1985) J. Lipid Res. 26,

104-114

94) Gross, R.W. and Sobel, B.E. (1982) J. Biol. Chem. 257, 6702-6708

95) Gilbert, V.A., Zebrowski, E.J. and Chan, A.C. (1983) Horm. Metab. Res. 15, 320-325

96) Man, R.Y.K., Slater, T.L., Pelletier, M.P. and Choy, P.C. (1983) Lipids 18, 677-681

97) Choy, P.C. and Arthur, G. (1988) in Phosphatidylcholine Metabolism (Vance, D.E. ed), CRC Press, Boca Raton, (In Press)

98) Devlin, T.M. (1986) in Biochemistry with Clinical Correlations (Devlin, T.M., ed.), John Wiley and Sons, New York, pp 177-210