CONTROL OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN HAMSTER HEART

by

Teresa A. Zelinski

A thesis submitted to the Faculty of Graduate Studies

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To Mom and Dad

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ACS	Aqueous counting scintillant
AdoMet	S-adenosyl-L-methionine
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
BHK	Baby Hamster Kidney
^{o}C	Degrees Celsius
cAMP	Adenosine 3':5'-monophosphate
CDP	Cytidine diphosphate
CHO	Chinese Hamster Ovary
Ci	Curie
CMP	Cytidine 5'-monophosphate
Co.	Company
CoA	Coenzyme A
cpm	Counts per minute
CTP	Cytidine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
dpm	Disintegrations per minute
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
Fig.	Figure
g	Gram
g	Gravitational force
h	Hour
K_m	Michaelis-Menten constant
l	Litre
M	Molar

Me	Methyl
min	Minute
mCi	Millicurie
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
nm	Nanometre
p	Statistical probability
s	Second
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
sn	Stereospecific numbering
TCA	Trichloroacetic acid
µCi	Microcurie
µl	Microlitre
µM	Micromolar
U.V.	Ultraviolet
v	Volume
V_{max}	Maximum velocity (of an enzyme reaction)
w	Weight

ABSTRACT

Phosphatidylcholine is the major phospholipid in the mammalian heart. The majority of this phospholipid is synthesized via the CDP-choline pathway. Although the control of phosphatidylcholine biosynthesis in other tissues has been extensively studied, control of this same process in the heart was largely unknown. Hence, this study was designed to elucidate some of the control mechanisms which may regulate phosphatidylcholine biosynthesis in the hamster heart.

Choline is a dietary requirement for most mammals. This metabolite is required for both acetylcholine and phosphatidylcholine biosynthesis in mammalian tissues. Since exogenous choline is necessary for normal cellular metabolism, the effects of some choline analogues on choline uptake and as a mode for the regulation of phosphatidylcholine biosynthesis in the heart were examined. Hamster hearts were perfused with [Me-^{3}H] choline in the presence of 0.05-0.5 mM ethanolamine. Incorporation of label into phosphatidylcholine was decreased 26-63% at 0.1-0.5 mM ethanolamine. Similar decreases in the labelling of the metabolites of the CDP-choline pathway were observed at these ethanolamine concentrations. The observed decrease in phosphatidylcholine labelling at 0.1-0.5 mM ethanolamine was attributed to an inhibition of labelled choline uptake by ethanolamine. The inhibitory role of ethanolamine on choline uptake was examined by comparison to hemicholinium-3. Both compounds inhibited choline uptake in a competitive manner. However, the intracellular concentration of choline and choline-containing metabolites was not altered despite a reduction in choline uptake. Hence, it can be concluded that exogenous ethanolamine

has no immediate effect on phosphatidylcholine biosynthesis. The reduced labelling of phosphatidylcholine is therefore a direct result of the reduction of labelled choline taken up by the isolated heart.

The corollary effect of choline on ethanolamine uptake and metabolism in the heart was also investigated. In this study, hearts were perfused with [2-^{14}C] ethanolamine in the presence of 0-0.5 mM choline. Incorporation of label into phosphatidylethanolamine was decreased 28% at 0.2-0.5 mM choline. Analysis of the intracellular metabolites of the CDP-ethanolamine pathway revealed that choline (0.2-0.5 mM) in the perfusate had no effect on the labelling of ethanolamine. However, a 28-30% reduction in the labelling of phosphoethanolamine and CDP-ethanolamine was observed. Concurrently there was a 2-fold increase in intracellular choline concentration. Although the formation of CDP-ethanolamine is regarded as the rate-limiting step for phosphatidylethanolamine biosynthesis, choline was found to inhibit ethanolamine kinase, but did not have any effect on CTP:phosphocholine cytidylyltransferase. Hence, choline may provide an additional mechanism (beyond the control provided by phosphoethanolamine cytidylyltransferase) for the regulation of phosphatidylethanolamine biosynthesis in the heart.

CTP:phosphocholine cytidylyltransferase is the enzyme which catalyzes the rate-limiting step of phosphatidylcholine formation. In order to understand the regulatory role of this enzyme in the control of phosphatidylcholine biosynthesis, cytidylyltransferase from hamster heart was partially purified and characterized. In fresh cytosol, three forms (H-, L- and S-form) of this enzyme were identified by gel filtration chromatography. The L-form was the major enzyme species present in fresh cytosol. When cytosol was incubated at 4^{o}C for 4 days,

a dramatic increase in cytidylyltransferase activity and conversion of L-form to H-form were observed. The H-form of cytidylyltransferase was partially dissociated by incubation with 0.025% sodium dodecyl sulfate. Application of this mixture to a Sepharose 6B column resulted in highly purified L- and S-forms of the enzyme. The cytosolic forms as well as the microsomal form of cytidylyltransferase were subsequently characterized. All enzyme forms displayed broad pH profiles, with optimal activity at pH 8.0. All forms of cytidylyltransferase were activated by Mg^{+2} in the same manner. The L- and S-forms required lipid for maximal activity whereas the H- and microsomal forms were found to have no lipid requirement. The true Michaelis constants for cytosolic and microsomal cytidylyltransferase were also determined. The H-form and microsomal form of the enzyme showed a true K_m of 1.0 mM for CTP and 0.44 mM for phosphocholine. The L-form of the enzyme is kinetically identical to the S-form, with a true K_m of 400 mM for CTP and 80 mM for phosphocholine. In view of the fact that the intracellular concentration of CTP (15 nmol/g heart) is much lower than the K_m values determined, intracellular CTP concentrations may regulate the reaction catalyzed by all forms of cytidylyltransferase. Additional regulation of the rate-limiting reaction may occur at the level of phosphocholine supply. Since the intracellular concentration of this metabolite is 0.25 mM changes in cellular phosphocholine levels may provide a facile mechanism for the regulation of phosphatidylcholine biosynthesis by modulating the reaction catalyzed by the L- and S-forms of cytidylyltransferase.

INTRODUCTION

I The Biological Membrane

Membranes are an integral part of any cell. In some instances, the different membrane systems may comprise up to 80% of the total dry cell mass. Membranes serve not only as barriers separating different compartments within the cell, but also as the structural basis of the cell.

The molecular organization of membrane components consists of a lipid bilayer in which biologically active proteins are embedded. Most membranes contain approximately 40% lipid and 60% protein, but there is considerable variation. For example, the inner mitochondrial membrane contains 20-25% lipid, whereas the myelin membrane surrounding certain nerves may contain up to 75% lipid.

The lipid bilayer is formed spontaneously by phospholipid self-interaction. That is, the hydrophilic head groups of the phospholipids arrange themselves toward the aqueous environment, whereas the hydrophobic fatty acyl groups occupy the interior of the bilayer. This spatial arrangement serves as the structural matrix for protein organization and movement as described in the fluid mosaic model of Singer and Nicolson (1). In addition to their structural function, phospholipids have recently been implicated in the control of various cellular processes including membrane fusion (2), metabolite transport (3,4) and the catalytic activity of membrane-bound enzymes (5). Hence it is readily apparent that phospholipids are not merely passive structural components of membranes, but rather play diverse and dynamic roles in membrane function.

II Phosphatidylcholine - Structure and Function

In eukaryotes, phosphatidylcholine accounts for nearly half of the membrane phospholipid. Although ubiquitous in higher plants and animals, the occurence of phosphatidylcholine in prokaryotes is rare.

Phosphatidylcholine assumes the general structure of a phospholipid with a polar headgroup region and a glycerol backbone region to which fatty acids are esterified. It is the choline headgroup which distinguishes phosphatidylcholines from other phospholipids. Saturated fatty acids are usually esterified at the C_1 position of the glycerol backbone, while unsaturated fatty acids are mainly located at the C_2 position.

The cellular processes in which phosphatidylcholine participates are as diverse as the molecular species of this phospholipid in various tissues. A few of these functions are listed below:

a) Phosphatidylcholine is the major component of pulmonary surfactant (6).

b) Phosphatidylcholine is a major component of bile and thereby aids the solubilization of cholesterol in bile (7).

c) Phosphatidylcholine serves as a major source of arachidonic acid for prostaglandin biosynthesis (8).

d) D-beta-hydroxybutyrate dehydrogenase activity has an absolute requirement for phosphatidylcholine (9).

Although these are just a few of the metabolic functions which involve phosphatidylcholine, it is obvious that the study of phosphatidylcholine biosynthesis and the regulation of this process is essential for understanding cellular function and membrane integrity in higher organisms.

III Phosphatidylcholine Biosynthesis

In mammalian tissues, there are several known pathways for the formation of phosphatidylcholine (10) (Fig.1). The majority of this phospholipid is formed from choline via the CDP-choline pathway (11). Alternatively, phosphatidylethanolamine is methylated to form phosphatidylcholine by the transfer of methyl groups from S-adenosylmethionine (12). Another pathway is the Ca^{+2}-mediated base exchange of choline with other phospholipid head groups (13). Each pathway's contribution to total phosphatidylcholine biosynthesis appears to be tissue specific. For example, the methylation of phosphatidylethanolamine contributes significantly to phosphatidylcholine biosynthesis in rat liver (10), but is completely absent in rat intestine (14). These pathways will be described in detail in the following sections.

a) CDP-choline Pathway

The major pathway for phosphatidylcholine biosynthesis was elucidated in the mid 1950's by Kennedy and his co-workers (15). Hence this pathway is also referred to as the Kennedy pathway or the *de novo* synthesis pathway.

The first step in the reaction sequence is a choline phosphorylation reaction which occurs at the expense of ATP and is catalyzed by choline kinase. Weinhold and Rethy described an ethanolamine kinase which also displayed choline kinase activity (16). Originally it was thought that both activities were shared by a single enzyme. However, studies in rat liver (17) demonstrated unambiguously that choline kinase and ethanolamine kinase were separate enzymes. In the same report the authors demonstrated that choline kinase was found

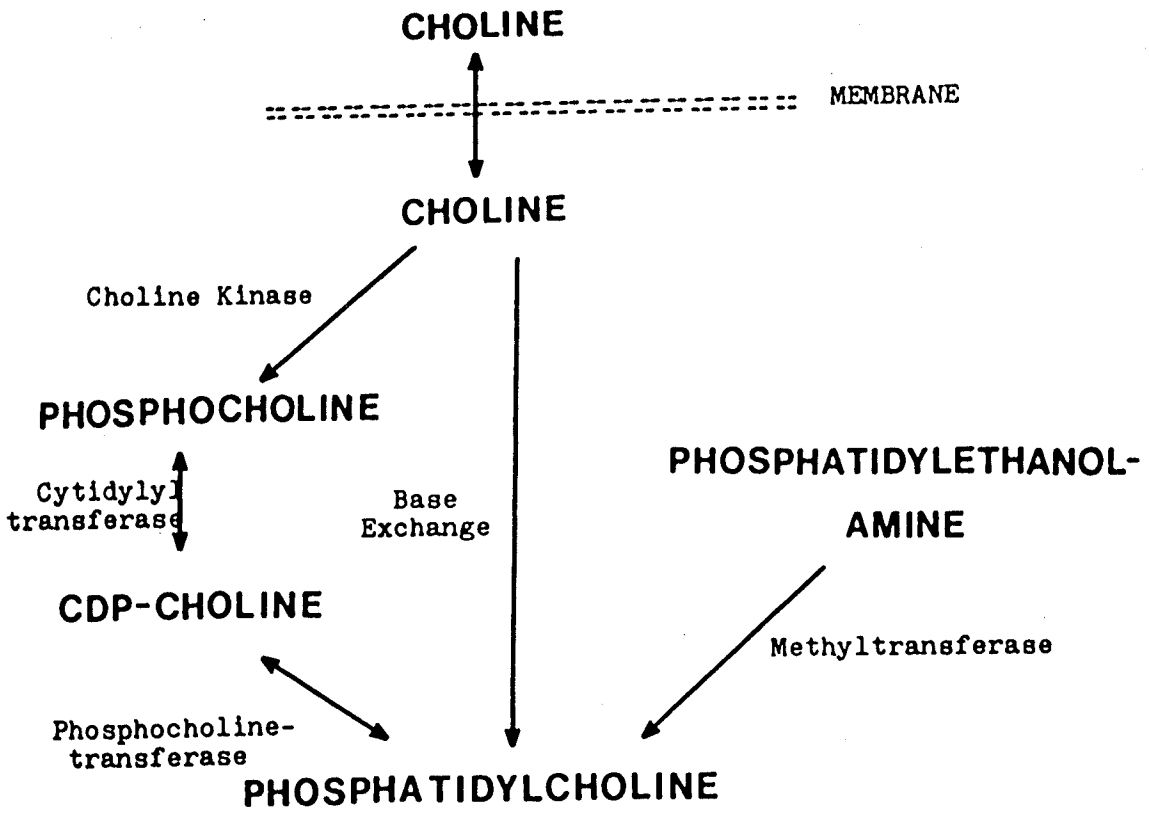


Figure 1 Major metabolic pathways for the biosynthesis of phosphatidylcholine.

exclusively in the cytosolic fraction.

It has been firmly established that CDP-choline is an essential intermediate in the de novo synthesis pathway described by Kennedy. CTP:phosphocholine cytidylyltransferase catalyzes the synthesis of CDP-choline and pyrophosphate from CTP and phosphocholine. This enzyme is ambiquitous (18) in that it is recovered in both the cytosolic and microsomal fractions of rat liver (19) homogenate. The enzyme is heat stable, requires Mg^{+2} or Mn^{+2} for activity and is specific for CTP or dCTP (20).

CTP:phosphocholine cytidylyltransferase has been extensively studied in rat liver (21). These investigators found that in fresh rat liver cytosol, cytidylyltransferase has a molecular weight of 200,000 and designated it as the L-form (low molecular weight). When cytosol is incubated at $4^{o}C$ for 5 days, the cytidylyltransferase activity is elevated 7-fold. In addition, the enzyme elutes from a Sepharose 2B column with a median molecular weight of 1.2×10^{6} (21). This form of cytidylyltransferase has been designated H-form (high molecular weight).

The L-form is markedly stimulated by liposomes prepared from total rat liver phospholipid, whereas, the microsomal and H-forms are much less sensitive (22). A number of acidic phospholipids can activate L-form, but the best activator reported to date is lysophosphatidyl-ethanolamine (22). The conversion of L-form to H-form in stored cytosol is not influenced by lysophosphatidylethanolamine but rather diglyceride present in rat liver cytosol was identified as the principal agent responsible for aggregation (23).

Feldman et al., have identified endogenous free fatty acids in rat lung cytosol as the major activating species for the cytidylyltransferase

(24). Removal of free fatty acids resulted in a corresponding decrease in enzyme activity. When the fatty acids were restored to the cytosol, the enzyme activity was also restored. Unsaturated fatty acids like oleate and linoleate were found to be the best activators of cytidylyltransferase (24).

Although cytidylyltransferase can be activated or aggregated by various agents, a functional relationship between the L-, H- and microsomal forms of the enzyme remains obscure. Choy et al., (25) have demonstrated that all three forms of the enzyme are immunologically identical. Additionally there is some support for the idea that the H- and microsomal forms represent the same species. That is, the H-form may be pelleted under conditions similar to those which will sediment microsomes (21) and these forms of the enzyme are usually insensitive to phospholipid activation (22). However, a direct functional link between all three forms of the cytidylyltransferase remains a mystery.

The final step in the CDP-choline pathway is the formation of phosphatidylcholine and CMP from CDP-choline and diglyceride. This step is catalyzed by CDP-choline : 1,2-diacylglycerol cholinephosphotransferase. This enzyme is located in the microsomal fraction (cytoplasmic side) (26) and is characterized by the need for Mg^{+2} or Mn^{+2}, inhibition by Ca^{+2} and the requirement for diacylglycerol which has been emulsified in a nonionic detergent such as Tween 20 (27,28). It has also been shown that this enzyme is specific for CDP-choline and other nucleotides linked to choline are ineffective in generating phosphatidylcholine. The CMP formed in the final reaction may be rephosphorylated at the expense of ATP to form CTP, which in turn can be used again to form CDP-choline. Hence, the cytidine nucleotides may

thus function in a catalytic cycle, with the net production of one mole of phosphatidylcholine in each cycle.

b) Progressive Methylation of Phosphatidylethanolamine

Bremer and Greenberg were the first to demonstrate that phosphatidylcholine could be formed from phosphatidylethanolamine via the addition of methyl groups from Ado Met, liberating S-adenosyl-homocysteine (12). This process is a stepwise one, in which one methyl group is transferred to the base moiety at a time (29) yielding phosphatidyl-N-monomethylethanolamine, phosphatidyl-N, N-dimethylethanolamine and phosphatidylcholine. These researchers, in the same report, identified two enzymes in the adrenal medulla that catalyze the methyl transfer.

The two phospholipid methyltransferases in the adrenal medulla have different properties. The first enzyme (methyltransferase I) converts phosphatidylethanolamine to phosphatidyl-N-monomethylethanolamine, requires Mg^{+2}, has an optimal pH about 7.0 and a low K_m for Ado Met. The second enzyme (methyltransferase II) catalyzes the stepwise methylation of phosphatidyl-N-monomethylethanolamine to phosphatidylcholine (30,31). This enzyme does not require Mg^{+2}, has a high K_m for Ado Met and an optimal pH of 10.0. The two methyltransferases are located in the microsomal and mitochondrial fractions of the adrenal medulla and were found in all tissues examined so far including brain (32), red cells (33), lymphocytes (34), mast cells (35) and basophils and neutrophils (36).

The ability to prepare erythrocyte ghosts either inside-out or right-side-out with respect to cellular contents (37) allowed Hirata and Axelrod (33) to study the localization of these enzymes in membranes. It was demonstrated that methylation of phospholipids began on the

cytoplasmic side of the membrane, where the substrate phosphatidylethanolamine for methyltransferase I is located. Subsequent to initial methylation, methyltransferase II added the additional methyl groups yielding phosphatidylcholine which faces the outside of the membrane (38). This enzymatically facilitated translocation of phospholipids was very rapid (less than two minutes) and was also reported in membranes of Bacillus megatherium (39).

The methylation and rapid rearrangement of phospholipids appears to influence membrane fluidity. When phospholipids are methylated, there is a measured decrease in microviscosity of the membrane (40). It is this translocation of phospholipids in the membrane, with the resultant decrease in microviscosity of the membrane which Hirata and Axelrod (41) believe to play an important role in signal transmission across the membrane. In this report, the authors conclude that neurotransmitters, peptides and immunoglobulins interacting with cell surface receptors initiate a cascade of biochemical and physical changes in local domains of the membrane. This leads to increased mobility of receptors, elevated phospholipid methylation, generation of cyclic AMP, histamine release, mitogenesis and chemotaxis. All of these processes in turn impart specialization of function and regulation to a variety of tissues and cell types. The authors point to phospholipid methylation as an initial common pathway for the transduction of many receptor mediated biological signals through membranes.

Although studies on the potential biological role of phosphatidylethanolamine methylation seem to present a rather convincing argument, recent reports from Audubert and Vance (42,43) questioned the number of enzymes involved in the conversion of phosphatidylethanolamine to

phosphatidylcholine. These investigators demonstrated in liver microsomes that all enzymes for the methylations of phosphatidylethanolamine showed similar K_m's for Ado Met and a single pH optimum of 10.25 (42). In addition, evidence has emerged which suggests that the conversion of phosphatidylethanolamine to phosphatidylcholine by successive methylation occurs entirely on the cytosolic side of the microsomal membrane (43). Hence the number of enzymes involved in the conversion of phosphatidylethanolamine to phosphatidylcholine in liver and other animal cells and tissues remains an important question. The only satisfactory answer will come from purification to homogeneity of the enzyme(s) involved in this conversion. Moreover, the biological functions of phosphatidylethanolamine methylation, as proposed by Hirata and Axelrod (40,41) may have to be reconsidered in view of these findings.

c) Base Exchange of Choline with other Phospholipids

The third pathway for the formation of phosphatidylcholine is the Ca^{+2}-mediated exchange of choline for another phospholipid head group described by Dils and Hübscher (13) in rat liver microsomes. This reaction is often simply referred to as base exchange and appears to be of minor importance in some tissues including liver and heart (44) where less than 10% of the newly synthesized phosphatidylcholine is formed by this route.

Bjerve (45) also investigated Ca^{+2}-stimulated base exchange in liver microsomes. He demonstrated that phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine could all act as substrates for this reaction. Furthermore, choline, ethanolamine and serine could exchange with phosphatidylcholine and phosphatidylserine, but only serine and ethanolamine could exchange with phosphatidylethanolamine. Bjerve

suggested (45) and others have since verified (46,47) that separate enzymes catalyze each of the reactions.

It should be mentioned here that although base exchange accounts for only a small percentage of net phosphatidylcholine biosynthesis in liver, this pathway is considered to be the major route of phosphatidylserine biosynthesis in this same tissue (45).

d) Reacylation Systems

Phospholipids, as previously mentioned, generally have saturated fatty acids at the C_1 position of their glycerol backbone and unsaturated fatty acids at position C_2. However in specialized cases, the fatty acyl moieties on the glycerol backbone of the phospholipid seem to be highly ordered to afford functional speciality. This restructuring of phospholipids occurs in many tissues but is most pronounced in lung, where dipalmitoylphosphatidylcholine (the major component of pulmonary surfactant) accounts for 70% of the total phosphatidylcholine (48). Evidence exists that newly synthesized phosphatidylcholine of lung does not possess this fatty acid arrangement (49) but rather through fatty acyl exchange, "tailor made" phosphatidylcholine is formed.

There are two ways by which the fatty acyl moiety at the C_2 position of phosphatidylcholine can be replaced. The initial removal of this chain by phospholipase A_2 is common to both mechanisms. The resulting 1-saturated-2-lysophosphatidylcholine is then either reacylated with acyl-CoA or transacylated with a second molecule of lysophosphatidylcholine.

The acylation of lysophosphatidylcholine with acyl-CoA by lysophosphatidylcholine-acyltransferase was first described by Lands (50) and subsequently studied in lung microsomes by Forsolono et al., (51).

These investigators found that the transfer of palmitoyl residues to either the 1 or 2 position of lysophosphatidylcholine occurred with similar effectiveness in the lung microsomes. This is in direct contrast to liver microsomes where the transfer of saturated fatty acid residues at the C_2 position was very slow compared to the C_1 position (51).

The transacylation between 2 molecules of lysophosphatidylcholine as catalyzed by lysophosphatidylcholine-lysophosphatidylcholine transacylase was first demonstrated by Marinetti (52). This enzyme catalyzed acyl group transfer from one molecule of lysophosphatidylcholine to another, producing phosphatidylcholine and sn-glycerol-3-phosphorylcholine (53,54). This reaction occurs in the cytosol (55) and has no requirement for ATP, CoA or palmitoyl-CoA (56). These energy-poor conditions suggest that this mechanism of synthesizing dipalmitoyl-phosphatidylcholine is not as important as the lysophosphatidylcholine-acyltransferase pathway in vivo. In this context, the endoplasmic reticulum, the site of the CDP-choline pathway and lysophosphatidylcholine-acyltransferase activity, is generally considered quantitatively more important in complex phospholipid biosynthesis than the cytosol, site of lysophosphatidylcholine-lysophosphatidylcholine transacylase activity.

IV Regulation of Phosphatidylcholine Biosynthesis via the CDP-choline Pathway

Since the CDP-choline pathway is the major route of phosphatidylcholine biosynthesis in many tissues (10,44,49) investigations into the regulation of this pathway may allow us to better understand the biological roles of this phospholipid. There are several ways in which phosphatidylcholine biosynthesis via the CDP-choline pathway might be controlled. The supply of substrates and cofactors for these reactions could be restricted at the level of choline uptake or by compartmentalization of metabolites. Alternatively, the phosphatidylcholine biosynthetic enzymes themselves might be regulated by modulators, covalent modification or by changes in the amount of enzyme protein. Any, or all of these factors might affect de novo phosphatidylcholine formation.

a) Choline Uptake

Choline is a dietary requirement for most mammals. Zeisel et al., (57) have studied the kinetics of choline uptake in the perfused liver. These investigators found that choline was transported by both a saturable and a non-saturable mechanism. The non-saturable mechanism was attributed to simple diffusion of choline across the plasma membrane. The saturable mechanism was reported to have an apparent K_m of 0.17 mM for choline (57). Since the rat plasma choline concentration normally ranges between 10-20 μM (58,59), the facilitated transport of choline was not expected to be saturated in vivo. However it should be mentioned that in hamster heart the plasma choline concentration was estimated at 0.18 mM and the K_m for choline uptake at 0.1 mM, hence plasma choline concentration may provide a mechanism for the regulation of choline uptake in this tissue (44).

Since choline is also a precursor for the neurotransmitter acetylcholine, brain choline transport has received considerable attention. Crude synaptosomes from rat cerebral cortex showed two choline uptake systems. One had a high affinity for choline and was sodium-dependent, while the other had a lower affinity for choline and was insensitive to sodium (60,61). The high affinity uptake of choline in brain cells was associated with a higher proportion of phosphocholine formation (62). However, at high choline concentrations in the medium, the added choline was increasingly recovered as acetylcholine or free choline, with a smaller proportion recovered as phosphocholine (62).

Although the rate of phosphatidylcholine biosynthesis is not thought to be influenced by choline uptake, there are some examples in the literature which suggest such a regulatory role. A 40% increase in the rate of phosphatidylcholine biosynthesis was reported in rat hepatocytes when the choline concentration in the medium was varied from 5-40 μM (63). Additional evidence for choline transport serving as the rate-limiting step in phosphatidylcholine formation has been obtained from Novikoff hepatoma cells (64). At concentrations below 20 μM, choline incorporation into phosphatidylcholine was limited by the rate of formation of phosphocholine which in turn was limited by the rate of choline entry into the cells. However, at concentrations exceeding 20 μM, the ultimate rate of choline incorporation into phosphatidylcholine was independent of the medium choline concentration and the intracellular level of phosphocholine (64).

b) Supply of Intracellular Metabolites

Not all of the intracellular choline is committed for phosphatidylcholine biosynthesis. In rat liver, (65) BHK-21 cells (66) and hamster

heart (44) there is ample evidence for a separate pool of choline which is active in phosphatidylcholine synthesis. Pulse-labelling studies with the isolated hamster heart suggest that [Me-^{3}H] choline does not equilibrate with the large intracellular choline pool, but is rapidly phosphorylated instead (44). This might be feasible if choline kinase phosphorylates choline just after transport across the plasma membrane (67). Another possible explanation is that some intracellular choline is sequestered and therefore is inaccessible to choline kinase (44).

The intracellular pool size of phosphocholine may also regulate the rate of phosphatidylcholine biosynthesis. In rooster liver, the pool size of phosphocholine is below the apparent K_m and may therefore be limiting (68). In addition the rate of phosphatidylcholine synthesis in BHK-21 cells may be influenced by the intracellular phosphocholine pool since this pool is approximately 10-fold lower than the apparent K_m for the cytidylyltransferase (66,69).

Regulation of phosphatidylcholine synthesis by the supply of CTP is an interesting hypothesis since CTP is required in the anabolism of all phospholipids. Studies with polio-virus infected HeLa cells have correlated a 2-fold stimulation of phosphatidylcholine biosynthesis with a 2-3-fold increase in CTP concentration (70). In 3-deaza-adenosine-treated rats, enhancement of phosphatidylcholine formation was accompanied by a 26% increase in hepatic concentration of CTP (71). Finally, in myopathic hamsters, the cardiac CTP level was 34% lower than found in controls (72). The rate of phosphatidylcholine synthesis in these hearts was comparable to controls because of a compensating increase in the microsomal cytidylyltransferase activity.

The supply of diglyceride may also affect the rate of phosphatidyl-

choline formation. Diglyceride is produced from phosphatidate in a reaction catalyzed by phosphatidate phosphohydrolase. Diglyceride is a substrate for cholinephosphotransferase, ethanolaminephosphotransferase, diglyceride lipase and diglyceride acyltransferase. All of these reactions compete for diglyceride, but during starvation diglyceride is channelled into rat hepatocyte phospholipids at the expense of triglyceride synthesis (73). Groener and van Golde have recently discounted the level of diglyceride as a rate-determining factor in the synthesis of phosphatidylcholine in rat liver (74). This conclusion was based on the insensitivity of the phosphatidylcholine pool size to alterations after a 48 h fast and 24 h refeeding on a high sucrose, fat-free diet, despite a 10-fold increase in the level of diglyceride.

c) CTP:Phosphocholine Cytidylyltransferase Catalyzes the Rate-Limiting Step of de novo Phosphatidylcholine Biosynthesis

In most tissues, the rate-limiting step of the CDP-choline pathway is catalyzed by CTP:phosphocholine cytidylyltransferase (10). This view is contrary to the general biochemical principle that the first committed reaction in a pathway is under metabolic control and is rate-limiting.

The evidence to support this theory is that the total pool size of choline is 5-10 fold lower than phosphocholine in liver (65) and HeLa cells (75). These relative pool sizes indicate a rapid conversion of choline to phosphocholine, with the rate-limiting step occurring subsequent to the choline kinase catalyzed reaction. Additionally the phosphocholine pool in rat liver was 150 times higher than the CDP-choline pool (65). Since CDP-choline is the only known metabolite of phosphocholine, these results suggest that once CDP-choline is formed, it is rapidly converted to phosphatidylcholine.

More definitive evidence for the rate-limiting role of this enzyme was obtained from pulse-chase studies in HeLa cells (75) and isolated hamster heart (44). The radioactivity of the exogenously added [Me-^{3}H] choline was rapidly converted to phosphocholine. Subsequently, as the radioactivity disappeared from phosphocholine, it was transiently associated with CDP-choline and was immediately recovered as phosphatidylcholine.

Further support was obtained by Choy et al., (70) studying poliovirus infected HeLa cells. These cells, when infected with virus, tend to stimulate the incorporation of [Me-^{3}H] choline into phosphatidylcholine. Pulse-chase studies showed that the observed increase in phosphatidylcholine formation was due solely to an acceleration of the CTP:phosphocholine cytidylyltransferase catalyzed reaction (70).

As previously mentioned, cytidylyltransferase exists in two subcellular compartments (cytosol and endoplasmic reticulum). Recent studies on the regulation of phosphatidylcholine biosynthesis have been directed at the functional significance of this distribution. To date, the prevailing theory is that the cytosol serves merely as a reservoir for inactive enzyme. When the cell requires phosphatidylcholine, a translocation from the cytosol to the endoplasmic reticulum occurs, with a resultant surge in phosphatidylcholine formation. This translocation process and the factors which may govern it will be discussed over the next several pages of this thesis.

Evidence for the conclusion that enzyme translocation governs the rate of phosphatidylcholine biosynthesis arises largely from correlation experiments. In particular experiments with rat hepatocytes and established cell lines have been most convincing.

Cyclic AMP analogues mediate a partial inhibition (35%) of phosphatidylcholine biosynthesis (76); this seems to fit into the general scheme of cAMP action, namely, as an inhibitor of biosynthetic reactions. The decreased rate of phosphatidylcholine biosynthesis correlated with a decrease (34%) in microsomal cytidylyltransferase activity (76).

In a different approach, cultured chick embryonic myoblasts and CHO cells were depleted of phosphatidylcholine by treatment with phospholipase C (77,78). Subsequently the rate of phosphatidylcholine formation was increased 3-fold and a similar increase in the microsomal activity was observed. Treatment of the myoblasts for 3 h with cycloheximide did not alter the cellular response to phospholipase C (77). Hence, protein synthesis was not required for translocation of the cytidylyltransferase or for the concomitant increase in phosphatidylcholine biosynthesis. Moreover, 6 h after removal of the phospholipase C, its effect on choline incorporation in CHO cells was fully reversed (79). However, a correlation between the reversal of choline incorporation and binding of the cytidylyltransferase to microsomes was not demonstrated (79).

Additional evidence for the translocation theory in intact animals has recently emerged. Several animal systems in which an increase in microsomal cytidylyltransferase activity has been correlated with a similar increased rate of phosphatidylcholine synthesis have been studied. Fetal rats were delivered prematurely after either 20 or 21 days gestation (80). The incorporation of choline into phosphatidylcholine was evaluated in lung slices at the time of delivery or 3 h after incubation of the fetus in a special chamber. During the 3 h period,

there was a 2-fold stimulation of phosphatidylcholine synthesis (80). During this same time there was a 2-3-fold redistribution of enzyme activity from cytosol to microsomes (80). Correspondingly, immediately after birth, the rate of phosphatidylcholine synthesis tripled in rat liver and this correlated with translocation of the enzyme to microsomes (81). In addition, a 3-fold increase in phosphatidylcholine synthesis has been induced in the livers of young rats fed a diet enriched in cholesterol and cholate (82). Once again a correlation between increased enzyme activity and its translocation to the microsomes was demonstrated. Taken together, the combined evidence from various cell cultures and animal studies make a strong case for regulation of the rate of phosphatidylcholine biosynthesis by reversible translocation of cytidylyltransferase between the cytosol and the endoplasmic reticulum.

The mechanism leading to the subcellular movement of cytidylyltransferase is still rather unclear. However there appears to be some evidence for two different modes of regulation for this process. The first involves reversible phosphorylation of the cytidylyltransferase (or possibly of an activator and/or inhibitor protein). Cyclic AMP analogues cause a decrease in the cytidylyltransferase activity associated with microsomes (76). Also, the activity of the enzyme appears to be regulated by cAMP-dependent protein kinases and protein phosphatases (83). Thus it seems likely that cAMP-dependent protein kinase causes a decrease in cytidylyltransferase activity associated with microsomes, whereas dephosphorylation promotes the binding of the enzyme to microsomes where it is activated.

A second mechanism involves the apparent effect of long chain fatty acids or acyl CoA's on cytidylyltransferase (24,84,85). The fatty acids

do not directly stimulate the enzyme activity. Instead they appear to increase the affinity of the enzyme for membranes which contain phospholipids, which in turn activate the cytidylyltransferase. The fatty acid can apparently reverse the phosphorylation-mediated decrease in cytidylyltransferase activity associated with microsomes (85).

The translocation of cytidylyltransferase and the processes which control this movement (phosphorylation and fatty acid modulation) seem to have considerable support. However, the complete understanding of this process has been hampered for a variety of reasons. The principal problem thus far has been a lack of purified cytidylyltransferase. Were this enzyme source available, reconstitution studies involving artificial membranes of known lipid composition would be made possible. The behaviour of cytidylyltransferase in these artificial membranes or in model membranes containing physiological concentrations of diglyceride (a potent membrane-binding promoter) could facilitate the understanding of the molecular mechanisms involved in translocation. Additionally, purified cytidylyltransferase with pure protein kinase and phosphatases would enable the demonstration of whether cytidylyltransferase is phosphorylated, and if so, how does the phosphorylation affect the enzymes affinity for membranes. Similarily, the mechanism by which fatty acids enhance binding of the enzyme to membranes might be studied.

It is clear that the regulation of phosphatidylcholine biosynthesis via the CDP-choline pathway is a complex process which we are only now beginning to comprehend.

V Research Aims

Phosphatidylcholine is the major phospholipid in the mammalian heart (86). It serves not only as a structural component of the cardiac membrane, but also as a modulator of a number of membrane-bound enzymes (5). Previous studies from our laboratory have demonstrated that choline is rapidly taken up by the isolated perfused heart and is subsequently incorporated into phosphatidylcholine (44). Additionally, the CDP-choline pathway has been shown to be the major route of phosphatidylcholine biosynthesis in this tissue and over 85% of the newly synthesized phosphatidylcholine in the heart is formed via this pathway (44). However, the control of phosphatidylcholine biosynthesis via the CDP-choline pathway in the mammalian heart remains unknown. Hence, the basic aim of the proposed research was to investigate various regulatory mechanisms for phosphatidylcholine biosynthesis in the hamster heart.

Two independent approaches were employed for this study. The first approach involved the study of the potential control afforded by various exogenous metabolites. Due to the absolute requirements of choline and ethanolamine for phospholipid biosynthesis and the structural similarities between these compounds, it was conceivable that these metabolites may regulate phospholipid biosynthesis. Initial studies determined the effect of ethanolamine on phosphatidylcholine biosynthesis and subsequently the corollary effect of choline on phosphatidylethanol-amine biosynthesis in the isolated perfused heart was studied. A second approach involved the *in vitro* characterization of CTP:phosphocholine cytidylyltransferase from the heart. As previously reported (44) this enzyme catalyzes the rate-limiting step of the CDP-choline pathway and

the reaction is regarded as an important control step for phosphatidylcholine biosynthesis. The mechanism involved in the regulation of this enzyme in the heart was completely unknown.

The proposed research is the first attempt to elucidate the control mechanism of phosphatidylcholine biosynthesis in the mammalian heart. In view of the fact that phosphatidylcholine is the major phospholipid in all mammalian cardiac tissues, an in-depth study on the control of the biosynthesis of this phospholipid is highly desirable. This study should enable us to understand some of the molecular events underlying the biosynthesis of phosphatidylcholine in the heart.

EXPERIMENTAL PROCEDURES

MATERIALS

I Experimental Animals

Syrian Golden hamsters (90-120 g, body weight) were maintained on Purina Hamster Chow and tap water, ad libitum, in a light- and temperature-controlled room.

II Enzymes, Chemicals and Radioisotopes

The commercial sources of many of the materials which were used during this investigation are listed in Table I. All other chemicals were of reagent grade and were obtained from Fisher Chemical Company. All solutions were prepared with distilled water and were adjusted to the desired pH.

TABLE I

Commercial suppliers of research materials

Chemical	Supplier
[2-^{14}C] ethanolamine	Amersham Corporation
[1-^{3}H] ethanolamine	Amersham Corporation
[γ-^{32}P] ATP	Amersham Corporation
Aqueous Counting Scintillant	Amersham Corporation
[Me-^{3}H] choline	Amersham Corporation
Sodium dodecyl sulfate	BDH Chemicals
Dowex AG1-X8 in formate or chloride form	Bio-Rad Laboratories
Silica G25 thin layer chromatography plates	Brinkmann
Phosphoethanolamine	Fisher Chemical Co.
2-Aminoethanol	Fisher Chemical Co.
Hemicholinium-3	Fisher Chemical Co.
Sodium Fluoride	Fisher Chemical Co.
Redi Plate Silica G thin layer chromatography plates	Fisher Chemical Co.
Sepharose 6B	Pharmacia Fine Chemicals
Sucrose	Schwartz-Mann
ATP	Sigma Chemical Co.
CDP-ethanolamine	Sigma Chemical Co.
Choline Chloride	Sigma Chemical Co.
Choline Kinase	Sigma Chemical Co.
CTP	Sigma Chemical Co.

cont'd

TABLE I (cont'd)

Chemical	Supplier
CDP-choline	Sigma Chemical Co.
Phosphocholine	Sigma Chemical Co.
Immunoglobulin G	Sigma Chemical Co.
Alkaline Phosphatase (Type IIIs)	Sigma Chemical Co.
Phosphodiesterase (Type II)	Sigma Chemical Co.
Bovine Serum Albumin	Sigma Chemical Co.
Phenylmethylsulfonylfluoride	Sigma Chemical Co.
Phosphatidylcholine	Serdary Research Laboratories
Phosphatidylethanolamine	Serdary Research Laboratories
Lysophosphatidylcholine	Serdary Research Laboratories
Lysophosphatidylethanolamine	Serdary Research Laboratories

GENERAL PREPARATIVE PROCEDURES

I Preparation of Perfusion Buffer

Krebs-Henseleit buffer was used in all perfusion studies. To prepare this buffer 70.1 g NaCl, 21 g $NaHCO_3$ and 9.91 g Dextrose (anhydrous) were combined and dissolved in 1 l distilled water to yield Solution A. Solution B was a mixture of 3.55 g KCl, 2.94 g $MgSO_4 \cdot 7H_2O$ and 1.63 g $NaH_2PO_4 \cdot H_2O$ in 100 ml distilled water. Solution C contained 3.734 g $CaCl_2 \cdot 2H_2O$ in 100 ml distilled water. These stock solutions were prepared and could be stored separately at 4^oC for up to two months. During each perfusion study fresh buffer was prepared by combining 100 ml Solution A, 10 ml Solution B and 5 ml Solution C with 885 ml distilled water. The buffer was subsequently saturated with 95% 0_2 - 5% CO_2. This solution could not be stored over 24 h and was therefore discarded immediately following perfusion.

II Perfusion of the Isolated Hamster Heart

Hamsters were sacrificed by decapitation and the hearts were rapidly removed and placed in Krebs-Henseleit buffer (87), saturated with 95% oxygen - 5% carbon dioxide at room temperature. The heart was cannulated via the aorta in the Langendorff mode (88) and the pulmonary artery was incised to ensure adequate coronary drainage. Perfusion was performed at 37^oC with a constant aortic pressure of 90-120 mm mercury and a coronary flow rate of 2.5-4 $ml \cdot min^{-1}$. A model of isolated heart perfusion in the Langendorff mode was described by Manning et al., (89). The viability of the hearts was maintained throughout the perfusion period as assessed by electrocardiac

recordings. One electrode was attached to the aortic cannula and the other was placed in the solution bathing the heart. The placement of electrodes in this manner allowed us to assess the atrial and ventricular activities simultaneously. The signals were amplified and recorded by a Sanborn paper recorder. No irregular electrocardiac recordings were observed throughout the perfusion periods employed in these studies. A typical electrocardiac recording of the isolated hamster heart was depicted in a previous report (89b).

III Preparation of Subcellular Fractions

Hamsters were sacrificed by decapitation and the hearts were rapidly removed and placed in ice-cold saline. The hearts were weighed, cut into pieces and homogenized in isotonic saline (0.145 M) for 30 s with a polytron homogenizer. The homogenates were centrifuged at 12,000 x g for 15 min and the post-mitochondrial supernatant was centrifuged at 150,000 x g for 60 min. The resultant supernatant was designated the cytosolic fraction. The microsomal pellet was resuspended in 0.145 M NaCl-5 mM Tris-HCl (pH 7.4) with a glass Dounce homogenizer. Since cytosolic CTP:phosphocholine cytidylyltransferase activity might change with the incubation of cytosol at $4^{\circ}C$ (21), the enzyme activities in the subcellular fractions were routinely assayed at 4 h after the tissue was homogenized.

IV Enzymatic Synthesis of [Me-^{3}H] Phosphocholine

Choline kinase was used to generate [Me-^{3}H] phosphocholine from [Me-^{3}H] choline. Choline kinase (baker's yeast, Grade II) was obtained from Sigma. One unit of enzyme was dissolved in 3 ml of distilled water and was subsequently placed in an Amicon CF-25 filtration cone.

The solution was centrifuged at 3,000 x g for 40 min. The filter was washed with 5 ml 0.2 M NaH_2PO_4 (pH 8.5) buffer and the solution was again centrifuged at 3,000 x g for 40 min. The choline kinase retained by the filter was in a volume of approximately 500 µl. Two mCi of labelled choline were added to a tube and the solvent evaporated under a stream of nitrogen. Fifty µl of $MgCl_2$ (100 mM) 50 µl of ATP (100 mM), 25 µl of 1 M Tris-HCl, pH 8.0 and 250 µl of choline kinase were added to the tube. The reaction mixture was incubated at 37°C for 1 h, put in boiling water for 3 min and centrifuged at low speed for 15 min. The supernatant was applied as two 4 cm bands to silica G-25 thin layer chromatography plates and developed in the solvent system containing CH_3OH/0.6% NaCl/NH_4OH (50/50/5;v/v/v). The synthesized phosphocholine was detected by an Autochron radioactivity scanner. Two distinct peaks or radioactivity were detected, one corresponding to the unreacted choline and the other to the newly synthesized phosphocholine. The silica that contained phosphocholine was extracted with three x 3 ml of distilled water and the washings were pooled and centrifugated at 12,000 x g for 60 min. The concentration of radioactivity was adjusted to 1 mCi/ml, and then unlabelled phosphocholine was added to prepare the desired final concentration. The yield was greater than 75%.

V Extraction and Preparation of Total Hamster Liver Phospholipid

Hamsters were sacrificed by decapitation and the livers were rapidly removed and placed on ice. A liver sample (5 g) was homogenized in 15 ml $CHCl_3/CH_3OH$ (1/2;v/v). The homogenate was centrifuged and the supernatant removed and placed in a separatory funnel. The pellet was extracted twice with 10 ml of $CHCl_3/CH_3OH/H_2O$ (1/2/0.8;v/v/v). The

extracts were pooled and a biphasic mixture was obtained by the addition of 10 ml $CHCl_3$ and 10 ml H_2O. The lower phase was removed and the upper phase was washed with 20 ml $CHCl_3/CH_3OH/H_2O$ (86/14/1;v/v/v). The lower phase from this wash was removed and combined with the extract from the first separation (90). The solvent was removed under reduced pressure and the dry extract was stored at -20°C in an atmosphere of nitrogen. The dry lipid extract was resuspended in $CHCl_3/CH_3OH$ (2/1;v/v) and quantitation of lipid phosphorus was determined by the method of Raheja (91). The phospholipid solution was adjusted to a final concentration of 1 mg/ml and stored at -20°C.

VI Partial Purification of CTP:Phosphocholine Cytidylyltransferase

A 20% heart homogenate from three male hamsters was prepared in 0.145 M NaCl. Cytosol was obtained by ultra-centrifugation as described in section III. The cytosol was stored at 4°C for 3-5 days. Following storage 4 ml of cytosol was applied to a Sepharose 6B column (1.5 x 45 cm) which had been equilibrated with a buffer containing 20 mM Tris-HCl, 100 mM NaCl and 1 mM DTT (pH 7.0). Cytidylyltransferase activity was measured and the fractions containing H-form were pooled. These fractions are milky in appearance and elute very near the void volume of the column. The pooled sample was subsequently incubated with 0.025% SDS for 5 min at 4°C and applied to another column of Sepharose 6B (1.5 x 45 cm) which had been equilibrated with the same buffer. Fractions (1.4 ml) were collected and three distinct peaks of enzyme activity were resolved (H-form, L-form and S-form). Cytidylyltransferase activity was assayed in these fractions in the presence of 1 µg total lipid phosphorus (liver). A 800-1800 fold

enrichment of enzyme was typically obtained under these conditions.

GENERAL ANALYTICAL PROCEDURES

I Uptake of Labelled Compounds by the Isolated Heart

a) General

The isolated heart was perfused for 5 min in Krebs-Henseleit buffer saturated with 95% oxygen - 5% carbon dioxide. The heart was then perfused with Krebs-Henseleit buffer containing [Me-^{3}H] choline for 45 min or [2-^{14}C] ethanolamine for 25 min. Subsequently, the heart was reperfused with 5 ml Krebs-Henseleit buffer to remove any labelled compound trapped in the vascular space. After perfusion, 10 ml of air was forced through the cannula to remove the buffer. The heart was cut open, blotted dry and the wet weight determined. The tissue was homogenized in 20 ml $CHCl_3/CH_3OH$ (2/1;v/v) and an aliquot of the homogenate was taken for radioactivity determination. The efficiency of lipid extraction by $CHCl_3/CH_3OH$ has been well-documented (90). The uptake of labelled compound was calculated from the specific radioactivity of the compound in the perfusate.

b) Analysis of Phospholipids

The homogenate obtained after perfusion was separated into two phases by the addition of 15 ml 0.1 M KCl to the homogenate. In studies where [Me-^{3}H] choline or [2-^{14}C] ethanolamine were the labelled precursors, phospholipids in the organic phase were separated by thin layer chromatography in a single dimension on Silica G-25 with $CHCl_3/CH_3OH/H_2O/CH_3COOH$ (70/30/4/2;v/v/v/v). This solvent system facilitates excellent separation of phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine. Unfortunately, phosphatidylcholine and lysophosphatidylethanolamine did not separate adequately with this solvent system, therefore quantitation of these two phospholipids were

confirmed by thin layer chromatography in a single dimension with a solvent system of $CHCl_3/CH_3OH/H_2O/NH_4OH$ (70/30/4/2;v/v/v/v) as described by Choy and Vance (22).

c) Analysis of Choline-containing Metabolites

In experiments with [Me-^{3}H] choline, the aqueous phase of the total heart homogenate was lyophilized and reconstituted in H_2O. Choline, phosphocholine and CDP-choline were resolved by thin layer chromatography in a single dimension by a solvent system of CH_3OH/0.6% NaCl/NH_4OH (50/50/5;v/v/v). Unlabelled choline, phosphocholine and CDP-choline were routinely chromatographed with the samples to improve recovery and resolution as well as to facilitate visualization of each compound. Choline and phosphocholine were detected by iodine staining (62), and CDP-choline was visualized with ultraviolet light.

d) Analysis of Ethanolamine-containing Metabolites

The aqueous soluble components, ethanolamine, phosphoethanolamine and CDP-ethanolamine were separated by thin layer chromatography with appropriate carriers in 96% CH_3CH_2OH/2% NH_4OH (1/2;v/v) as described by Sundler (92). The amino groups of ethanolamine, phosphoethanolamine and CDP-ethanolamine were detected by spraying with 0.1% ninhydrin (triketohydrindene hydrate).

II Metabolite Pool Size Measurements

a) Choline-containing Metabolites

In these studies, hamster hearts were perfused with Krebs-Henseleit buffer without choline, with choline, or with choline and ethanolamine at various concentrations and times as described in Tables VI and X. Immediately after perfusion two hearts from each group were weighed and

homogenized in 30 ml $CHCl_3/CH_3OH$ (1/1;v/v). Fifteen ml H_2O was added to the homogenate and the aqueous phase was removed and evaporated under reduced pressure. Yields of the aqueous soluble choline-containing metabolites were estimated by the recovery of the [Me-^{3}H] choline, [Me-^{14}C] phosphocholine and [Me-^{14}C] CDP-choline which were added to each homogenate.

The procedure for quantitation of the choline-containing compounds in the aqueous extracts was adapted from Vance et al., (73). The aqueous phase was redissolved in 5 ml H_2O and applied to a column (1 cm x 25 cm) that contained Dowex AG-1-x8 (formate form) which had been equilibrated with CH_3OH/H_2O (1/1;v/v). Choline was eluted from the column with 100 ml CH_3OH/H_2O (1/1;v/v) followed by 10 ml H_2O. Phosphocholine and CDP-choline were eluted with 200 ml of 0.4 M NH_4HCO_3. These fractions were evaporated under reduced pressure.

The phosphocholine and CDP-choline samples were redissolved in 5 ml H_2O and applied to a Norite A-cellite (1/2;v/v) column (1 cm x 7.5 cm). Phosphocholine was eluted with 20 ml H_2O and 15 ml 2% ethanol. CDP-choline was eluted from the column with the addition of 30 ml 40% ethanol containing 1% NH_4OH. These solutions were evaporated under reduced pressure. The fractions containing choline, phosphocholine and CDP-choline were reconstituted in H_2O and an aliquot from each fraction was taken for radioactivity determination. The fraction containing phosphocholine was digested with 10 units of E. coli alkaline phosphatase for 2 h at 37°C. The fraction containing CDP-choline was digested with 0.6 units of Crotalus adamenteus phosphodiesterase and 10 units of E. coli alkaline phosphatase for 2 h at 37°C. Subsequently, both groups of reaction mixtures were frozen and lyophilized. The resulting residue was extracted three times with 2 ml 70% ethanol. The ethanol in the

extracted sample was evaported under a stream of nitrogen.

The choline, digested phosphocholine and CDP-choline were separately dissolved in 3 ml H_2O. Tetraphenylboron (10 mg/ml) in 2 ml of 3-heptanone was used to extract choline from each pool. A second extraction was performed with 2 ml of tetraphenylboron in 3-heptanone. The choline in each sample was back-extracted from the tetraphenylboron solution with 4 ml of 0.4 N HCl, followed by a second extraction with 2 ml of 1 N HCl. The HCl solutions were combined, frozen and lyophilized. Each pool was redissolved in 0.5 ml H_2O and an aliquot was taken for radioactivity recovered in each pool.

The reaction of each sample with choline kinase and [γ-^{32}P] ATP allowed quantitation of the amount of extracted choline, by the production of [^{32}P] phosphocholine (93). A standard choline curve was established and the choline concentration in each pool was calculated form the standard curve.

b) Ethanolamine-containing Metabolites

In these studies, normal unperfused hearts were used for the estimation of the pool sizes of various ethanolamine-containing metabolites. As in the measurement of the choline-containing metabolites, the hearts were rapidly removed from the animal, weighed and homogenized in $CHCl_3/CH_3OH$ (2/1;v/v). After the addition of H_2O the aqueous phase was separated from the organic phase and was evaporated under reduced pressure. [1-^{3}H] ethanolamine was added to estimate the recovery of the ethanolamine-containing compounds. The aqueous phase was subsequently dissolved in 2 ml H_2O. An aliquot of the aqueous phase was combined with trichloroacetic acid (TCA), to make a final concentration of 55% TCA. This solution was centrifuged at 3,000 x g for 20 min to

pellet any protein in the sample. An aliquot of the supernatant was taken for radioactivity determination and a separate aliquot was applied to an amino acid analyzer.

Analysis of the ethanolamine-containing compounds was adapted from the procedure of van Sande and van Camp (94). The amino acid analyzer was equipped with a W-2 (30 cm x 0.75 cm) column. The samples were eluted with 0.2 M sodium citrate buffer (pH 4.75) at a constant flow rate of 78 ml/h at 40°C. The column was standardized against norleucine, ethanolamine, phosphoethanolamine and CDP-ethanolamine. Ethanolamine was retained under these conditions (32 min), whereas phosphoethanolamine and CDP-ethanolamine co-eluted at 10 min. Since these two compounds were poorly resolved, an identical aliquot of the aqueous phase was taken and digested with 10 units of alkaline phosphatase in 50 mM Tris-HCl buffer (pH 8.5) for 2 h at 37°C. Subsequent to digestion, TCA was added to precipitate the protein in the samples. An aliquot of this mixture was taken for calculation of recovery and another aliquot for application to the analyzer. In this case, the peak at 10 min is CDP-ethanolamine, and the other peak (32 min) is ethanolamine, which originates from phosphoethanolamine hydrolysis and ethanolamine which was in the original (non-digested) sample. The decrease in peak area at 10 min, corresponded to the increase in peak area at 32 min. By this procedure, the pool sizes of ethanolamine, phosphoethanolamine and CDP-ethanolamine were calculated by the simple equation:

$$\text{Concentration} = \frac{\text{area under the peak}}{\text{KF} \times 10^{-4}}$$

Where KF is the calibration factor and was determined by the ratio of peak area to a known amount of amino acid (95).

III Enzyme Assays

a) Ethanolamine Kinase

Ethanolamine kinase activity is found exclusively in the cytosolic fraction (96) and was assayed as described by Schneider and Vance (97). A typical reaction mixture contained 80 mM sodium glycylglycine (pH 8.5), 1 mM [1-^{3}H] ethanolamine, 3 mM $MgCl_2$, 3 mM ATP, H_2O and heart cytosol in a final volume of 100 µl. The reaction mixture was incubated for 30 min at 37°C and was subsequently placed in boiling water for 3 min. The protein was pelleted by centrifugation and 50 µl of the mixture was applied to a thin-layer chromatography plate with phosphoethanolamine carrier (unlabelled). The plate was developed in 96% ethanol/2% NH_4OH (1/2;v/v). The phosphoethanolamine band was visualized with ninhydrin spray as previously described, removed from the plate and analyzed for radioactivity associated with phosphoethanolamine. Under these assay conditions product formation was linear to 60 min.

b) CTP:Phosphocholine Cytidylyltransferase

CTP:phosphocholine cytidylyltransferase is located in both the cytosolic and microsomal fractions (21,44). Enzyme assays were performed as described by these investigators. A typical reaction mixture contained 100 mM Tris-succinate (pH 8.0), 12 mM magnesium acetate, 2.5 mM CTP, 1 mM [Me-^{3}H] phosphocholine, 1 µg lipid phosphorus (in suspension) and either heart cytosol or heart microsomes in a total volume of 100 µl. Cytosolic fractions were incubated for 30 min at 37°C and microsomal fractions for 15 min at 37°C. The reactions were terminated by placing the tubes in boiling H_2O for 3 min. An aliquot of the reaction mixture was applied to a thin layer chromatography plate with unlabelled CDP-choline as carrier. The plate was developed in

CH_3OH/0.6% NaCl/NH_4OH (50/50/5;v/v/v). The CDP-choline band was visualized by U.V. light, and the spot was removed and analyzed for radioactivity associated with CDP-choline.

IV Protein Assays

a) Lowry Protein Assay

For some studies, the protein content was estimated by a modified method of Lowry et al., (98). Aliquots of subcellular fractions were incubated in 1.5 ml of 0.66 N NaOH at 37°C overnight. To each sample 1.5 ml of solution A (100 ml of 13% sodium carbonate; 3 ml of 4% sodium potassium tartrate; and 3 ml of 4% copper sulfate) was added and the entire mixture was vortexed vigorously. Subsequently, 0.5 ml of 2 N phenol reagent was added to each sample. The final volume of the assay was 3.5 ml. This mixture was vortexed and allowed to sit at room temperature for 1 h. Absorbance of the blue solution was measured at 625 nm against a blank. Fatty acid free albumin (1 mg/ml) was used as a standard, absorbance was linear to 100 µg of protein.

b) Bio-Rad Protein Assay

For studies involving cytidylyltransferase, the Bio-Rad protein assay based on the method of Bradford (99) provided rapid and reproduceable determinations. Two ml of diluted protein reagent (Bio-Rad Stock Reagent/H_2O; 1/4;v/v) were added to 0.5 ml of protein sample. After 10-15 min, the absorbance of the blue-brown solution at 595 nm was measured. Fatty acid free albumin was used as the protein standard and the absorbance of the reaction was linear to 100 µg of protein.

V Phospholipid Assays

a) Determination of Lipid Phosphorus Without Acid Digestion

Phospholipid concentrations were determined by the method of Raheja et al., (91). In this procedure, 16 g of ammonium molybdate was added to 120 ml of water to yield solution I. Subsequently, 40 ml of concentrated hydrochloric acid and 10 ml mercury were shaken with 80 ml of solution I for 30-60 min to yield, after filtration, solution II. Two hundred ml of concentrated sulfuric acid was then added to the remainder of solution I, after which solution II was added to this solution to yield solution III. Forty-five ml of methanol, 5 ml of chloroform and 20 ml of water were added to 25 ml of solution III to give the chromogenic solution. Chloroform (0.4 ml), and an aliquot of the chromogenic solution (0.1 ml) was added to phospholipid samples or standards. The tubes were placed in a boiling water bath for 1-1.5 min. After cooling to room temperature, 5 ml of chloroform was added and each tube was vortexed vigorously. The chloroform layer was removed and absorbance of the blue solution was measured at 710 nm. Phospholipid concentrations in different samples were calculated from the standard phospholipid curve. Absorbance was linear to 20 µg of lipid phosphorus.

b) Determination of Lipid Phosphorus by Acid Digestion

In order to increase the sensitivity of the assay for lipid phosphorous, phospholipid concentrations were also determined by the method of Bartlett, (100). In these determinations phospholipid samples or standards were placed in test tubes to which 0.5 ml of sulfuric acid was added. The mixture was incubated at 160°C overnight and then allowed to cool to room temperature. At this time 0.2 ml of hydrogen peroxide was added to each tube. The samples were heated at 160°C for an additional

2 h. Subsequent to cooling, 3 ml of water and 6 ml of 0.4% ammonium molybdate were added. The tubes were vortexed and 0.4 ml of freshly prepared 1-amino-2 naphtol 4-sulfonic acid was added. The tubes were then placed in a boiling water bath for 10-15 min and absorbance of the blue solution was measured at 820 nm against a blank. Phospholipid concentrations in the samples were similar to those described in section (a).

VI Liquid Scintillation Counting

Lipid and aqueous samples were placed in scintillation vials to which 2 ml of water, 10 ml ACS and 0.2 ml acetic acid were added. Radioactivity was measured by liquid scintillation counting in a LKB mini beta scintillation counter with channels' ratio calibration method.

VII Statistical Analysis

The student's test was used for the determination of significance. The level of significance was defined as $p<0.05$. All results were tabulated as mean $\pm$ standard deviation (number of experiments).

E X P E R I M E N T A L R E S U L T S

THE EFFECT OF ETHANOLAMINE ON PHOSPHATIDYLCHOLINE BIOSYNTHESIS

I Incorporation of Radioactivity into Phosphatidylcholine

Although choline was shown to be taken up and subsequently incorporated into phosphatidylcholine by the isolated hamster heart (44), the effect of exogenous ethanolamine on phosphatidylcholine biosynthesis was not known. In order to elucidate the possible regulatory role of ethanolamine, isolated hamster hearts were perfused with Krebs-Henseleit buffer containing 0.05 mM [Me-^{3}H] choline in the presence of 0.05-0.5 mM ethanolamine. Subsequent to perfusion, the hearts were homogenized in $CHCl_3/CH_3OH$ (2/1;v/v) and the homogenate was analyzed for radioactivity associated with various labelled metabolites. In all experiments more than 90% of the radioactivity in the lipid extract was recovered as phosphatidylcholine. Less than 7% of the radioactivity was recovered as sphingomyelin and lysophosphatidylcholine. When equimolar (0.05 mM) choline and ethanolamine were present in the perfusate, no significant reduction in phosphatidylcholine labelling was observed (Table II). However, higher concentrations of ethanolamine (0.1-0.5 mM) caused a 26-63% reduction in phosphatidylcholine labelling as compared to the control.

One possible explanation for reduced phosphatidylcholine labelling in the presence of 0.1-0.5 mM ethanolamine is that ethanolamine affects the uptake of choline by the heart. Therefore, total uptake of labelled choline in the presence of ethanolamine (0.05-0.5 mM) was determined. At 0.05 mM, ethanolamine had no effect on the uptake of labelled choline, whereas 0.1-0.5 mM ethanolamine caused a 23-65% reduction of total uptake (Table II). The percentage decrease in

TABLE II

Effect of ethanolamine on choline uptake and phosphatidylcholine labelling

Hamster hearts were perfused with 0.05 mM [Me-^{3}H] choline in Krebs-Henseleit buffer for 45 min in the presence of 0-0.5 mM ethanolamine. Incorporation of [Me-^{3}H] choline into the heart was linear to 60 min of perfusion. Subsequent to perfusion, the hearts were homogenized in 20 ml $CHCl_3/CH_3OH$ (2/1;v/v). An aliquot of the homogenate was used for total radioactivity determination. An additional aliquot of the homogenate was analyzed by thin layer chromatography for radioactivity in phosphatidylcholine.

Ethanolamine concentration in the perfusate	Uptake of radioactivity	Radioactivity in phosphatidylcholine
	dpm·g heart^{-1} x 10^{-7}	dpm·g heart^{-1} x 10^{-6}
Control (no ethanolamine)	3.10 ± 0.19 (6)	3.33 ± 0.21 (6)
0.05 mM ethanolamine	2.90 ± 0.33 (5)	3.22 ± 0.42 (5)
0.1 mM ethanolamine	2.39 ± 0.15 (5)*	2.47 ± 0.29 (5)*
0.2 mM ethanolamine	1.85 ± 0.03 (5)*	2.09 ± 0.15 (5)*
0.5 mM ethanolamine	1.10 ± 0.14 (3)*	1.24 ± 0.10 (3)*

*$p < 0.01$ as compared to control

labelled choline uptake appears to be closely related to the percentage decrease in phosphatidylcholine labelling under identical experimental conditions (Table II). Hence, it is possible that the decrease in phosphatidylcholine labelling results from the inhibition of choline uptake by ehtanolamine in a concentration dependent manner.

High concentrations of ethanolamine in the perfusate may also cause an increase in the intracellular ethanolamine pool. Such an increase may subsequently affect the conversion of choline to phosphocholine since ethanolamine has been shown to be an inhibitor of choline kinase (17). However, this does not seem to be the case, as intracellular ethanolamine concentrations were unchanged (5-6 μmol/g heart) when hearts were perfused with ethanolamine or when hearts were removed from hamsters without perfusion (96). In order to evaluate other possible effects of ethanolamine on phosphatidylcholine labelling, the radioactivity associated with the metabolites of the CDP-choline pathway was analyzed. When hamster hearts were perfused with [Me-^{3}H] choline in the presence of 0.05 mM ethanolamine, no significant change in labelling of the choline-containing metabolites was detected (Table III). However, at 0.1-0.5 mM ethanolamine a concomitant reduction in choline, phosphocholine and CDP-choline labelling was observed. The percentage reductions correspond very well with the decrease in total incorporation of label from choline by the heart and phosphatidylcholine labelling (Table II). The results indicate that 0.1-0.5 mM ethanolamine in the perfusate has no effect on the conversion of labelled choline to CDP-choline and subsequently phosphatidylcholine. The 26-63% reduction in phosphatidylcholine labelling appears to be caused solely by an inhibition of choline uptake (23-65%) in the presnece of 0.1-0.5 mM ethanolamine.

TABLE III

Effect of ethanolamine on incorporation of radioactivity into choline-containing metabolites

Hamster hearts were perfused as described in Table II. Subsequent to perfusion, hearts were homogenized in 20 ml $CHCl_3/CH_3OH$ (2/1;v/v), to which 15 ml of 0.1 M KCl was added. The aqueous phase was analyzed by thin layer chromatography for labelled metabolites of choline.

Ethanolamine concentration in the perfusate	Choline	Phosphocholine	CDP-choline
		dpm·g heart^{-1}x10^{-6}	
Control (no ethanolamine)	0.28 ± 0.02 (5)	16.96 ± 1.50 (5)	0.32 ± 0.05 (5)
0.05 mM ethanolamine	0.32 ± 0.06 (5)	16.68 ± 1.65 (5)	0.29 ± 0.04 (5)
0.1 mM ethanolamine	0.18 ± 0.02 (5)*	13.00 ± 0.71 (5)*	0.23 ± 0.02 (5)*
0.2 mM ethanolamine	0.13 ± 0.03 (5)*	9.53 ± 1.52 (5)*	0.19 ± 0.01 (5)*
0.5 mM ethanolamine	0.07 ± 0.01 (3)*	4.61 ± 0.15 (3)*	0.12 ± 0.01 (3)*

*$p<0.05$ as compared to control

II The Effect of Hemicholinium -3 on the Labelling of Phosphatidylcholine

Hemicholinium -3 is a known competitive inhibitor of choline uptake (101). It also appears that hemicholinium -3 is not actively transported across the membrane (102). In order to obtain additional support for the inhibitory role of ethanolamine, the effect of hemicholinium -3 on choline uptake and metabolism was studied. Isolated hamster hearts were perfused with [Me-^{3}H] choline in the presence of 0.01-0.05 mM hemicholinium -3 for 45 min as described previously. As depicted in Table IV, choline uptake was inhibited by hemicholinium -3 (0.01-0.05 mM) in a concentration dependent fashion. Corresponding decreases in labelling of phosphatidylcholine were also observed. It appears that hemicholinium -3 is a more potent inhibitor (10-fold) of choline uptake than ethanolamine. At higher concentrations of hemicholinium -3 (>0.05 mM) in the perfusate, irregular heart rates and abnormal electrocardiac recordings were observed, which might interfere with the biosynthesis of phosphatidylcholine under undefined experimental conditions. Hence, only 0.01-0.05 mM hemicholinium -3 was used in this study.

Analysis of the CDP-choline pathway intermediates after perfusion with hemicholinium -3 revealed a general decrease in labelling of choline, phosphocholine and CDP-choline (Table V). This reduction in labelling was similar and closely resembled the reduction caused by ethanolamine. Hence, both ethanolamine and hemicholinium -3 appear to inhibit choline uptake in the isolated hamster heart. Additional effects by these compounds at an enzymatic level were not detected since the percentage reductions in phosphatidylcholine labelling and the metabolites of the CDP-choline pathway were very similar to the observed reductions in labelled choline uptake.

TABLE IV

Effect of hemicholinium-3 on choline uptake and phosphatidylcholine labelling

Hamster hearts were perfused with 0.05 mM [Me-^{3}H] choline in Krebs-Henseleit buffer containing 0-0.05 mM hemicholinium-3 for 45 min. Subsequent to perfusion, the hearts were homogenized in 20 ml $CHCl_3$/CH_3OH (2/1;v/v). An aliquot of the homogenate was used for total radioactivity determination. An additional aliquot of the chloroform-methanol homogenate was analyzed by thin layer chromatography for radioactivity in phosphatidylcholine.

Hemicholinium-3 concentration in the perfusate	Uptake of radioactivity	Radioactivity in phosphatidylcholine
	dpm·g heart^{-1}x10^{-7}	dpm·g heart^{-1}x10^{-6}
Control (no hemicholinium-3)	3.10 ± 0.19 (6)	3.33 ± 0.21 (6)
0.01 mM hemicholinium-3	1.35 ± 0.17 (3)*	1.33 ± 0.14 (3)*
0.025 mM hemicholinium-3	1.07 ± 0.06 (3)*	1.02 ± 0.04 (3)*
0.05 mM hemicholinium-3	0.73 ± 0.10 (3)*	0.62 ± 0.07 (3)*

*$p<0.01$ as compared to control

TABLE V

Effect of hemicholinium-3 on incorporation of radioactivity into choline-containing metabolites

Hamster hearts were perfused as described in Table IV. Subsequent to perfusion, hearts were homogenized in 20 ml $CHCl_3/CH_3OH$ (2/1;v/v), to which 15 ml of 0.1 M KCl was added. The aqueous phase was analyzed by thin layer chromatography for labelled metabolites of choline.

Hemicholinium-3 concentration in the perfusate	Choline	Phosphocholine	CDP-choline
	dpm·g heart^{-1}x10^{-6}		
Control (no hemicholinium-3)	0.281±0.033(5)	16.96±1.50(5)	0.320±0.047(5)
0.01 mM hemicholinium-3	0.126±0.025(3)*	6.77±0.91(3)*	0.119±0.022(3)*
0.025 mM hemicholinium-3	0.092±0.006(3)*	5.22±0.21(3)*	0.088±0.004(3)*
0.05 mM hemicholinium-3	0.061±0.006(3)*	3.76±0.04(3)*	0.054±0.003(3)*

*$p<0.05$ as compared to control

III Competitive Inhibition of Choline Uptake

It is clear that ethanolamine and hemicholinium -3 inhibit choline uptake in the isolated hamster heart in a similar fashion. The nature of the inhibition exhibited by these compounds was studied in the perfused heart with varying concentrations of labelled choline in the presence of ethanolamine (0.2 mM) or hemicholinium -3 (0.025 mM). Subsequent to perfusion, total uptake of choline by the heart was determined. When the results were analyzed by double reciprocal plot of the rate of choline uptake versus choline concentration, a simple competitive type of inhibition by both ethanolamine and hemicholinium -3 was obtained (Fig.2). The competitive nature of these inhibitors was confirmed by a Hoffstee's plot as depicted in Fig. 2 (inset).

IV Intracellular Choline, Phosphocholine and CDP-Choline Concentrations

In all of the preceding studies, a high ethanolamine to choline ratio (2/1 or higher) caused a reduction in phosphatidylcholine labelling. If exogenous ethanolamine interfered with the metabolism of the CDP-choline pathway, the pool size of one or more of the choline-containing metabolites during perfusion may change. Hence, the intracellular concentrations of choline, phosphocholine and CDP-choline were determined in hearts perfused without choline, with 0.05 mM choline or with 0.05 mM choline in the presence of 0.5 mM ethanolamine. As depicted in Table VI, no significant change in the pool size of these metabolites was observed. The amount of newly synthesized phosphocholine, CDP-choline or phosphatidylcholine was calculated by the specific radioactivity of its immediate precursor in the presence or absence of ethanolamine. Based on this analysis, exogenous ethanolamine has no effect on the conversion of choline to CDP-choline and subsequently phosphatidylcholine.

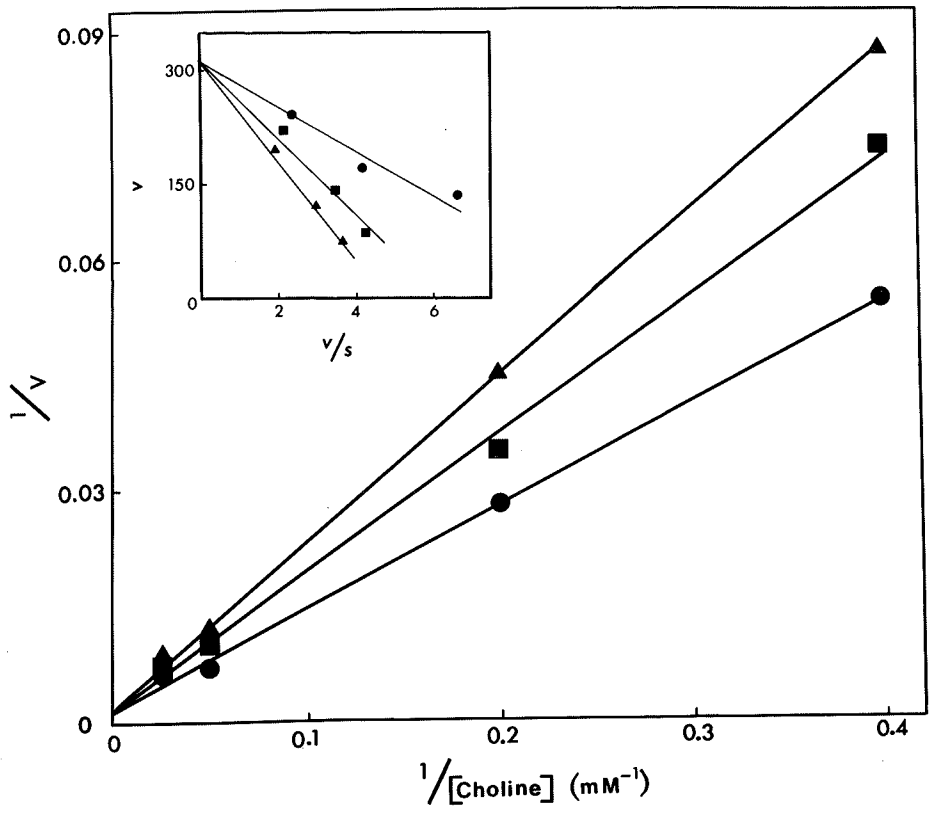


Figure 2 Double reciprocal plot and Hoffstee's plot of choline uptake versus choline concentration in the presence of ethanolamine or hemicholinium-3.

The inhibitory effects of ethanolamine and hemicholinium-3 were studied in the perfused heart with varying concentrations of labelled choline. Subsequent to perfusion, total uptake of choline by the heart in the presence of these inhibitors was determined. (●) No addition; (■) 0.2 mM ethanolamine and (▲) 0.025 mM hemicholinium-3. Each point represents the mean of two separate experiments. The competitive nature of the inhibition of choline uptake by ethanolamine and hemicholinium-3 was confirmed by a Hoffstee's plot (inset). Total choline uptake (v) is expressed as nmol·g heart^{-1}.

TABLE VI

Concentration of choline, phosphocholine and CDP-choline in hamster heart

The pool sizes of the choline-containing metabolites were determined in hearts perfused without choline, with 0.05 mM choline or with 0.05 mM choline in the presence of 0.5 mM ethanolamine as described under "General Analytical Procedures". Each value is expressed as the mean ± standard deviation (number of experiments).

	Perfusion without choline	Perfusion with	
		0.05 mM choline	0.05 mM choline and 0.5 mM ethanolamine
		nmol·g heart^{-1}	
Choline	283 ± 44 (3)	285 ± 59 (3)	242 ± 46 (3)
Phosphocholine	236 ± 22 (3)	176 ± 32 (3)	190 ± 36 (4)
CDP-choline	91 ± 2 (3)	92 ± 9 (3)	103 ± 9 (4)

THE EFFECT OF CHOLINE ON PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS

I Incorporation of Radioactivity into Phosphatidylethanolamine

Results from the previous section demonstrated that the concentration of exogenous ethanolamine had no effect on phosphatidylcholine biosynthesis, beyond the level of choline uptake. However, the corollary effect of choline on phosphatidylethanolamine biosynthesis was not known. Therefore, to elucidate the possible role of choline in this regulation, isolated hamster hearts were perfused with Krebs-Henseleit buffer containing [2-^{14}C] ethanolamine and 0-0.5 mM choline for 25 min. Subsequent to perfusion, the hearts were homogenized in $CHCl_3/CH_3OH$ (2/1;v/v). An aliquot of the homogenate was analyzed by thin layer chromatography for radioactivity associated with various phospholipids. In all experiments, more than 90% of the radioactivity in the lipid extract was recovered in phosphatidylethanolamine and less than 6% of the radioactivity was found in phosphatidylcholine and lysophosphatidylethanolamine. When 0-0.1 mM choline was present in the perfusate, no significant reduction of phosphatidylethanolamine labelling was observed (Table VII). However, a 28% reduction in phosphatidylethanolamine labelling was observed when 0.2-0.5 mM choline was added to the perfusate.

II The Effect of Choline on Ethanolamine Uptake

One possible explanation for the reduction of phosphatidylethanolamine labelling was that high choline concentrations (0.2-0.5 mM) inhibited ethanolamine uptake by the heart. Therefore, total uptake of radioactivity by the heart in the presence of 0-0.5 mM choline was

TABLE VII

Radioactivity incorporated into phosphatidylethanolamine

Hamster hearts were perfused with 0.05 mM [2-^{14}C] ethanolamine in Krebs-Henseleit buffer for 25 min in the presence of 0-0.5 mM choline. Subsequent to perfusion, the hearts were homogenized in 20 ml $CHCl_3$/CH_3OH (2/1;v/v). An aliquot of the homogenate was analyzed by thin layer chromatography for radioactivity in phosphatidylethanolamine.

Choline concentration in the perfusate	Radioactivity in Phosphatidylethanolamine		
	dpm·g heart^{-1} x 10^{-6}		
Control (no choline)	0.46 ± 0.04	(8)	
0.05 mM Choline	0.40 ± 0.09	(8)	
0.1 mM Choline	0.40 ± 0.09	(4)	0.5>p>0.1
0.2 mM Choline	0.33 ± 0.07	(4)	p<0.01
0.5 mM Choline	0.31 ± 0.02	(4)	p<0.01

determined. As depicted in Table VIII, the total amount of radioactivity taken up by the heart was not affected by the presence of choline. Hence, it appears that choline does not affect the transport of ethanolamine across the sarcolemma.

III Analysis of Ethanolamine-containing Metabolites

In order to elucidate the cause(s) of reduced phosphatidylethanolamine labelling by 0.2-0.5 mM choline, the amount of radioactivity associated with ethanolamine, phosphoethanolamine and CDP-ethanolamine was determined. No significant difference in ethanolamine labelling was observed at all choline concentrations tested (Table IX). The radioactivity associated with phosphoethanolamine and CDP-ethanolamine was unchanged at 0-0.1 mM choline, but a 28-30% reduction in the labelling of these metabolites was observed at 0.2-0.5 mM choline (Table IX). Hence, the change in phosphatidylethanolamine labelling in the presence of choline (0.2-0.5 mM) may be caused by a reduction in labelled phosphoethanolamine formation, which subsequently results in reduced CDP-ethanolamine labelling.

IV Hamster Heart Ethanolamine Kinase Assay

The reduction in phosphoethanolamine labelling *in vivo* may result from inhibition of ethanolamine kinase by choline. Thus, the inhibitory effect of choline on ethanolamine kinase activity was studied. We have shown previously that this enzyme is located exclusively in the cytosolic fraction of the hamster heart (96). The assay for ethanolamine kinase activity was optimized with respect to pH (8.5) and K_m for ethanolamine (0.91 mM). These values are comparable to those reported

TABLE VIII

Total uptake of [2-^{14}C] ethanolamine by the isolated hamster heart

Hamster hearts were perfused as described in Table VII. An aliquot of the chloroform-methanol homogenate was used for total radioactivity determination.

Choline concentration in the perfusate	Uptake of radioactivity		
	dpm·g heart^{-1} x 10^{-6}		
Control (no choline)	1.87 ± 0.34	(8)	
0.05 mM Choline	2.29 ± 0.58	(8)	
0.1 mM Choline	2.06 ± 0.57	(4)	
0.2 mM Choline	1.55 ± 0.34	(4)	0.5>p>0.1
0.5 mM Choline	1.55 ± 0.37	(4)	0.5>p>0.1

TABLE IX

Radioactivity incorporated into ethanolamine-containing metabolites

Hamster hearts were perfused as described in Table VII. Subsequent to perfusion, hearts were homogenized in 20 ml $CHCl_3/CH_3OH$ (2/1;v/v), to which 15 ml 0.1 M KCl was added. The aqueous phase was analyzed by thin layer chromatography for labelled metabolites of ethanolamine.

Choline concentration in the perfusate	Ethanolamine	Phosphoethanolamine	CDP-ethanolamine
		dpm·g heart^{-1}x10^{-6}	
Control (no choline)	0.12±0.02(8)	1.45±0.24(8)	0.47±0.06 (8)
0.05 mM Choline	0.10±0.02(4)	1.33±0.13(4)	0.44±0.01 (4)
0.1 mM Choline	0.09±0.02(4)	1.37±0.22(4)	0.46±0.07 (4)
0.2 mM Choline	0.10±0.03(4)	1.04±0.27(4)[a]	0.33±0.06 (4)[a]
0.5 mM Choline	0.09±0.01(4)	1.06±0.10(4)[a]	0.28±0.03 (4)[a]

[a] $p<0.05$

for rat liver ethanolamine kinase (16). In order to study the effect of choline on ethanolamine kinase in hamster heart, enzyme activities were assayed under optimal conditions in the presence of 0-0.2 mM choline. As depicted in Fig. 3, choline inhibited enzyme activity in a linear fashion. At 0.2 mM choline, approximately 85% of the ethanolamine kinase activity was inhibited. The nature of this inhibition was analyzed by a double reciprocal plot of enzyme activity versus ethanolamine concentration in the presence of 0-0.1 mM choline. The results show (Fig.4) that choline inhibits ethanolamine kinase in a competitive manner. The kinetic data obtained in this study are different from those reported in rat liver where a "mixed" type of inhibition by choline was observed (17).

V Intracellular Choline Concentration

The data obtained in the preceding sections suggest that the reduction in phosphatidylethanolamine labelling may be caused by the inhibitory effect of choline on ethanolamine kinase. However, this hypothesis can only be substantiated if there were a dramatic elevation of intracellular choline concentration after the heart was perfused with 0.2-0.5 mM choline. Hence, intracellular choline concentrations were determined after perfusion with 0-0.5 mM choline (Table X). Intracellular choline concentration was unchanged when hearts were perfused with 0-0.05 mM choline. However, in the presence of 0.2-0.5 mM choline, a 2-fold increase in intracellular choline concentration was observed. This elevation correlates very well with the observed reduction in phosphatidylethanolamine labelling. The results also provide a plausible explanation for the 28% reduction of radioactivity incorporated into phosphoethanolamine under high choline concentrations.

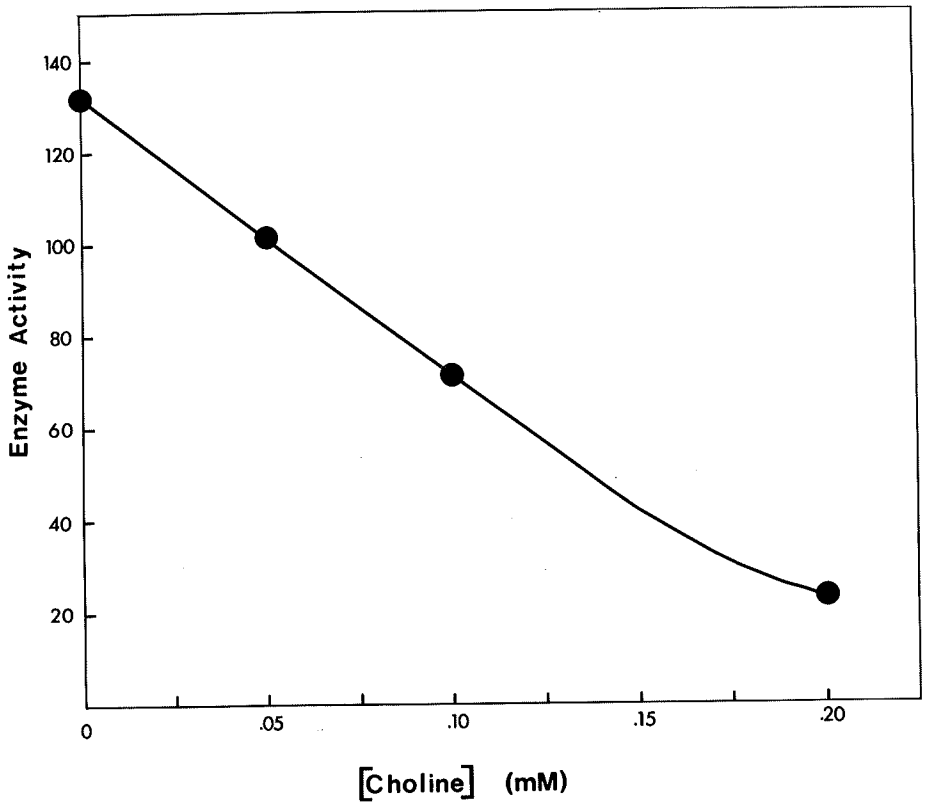


Figure 3 The effect of choline concentration on ethanolamine kinase activity.

Ethanolamine kinase was assayed as described by Schneider and Vance (97), in the presence of 0-0.2 mM choline. Enzyme activity is expressed as $pmol \cdot min^{-1} \cdot mg^{-1}$. All assays were performed in duplicate.

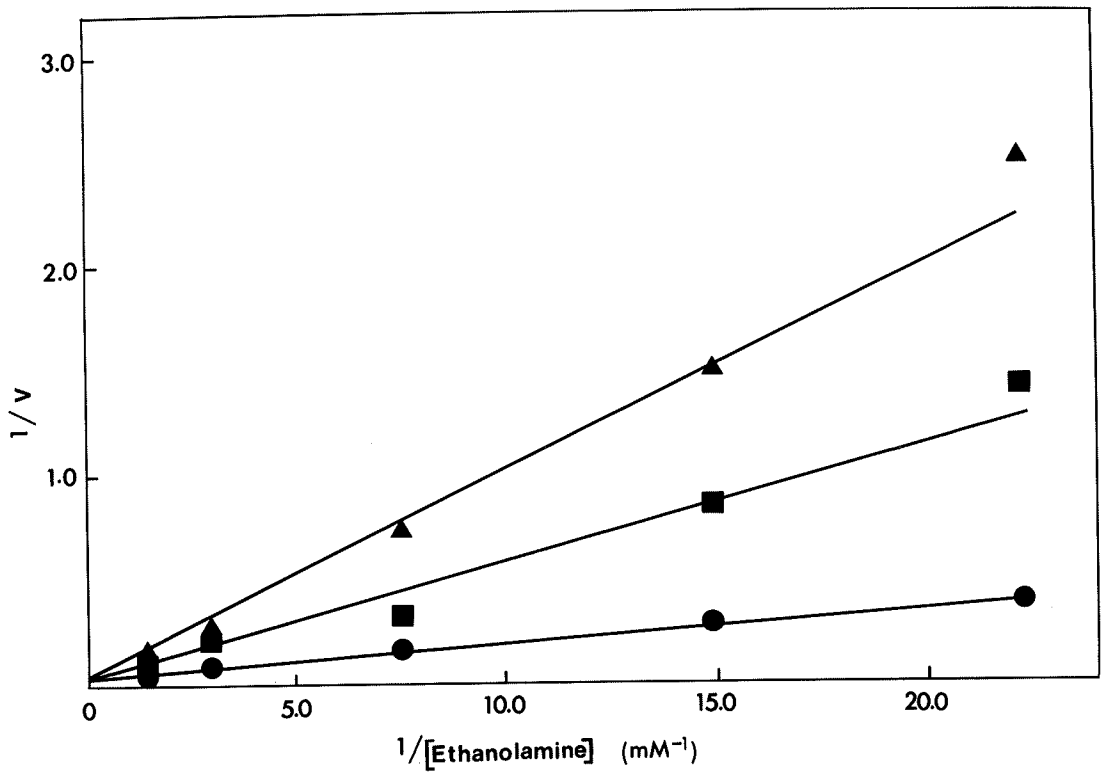


Figure 4 Double reciprocal plot of ethanolamine kinase activity versus ethanolamine concentration in the presence of choline.

Ethanolamine kinase activity was assayed as described in Fig. 3. All assays were performed in triplicate in the absence (●) and presence of 0.025 mM (■) and 0.10 mM (▲) choline. The apparent K_m of the enzyme in the absence of choline was 0.91 mM.

TABLE X

Intracellular choline concentration in hamster heart

The pool size of choline in hamster hearts perfused with [2-^{14}C] ethanolamine in the presence of 0-0.5 mM choline was determined as described under "General Analytical Procedures". Each value is the mean of three separate experiments.

Perfusion without choline	Perfusion with choline			
	0.1 x 10^{-3} mM	0.05 mM	0.2 mM	0.5 mM
		nmol·g heart^{-1}		
283 ± 44	245 ± 22	285 ± 59	621 ± 80	608 ± 80

CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE FROM HAMSTER HEART

I General

It was previously demonstrated that cytidylyltransferase is located in both the cytosolic and microsomal fractions of heart homogenates (44). Additionally, this enzyme catalyzes the rate-limiting step in phosphatidylcholine biosynthesis in heart (44) and several other tissues (10). Although many studies on cytidylyltransferase have been undertaken in other tissues (103), the characterization and regulation of this enzyme in the heart was not known. Hence, studies of hamster heart cytidylyltransferase are described in the next several sections of this thesis.

II Cytosolic and Microsomal Cytidylyltransferase Activity with Time and Protein

The cytosolic cytidylyltransferase reaction was linear with time for up to 60 min (Fig.5A), and with up to 0.3 mg of protein in the assay (Fig.5B). However, non-linearity with respect to protein was observed at concentrations greater than 0.3 mg (Fig.5B). The microsomal cytidylyl-transferase was non-linear after 25 min and 15 µg of protein (Fig.6A and B). The CDP-choline produced in the reaction was presumably converted to phosphatidylcholine by cholinephosphotransferase which is also present in the microsomes or was degraded by a microsomal CDP-choline hydrolase. The non-linearity was not due to product inhibition since similar results were obtained when enzyme activity was measured in the presence of excess CDP-choline (10 nmol).

In view of these findings, all subsequent cytosolic enzyme activities

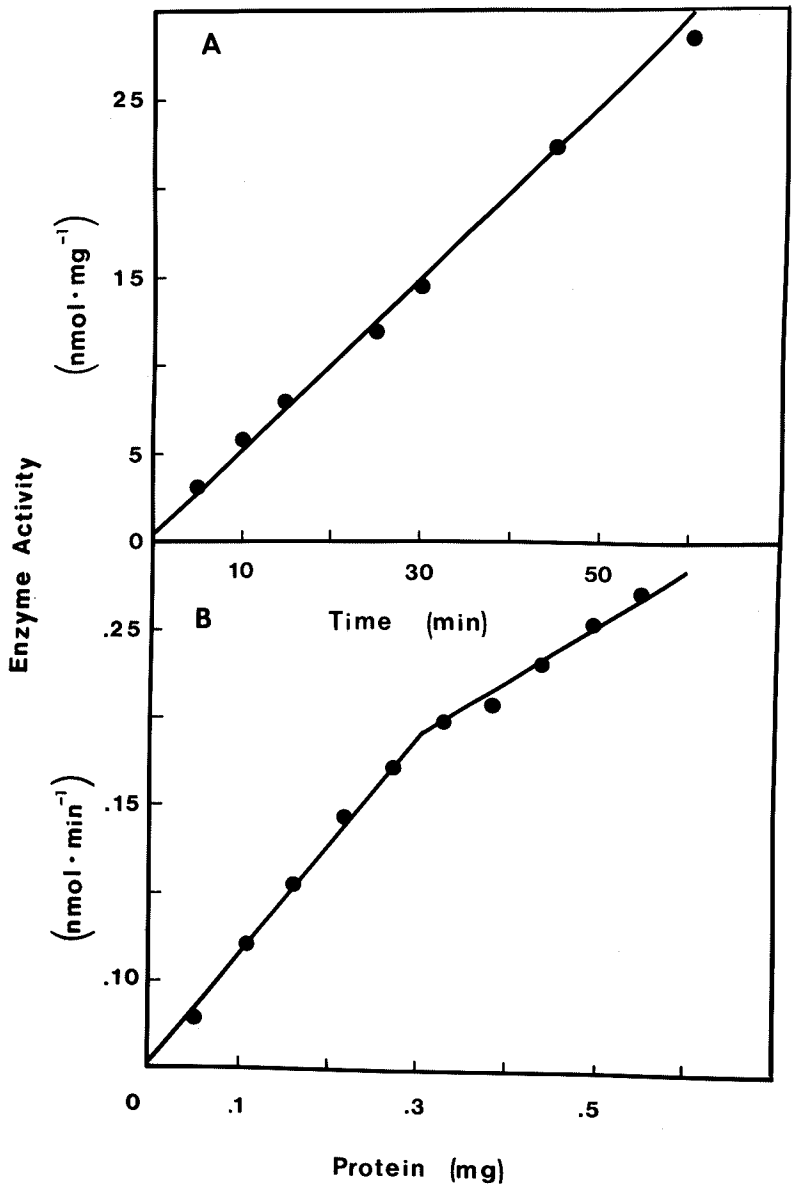


Figure 5 Cytosolic cytidylyltransferase activity as a function of time and protein concentration.

Cytidylyltransferase activity was measured in fresh hamster heart cytosol for up to 60 min with 0.22 mg of cytosolic protein (Panel A) or for 30 min with up to 0.55 mg of cytosolic protein (Panel B). All enzyme assays were performed in the absence of phospholipid. Each point is the mean of two separate experiments.

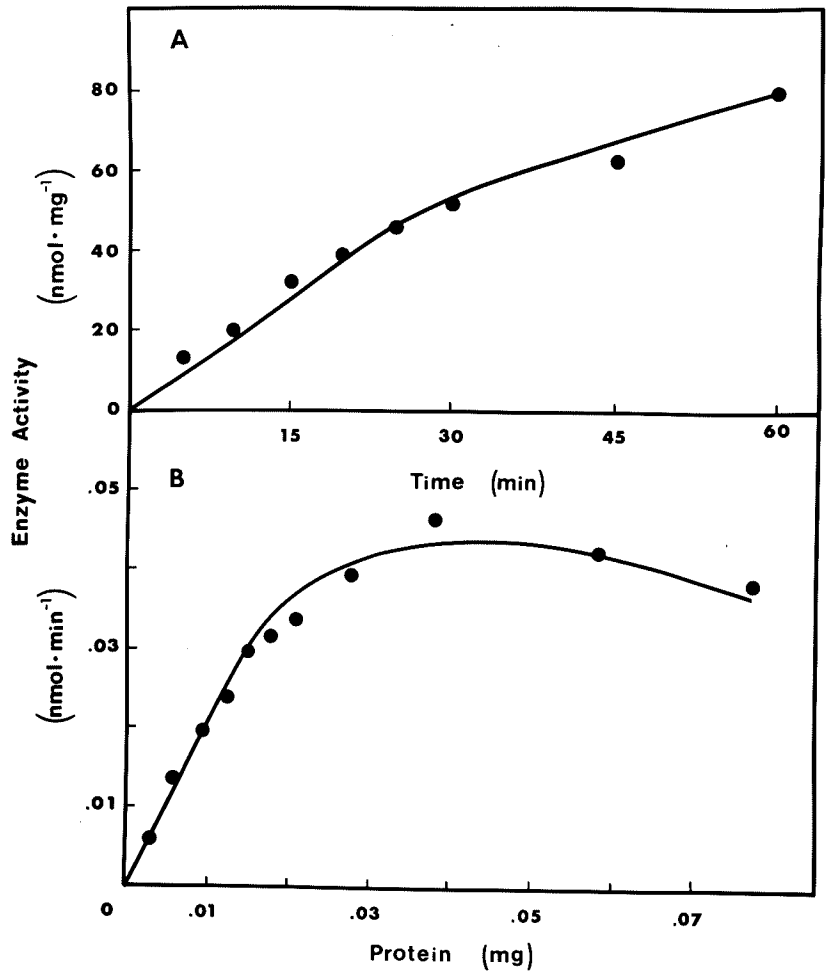


Figure 6 Microsomal cytidylyltransferase activity as a function of time and protein concentration.

Cytidylyltransferase activity was measured in hamster heart microsomes. The enzyme was assayed for up to 60 min with 12 µg of microsomal protein (Panel A). The enzyme was assayed with up to 75 µg of microsomal protein for 15 min (Panel B). Each point represents the mean of two separate experiments.

were determined with 0.22 mg protein for 30 min. The microsomal cytidylyltransferase activity was measured in the presence of 12 µg protein for 15 min.

III pH Profile of Cytosolic and Microsomal Cytidylyltransferase

Fresh heart cytosol was assayed over the pH range of 4-11. As depicted in Fig. 7A, cytosolic cytidylyltransferase displayed a pH optimum of 8.0. However, enzyme activity extended over a broad range of pH. The pH profile of the microsomal enzyme (Fig.7B) was very similar to cytosolic cytidylyltransferase.

IV Magnesium Requirement of the Cytidylyltransferases

Enzymes which utilize high energy nucleotides (such as ATP and CTP) normally require magnesium for maximal activity. The magnesium requirement of both the cytosolic and microsomal forms of cytidylyltransferase was investigated. Cytidylyltransferase from fresh cytosol displayed maximal activity at a magnesium concentration of 10 mM (Fig.8A). Similar results were obtained for the microsomal form of the enzyme (Fig.8B). Optimal Mg^{+2} concentration was dependent on CTP concentration but was not affected by the amount of enzyme protein in the assay. In the presence of 1 mM EDTA, cytosolic activity was reduced approximately 4-fold and microsomal activity 3-fold.

V Hamster Heart Cytosolic Cytidylyltransferase Activation and Aggregation are Time Dependent

Cytidylyltransferase from liver cytosol, upon storage, is known to aggregate with a corresponding increase in enzyme activity (21).

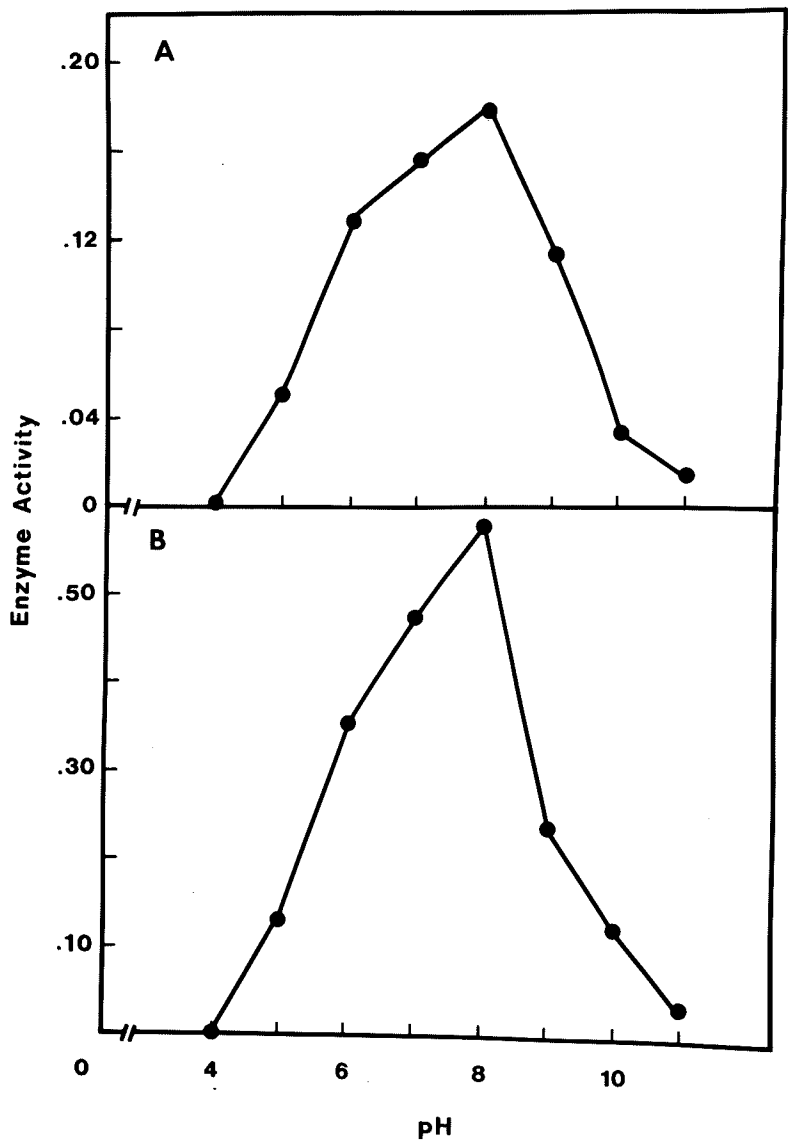


Figure 7 pH profile of cytosolic and microsomal cytidylyltransferases.

Cytidylyltransferase activity was measured in fresh heart cytosol (Panel A) or in heart microsomes (Panel B). Both forms of the enzyme displayed a broad pH profile, with maximal activity at pH 8.0. Enzyme activity is expressed as $nmol \cdot min^{-1} \cdot mg^{-1}$. Each point is the mean of two separate experiments.

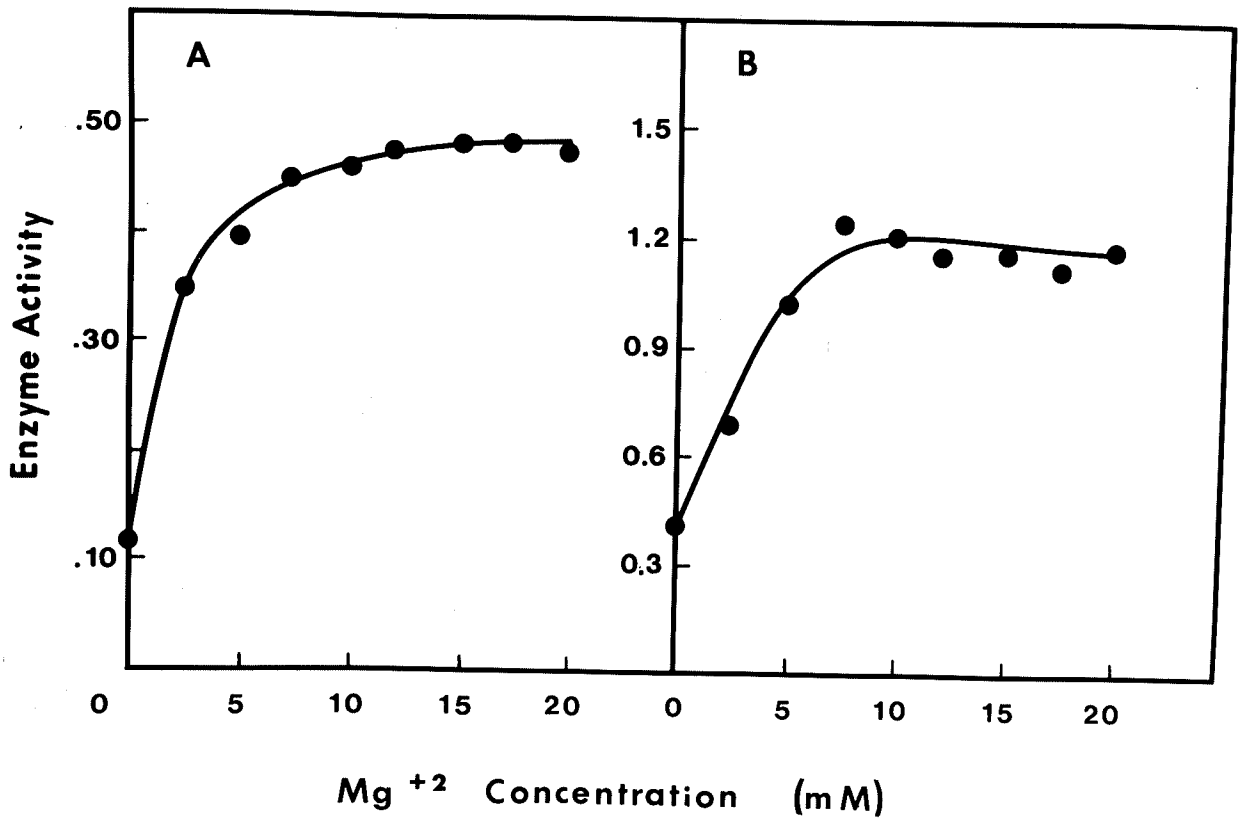


Figure 8 The effect of magnesium on cytidylyltransferase activity.

Cytidylyltransferase activity was determined in the presence of 0-20 mM magnesium acetate. Cytosolic cytidylyltransferase displayed maximal activity at 10 mM magnesium (Panel A). The microsomal form of the enzyme had maximal activity at 7.5 mM magnesium (Panel B). Enzyme activity is expressed as $nmol \cdot min^{-1} \cdot mg^{-1}$. Each point represents the mean of two separate experiments.

In an attempt to determine whether heart cytidylyltransferase also behaved in this manner, hamster heart cytosol was stored at 4°C for 1-7 days. As depicted in Fig. 9A, cytidylyltransferase from heart cytosol displayed a 6.5-fold increase in activity after 2 days of storage. This elevated enzyme activity was maintained over the subsequent 4 days of storage. As a comparison, the behaviour of hamster liver cytidylyltransferase was determined under the same conditions. The liver enzyme also displayed increased enzyme activity versus storage (Fig.9B). Although activation of the hamster liver enzyme was only 2-fold, a 7-8-fold increase of enzyme activity was observed with rat liver cytosol (21). The results suggest that the heart enzyme in the cytosol behaves like the liver enzyme (22,23), which is subject to modulation by various effectors. These modulators are either endogeneous to the cytosol or are generated in the cytosol upon storage.

In order to compare cytosolic cytidylyltransferase from heart with the liver enzyme (21), the behaviour of fresh and stored cytosol on Sepharose 6B was examined. Freshly prepared hamster heart cytosol or cytosol that had been stored at 4°C for 4 days was applied to a column of Sepharose 6B. The activity from fresh cytosol was resolved into three distinct peaks (Fig.10). These three forms were designated H, L and S. The H-form eluted very near the void volume of the column, the L-form eluted over a wide range of molecular weights (178,000-316,000) with a mean molecular weight of 209,000 and the S-form had a molecular weight of less than 100,000. The H- and L-forms of cytidylyltransferase are present in liver cytosol, but the S-form of the enzyme was absent from this tissue (21). Although this form of the enzyme may result from proteolysis of the H- or L-forms, this does not seem to be

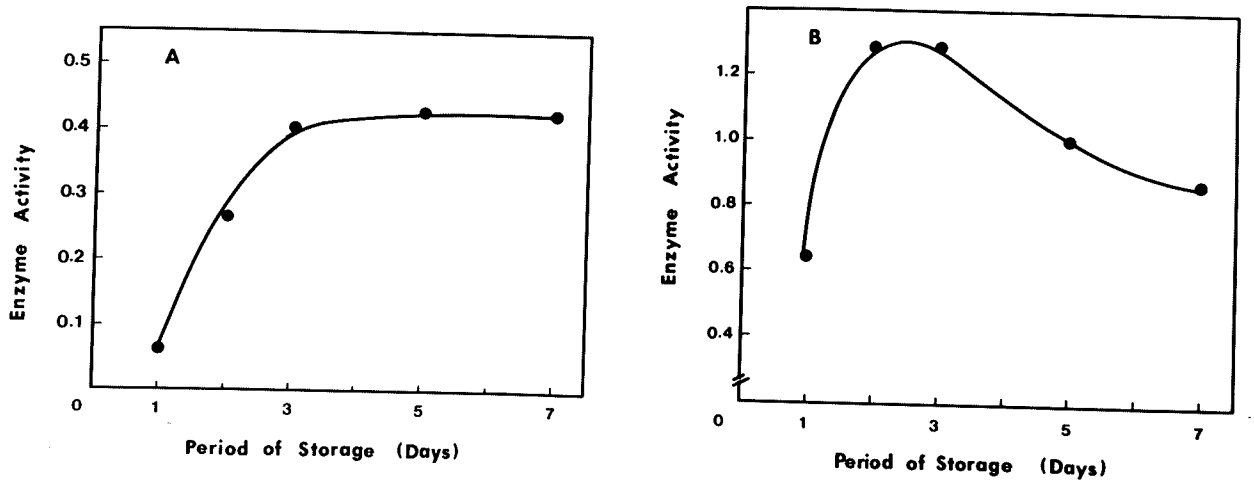


Figure 9 The effect of storage on cytidylyltransferase from hamster heart and liver cytosol.

A 20% homogenate of hamster heart and liver was prepared. The heart (Panel A) and liver (Panel B) cytosols were incubated at $4^{\circ}C$ for various time periods. Enzyme activity is expressed as $nmol \cdot min^{-1} \cdot mg^{-1}$. Each point is the mean of two experiments.

the case since similar elution profiles were obtained when 1 mM phenylmethylsulfonylfluoride was included in the heart homogenate.

Storage of heart cytosol for 4 days resulted in a dramatic shift in enzyme activity to predominantly H-form (Fig.10). This shift corresponded with an approximate 5-fold increase in enzyme activity. Although the elution profile did not display three distinct enzyme forms after storage, there was still appreciable activity in the fractions corresponding to L- and S-forms of cytidylyltransferase. One possible explanation for this heterogeneous distribution is that upon storage an aggregation promoter (perhaps diglyceride) is produced, thereby enhancing the conversion of the low molecular weight forms of cytidylyltransferase to H-form. Heart cytidylyltransferase, at the incubation time tested, may be in various states of aggregation and hence activity is detected in several column fractions.

VI Dissociation of H-form of Cytidylyltransferase

Choy et al., (21) have reported the only successful purification of cytidylyltransferase to date. These investigators demonstrated that dissociation of H-form with SDS resulted in a highly purified cytidylyltransferase preparation (L-form) which migrated as a single protein band on a 5% polyacrylamide gel at pH 7.5. In order to determine the characteristics of H-, L- and S-forms of cytidylyltransferase from heart cytosol, a similar approach to enzyme purification was employed.

The H-form of the enzyme obtained from stored cytosol (Fig.10) was incubated for 5 min at $4^{o}C$ in the presence of 0.025% SDS. This sample was applied to a Sepharose 6B column and enzyme activity was

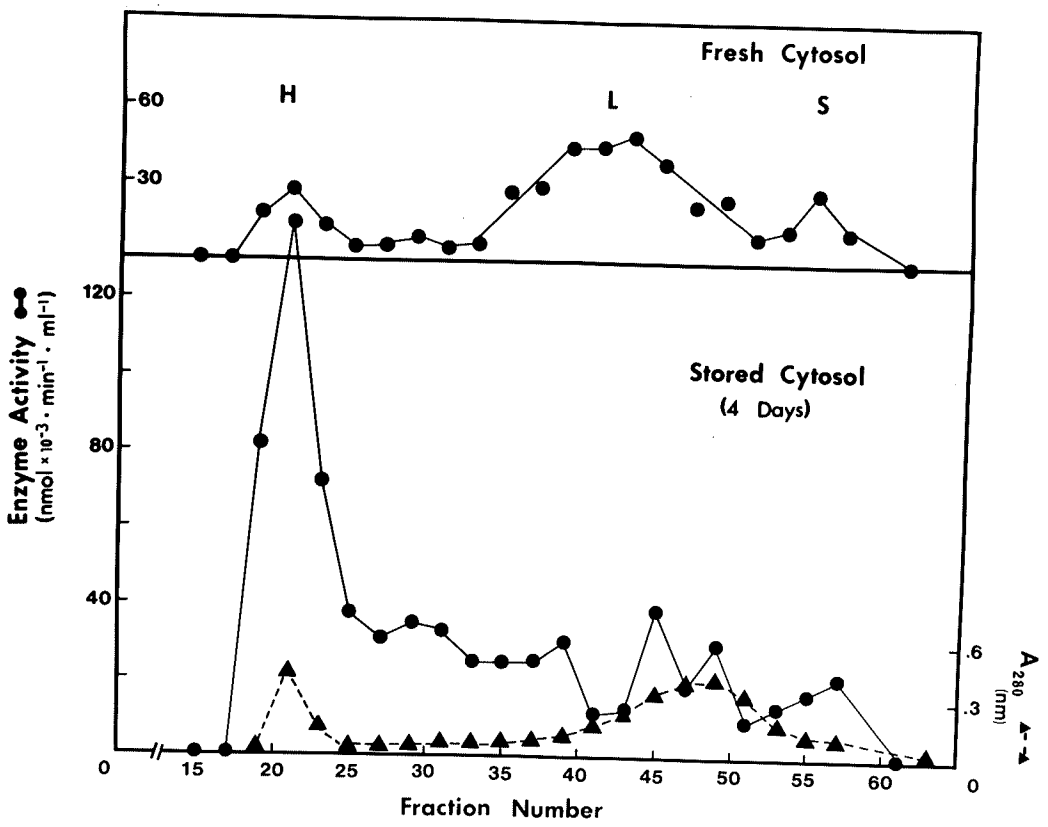


Figure 10 Sepharose 6B chromatography of cytidylyltransferase from hamster heart cytosol.

Two ml of freshly prepared hamster heart cytosol or cytosol that had been stored at 4°C for 4 days was applied to a column of Sepharose 6B (1.5 x 45 cm). Fractions (1.4 ml) were collected and assayed for enzyme activity in the presence of hamster liver phospholipid. Enzyme activity is expressed per ml of column eluant.

measured. Enzyme activity was resolved into three distinct peaks (Fig.11) which corresponded to H-, L- and S-forms in fresh cytosol (Fig.10). The results clearly demonstrate that the use of SDS under the appropriate conditions can dissociate H-form of cytidylyltransferase to L- and S-forms.

Although electrophoretic purity of these preparations was not demonstrated, several hundred fold enrichments of L-form (1800-fold) and S-form (800-fold) were achieved (Table XI). These highly purified forms of cytidylyltransferase from hamster heart were subsequently characterized.

VII Characterization of Highly Purified Cytidylyltransferase from Hamster Heart Cytosol

The L- and S-forms of the enzyme displayed a pH profile similar to fresh cytosol (Fig.7A), with a pH optimum of 8.0. The H-form of the enzyme had a pH profile and optimum similar to the microsomal form of cytidylyltransferase (Fig.7B). The magnesium requirement for the L- and S-forms paralleled that for the cytosolic enzyme and the H-form compared to the microsomal form (Fig.8A and B). These results suggest that cytosolic H-form is similar to the microsomal form of the enzyme and that L- and S-forms represent the cytosolic forms of cytidylyl-transferase.

The most compelling evidence in support of this hypothesis was obtained from kinetic analysis of the three cytosolic forms and the microsomal form of the enzyme. In these experiments, enzyme activity was determined with varying concentrations of phosphocholine in the presence of fixed CTP concentrations or with varying concentrations

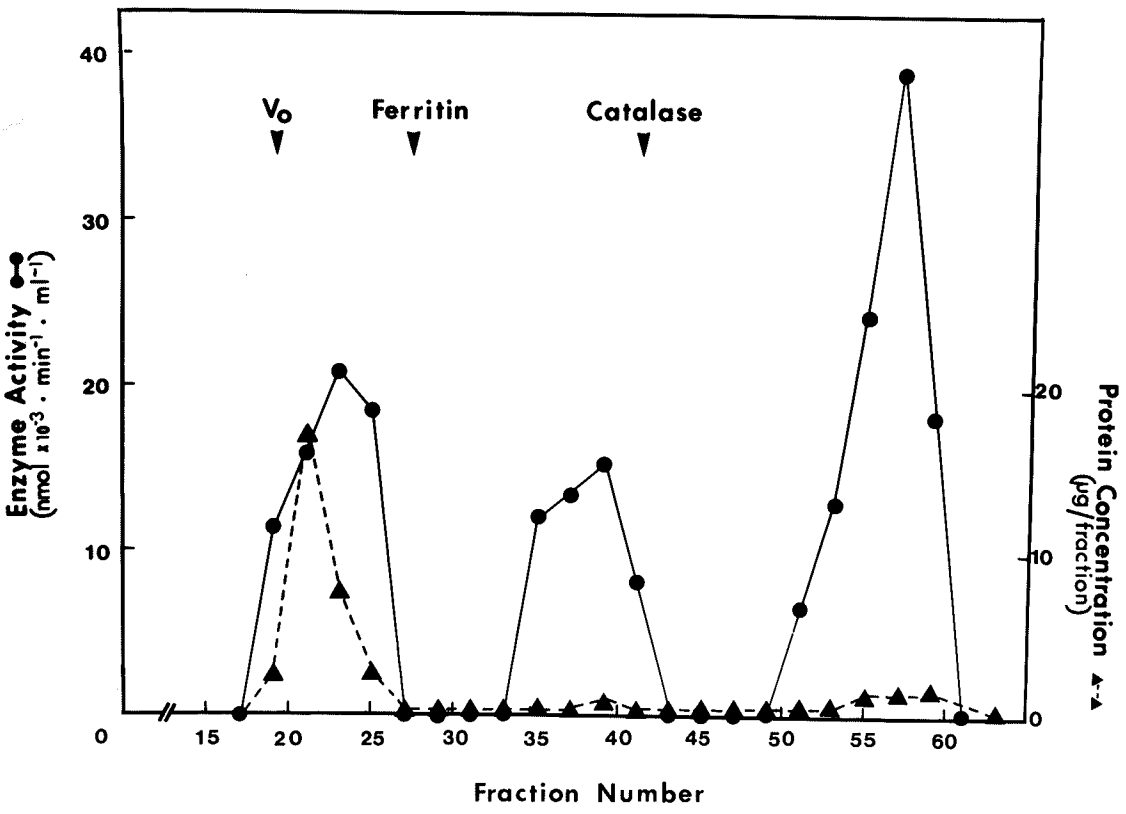


Figure 11 Dissociation of the high molecular weight form (H-form) of cytidylyltransferase.

The H-form of the enzyme (Fig.10) was incubated for 5 min at 4°C in the presence of 0.025% sodium dodecyl sulfate. Four ml of the sample was applied to a Sepharose 6B column (1.5 x 45 cm). Fractions (1.4 ml) were collected and assayed in the presence of hamster liver phospholipid. The H-form of the enzyme eluted very close to V_o, the L-form slightly ahead of catalase and the S-form later. Enzyme activity is expressed per ml of column eluant.

TABLE XI

Purification of CTP:phosphocholine cytidylyltransferase from hamster heart

Fraction	Volume (ml)	Protein (mg)	Total Activity (nmol/ml)	Specific Activity (nmol/min/mg)	Purification (Fold)
Cytosol	4.085	28.6	1.239	0.0433	—
Stored Cytosol	4.085	26.6	8.919	0.3359	7.75
1st Sepharose 6B Column (H Form)	4.0	0.168	0.737	4.386	101.3
2nd Sepharose 6B Column after SDS dissociation (L Form)*	2.8	0.0005	0.0403	80.6	1861
(S Form)*	2.8	0.0026	0.0886	34.1	788

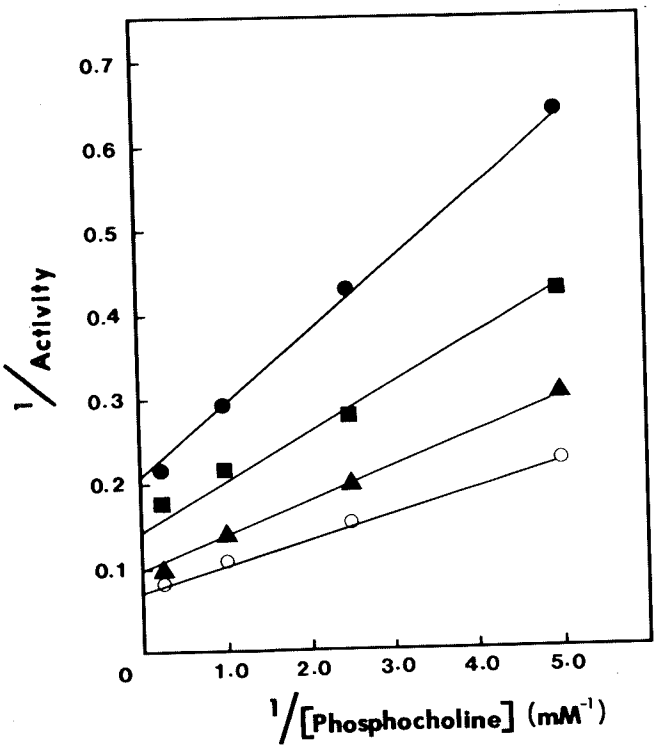
*Assayed in the presence of 1 µg total lipid phosphorus (liver).

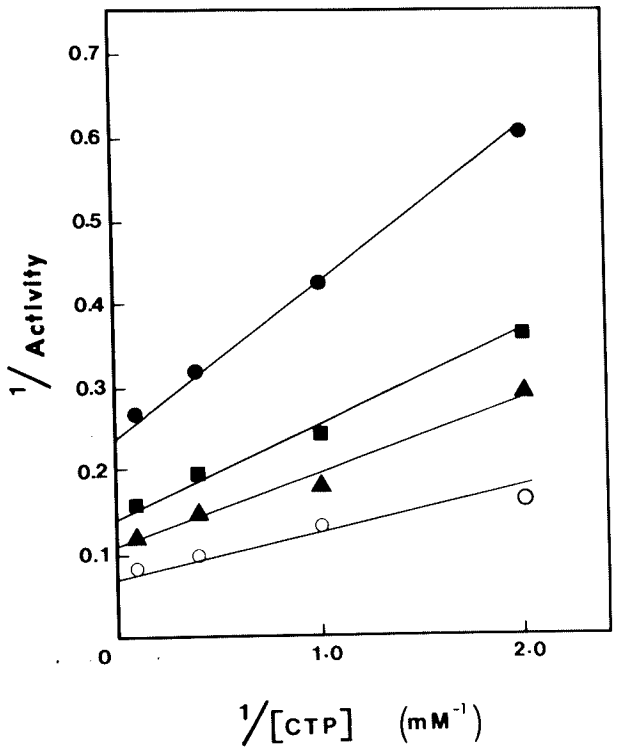
of CTP in the presence of fixed phosphocholine concentrations. A replot of the apparent V_{max} of CTP and phosphocholine versus the reciprocal of substrate concentration would yield the true K_m for CTP and phosphocholine.

The microsomal form of cytidylyltransferase appears to be kinetically similar to cytosolic H-form. The true K_m for phosphocholine is 0.44 mM and for CTP 1.0 mM (Fig.12). The L-form of the enzyme is kinetically identical to the S-form with a K_m for phosphocholine of 80 mM and for CTP of 400 mM (Fig.13).

Figure 12 Double reciprocal plots of initial velocity of high molecular weight form (H-form) of cytidylyltransferase.

The fixed concentrations of CTP in the upper left hand panel were 0.5 (●), 1.0 (■), 2.5 (▲) and 10 (○) mM. The fixed concentrations of phosphocholine in the upper right hand panel were 0.2 (●), 0.4 (■), 1.0 (▲) and 4.0 (○) mM. In the lower panel, the apparent V_{max} of CTP and phosphocholine were replotted to obtain the true K_m. Activity is expressed as $nmol \cdot min^{-1} \cdot mg^{-1}$. Each point is the mean of duplicate determinations.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.0
2.0
3.0
4.0
5.0
1/Activity
1/[Phosphocholine] (mM⁻¹)


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.0
2.0
1/Activity
1/[CTP] (mM⁻¹)

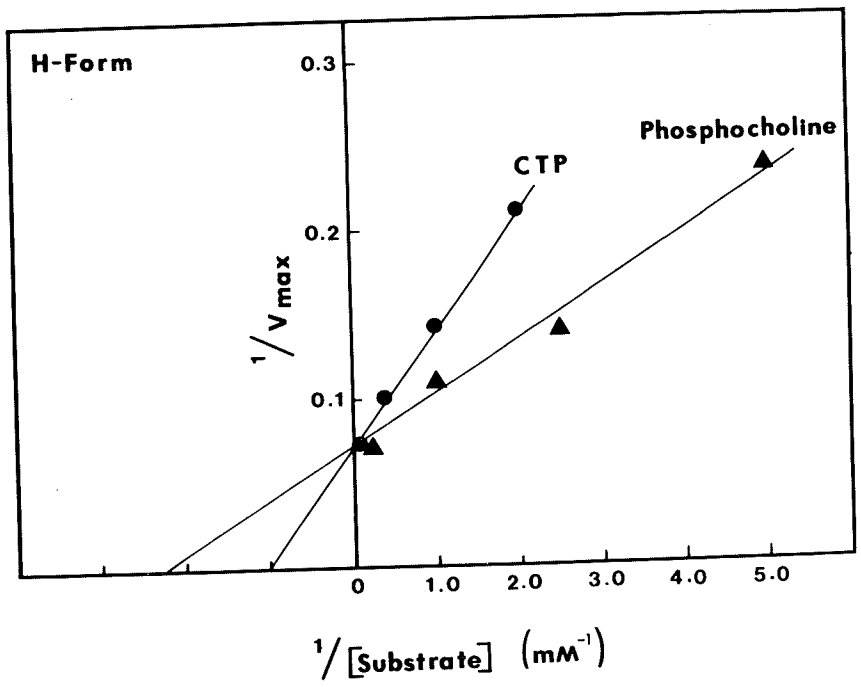
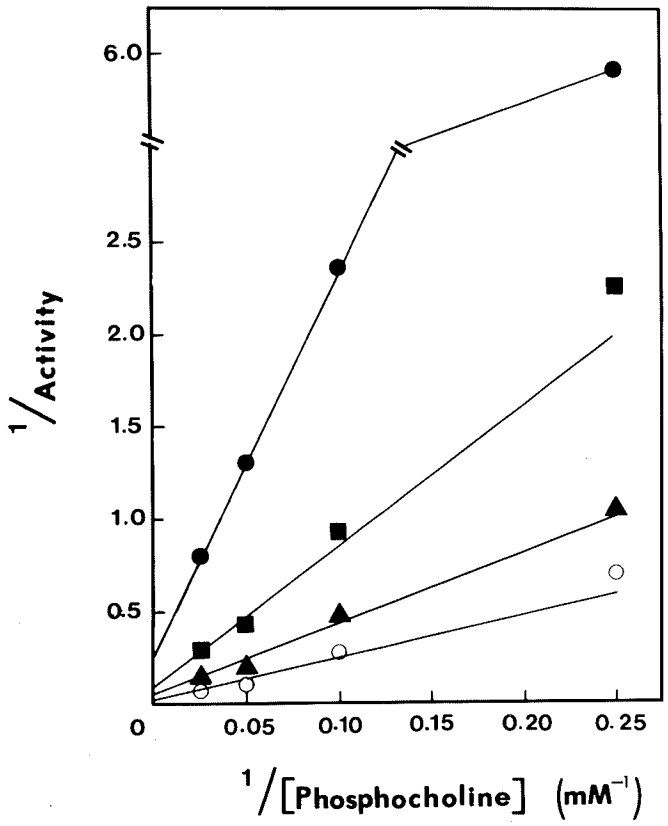
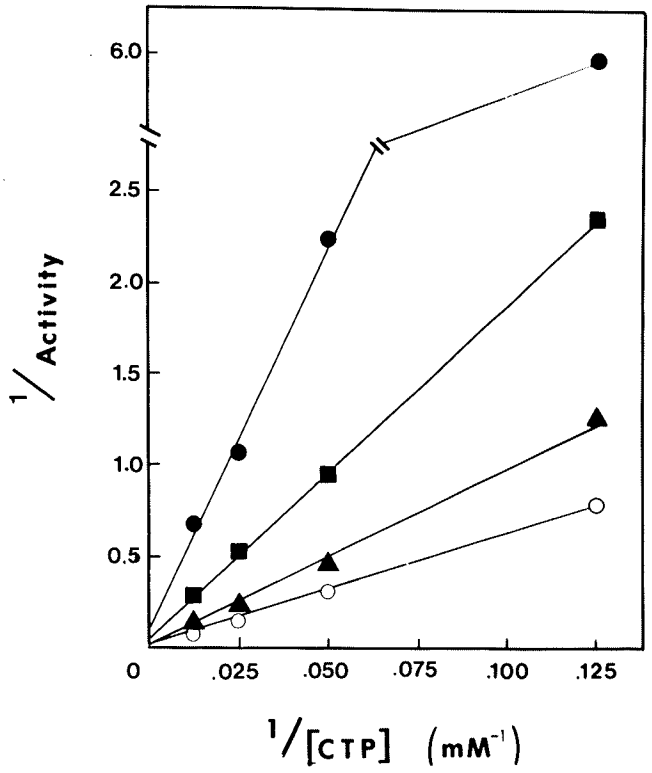
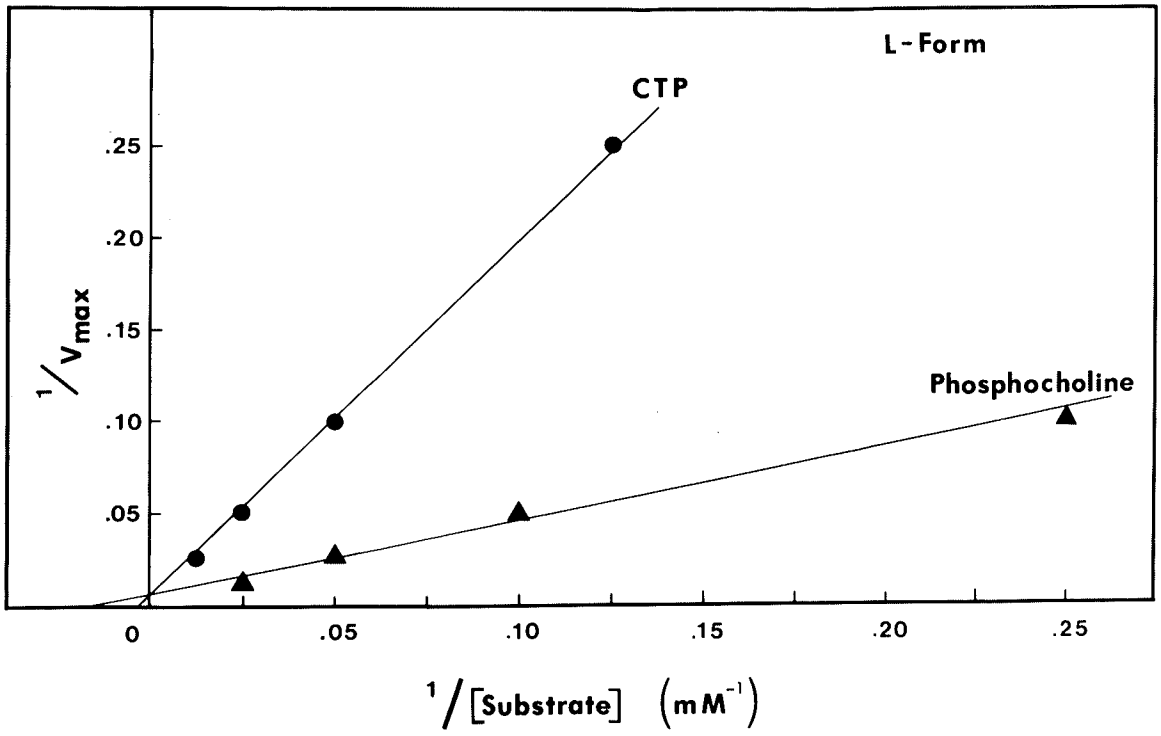

H-Form
0.3
0.2
0.1
0
1.0
2.0
3.0
4.0
5.0
1/Vmax
CTP
Phosphocholine
1/[Substrate] (mM⁻¹)

Figure 13 Double reciprocal plots of initial velocity of low molecular weight form (L-form) of cytidylyltransferase.

The fixed concentrations of CTP in the upper left hand panel were 8 (●), 20 (■), 40 (▲) and 80 (○) mM. The fixed concentrations of phosphocholine in the upper right hand panel were 4 (●), 10 (■), 20 (▲) and 40 (○) mM. In the lower panel, the true K_m for CTP and phosphocholine were determined as described in (Fig.12). Activity is expressed as $nmol \cdot min^{-1} \cdot mg^{-1}$. Each point is the mean of duplicate determinations.


6.0
2.5
2.0
1.5
1.0
0.5
0
0.05
0.10
0.15
0.20
0.25
1/Activity
1/[Phosphocholine] (mM⁻¹)


6.0
2.5
2.0
1.5
1.0
0.5
0
.025
.050
.075
.100
.125
1/Activity
1/[CTP] (mM⁻¹)


L-Form
CTP
Phosphocholine
.25
.20
.15
.10
.05
0
.05
.10
.15
.20
.25
1/Vmax
1/[Substrate] (mM⁻¹)

DISCUSSION

I The Effect of Ethanolamine on Phosphatidylcholine Biosynthesis

Although the requirement for ethanolamine in mammals has not been demonstrated, it is obvious that ethanolamine is the precursor for *de novo* synthesis of phosphatidylethanolamine (11). From the results section of this thesis, exogenous ethanolamine also inhibits choline uptake by the isolated heart in a competitive fashion. The nature of inhibition is very similar to that elicited by hemicholinium-3 (101,104,105). However, exogenous ethanolamine does not seem to affect the biosynthesis of phosphatidylcholine via the CDP-choline pathway. Although *in vitro* inhibition of choline kinase by ethanolamine has been well-documented (16,17) the results from this study indicate that the conversion of choline to phosphocholine in the heart was not affected by exogenous ethanolamine in the perfusate. This is not surprising since the intracellular pool of ethanolamine was not altered by exogenous ethanolamine concentrations (0-0.8 mM) (96). Moreover the pool sizes of the metabolites of the CDP-choline pathway were not changed. Hence the reduction in labelling of choline, phosphocholine, CDP-choline and subsequently phosphatidylcholine was probably a reflection of decreased labelled choline uptake at high ethanolamine concentrations.

This study indicates that ethanolamine competitively inhibits choline uptake by the heart. However, there was no decrease in the intracellular choline concentration in the presence of ethanolamine. Therefore it is conceivable that choline is conserved by recycling. By maintaining the intracellular choline levels, the heart would also maintain the phosphocholine and CDP-choline levels and consequently, the rate of phosphatidylcholine biosynthesis. This release of choline may result from the action of phospholipase D (106) on phosphatidlycholine,

or from the hydrolysis of other choline-containing compounds (44). Moreover, it is not known if intracellular choline levels can be maintained throughout an extended period of perfusion in the presence of high ethanolamine. The fact that a substantial amount of choline is released into the perfusate (44) may result in depletion of the intracellular choline pool over a sustained period of low choline uptake. The result of low choline uptake may be altered phosphatidylcholine biosynthesis.

II The Effect of Choline on Phosphatidylethanolamine Biosynthesis

The requirement of choline for the biosynthesis of acetylcholine and phosphatidylcholine is well-documented (107). The pathways for the incorporation of this metabolite into phosphatidylcholine in hamster heart have been studied (44). However, the effect of choline on phosphatidylethanolamine biosynthesis in the isolated hamster heart was unknown. Hence, two questions being addressed by this study were: (a) Does choline regulate phosphatidylethanolamine biosynthesis? and (b) If so, how?

The results demonstrate unambiguously that choline (0.2-0.5 mM) in the perfusate causes a 28% reduction in the rate of phosphatidylethanolamine biosynthesis. This decrease does not appear to be mediated by impaired transport of ethanolamine across the sarcolemma, since no significant difference in uptake of labelled ethanolamine was observed at any choline concentration tested (Table VIII).

Investigations into radioactivity incorporated into the ethanolamine-containing compounds (Table IX) suggested that choline was regulating phosphatidylethanolamine biosynthesis at the ethanolamine kinase level.

CTP:phosphoethanolamine cytidylyltransferase, which was shown to be the rate-limiting enzyme for phosphatidylethanolamine biosynthesis (96,108) was not affected by similar choline concentrations (data not shown). Therefore, the reduction in phosphoethanolamine, CDP-ethanolamine and phosphatidylethanolamine labelling was probably caused by competitive inhibition of cardiac ethanolamine kinase by high intracellular choline concentrations. This hypothesis is supported by the fact that intracellular choline concentration is elevated 2-fold after perfusion with 0.2 mM choline. In addition, perfusion with 0.5 mM choline did not further increase the intracellular choline pool and hence did not cause any further reduction in phosphatidylethanolamine labelling.

It was expected that when the hearts were perfused with 0.2 mM choline, intracellular choline levels should increase since the K_m of choline uptake is 0.1 mM (44). Accordingly, perfusion with 0.5 mM choline should not further increase the intracellular choline pool because the K_m of choline uptake has already been greatly exceeded. Hence exogenous choline levels above 0.2 mM will have little effect on intracellular choline concentrations. Based on these observations, it is not unreasonable to assume that only 30% of total phosphatidylethanolamine biosynthesis is subject to regulation by exogenous choline.

It is also clear from this study that choline concentration in the plasma has an important role in the regulation of phosphatidyl-ethanolamine biosynthesis. In hamster plasma, the concentration of choline is 0.18 mM (44). This choline concentration should cause a 28-30% inhibition of phosphatidylethanolamine biosynthesis. Plasma choline levels above 0.18 mM should have little additional effect. Conversely, phosphatidylethanolamine biosynthesis should be greatly

enhanced with a decrease in plasma choline levels. This hypothesis is supported by an observed increase in phosphatidylethanolamine biosynthesis during choline deficiency (109). Hence, plasma choline levels may provide an additional mechanism (beyond the control provided by CTP: phosphoethanolamine cytidylyltransferase) for transient regulation of phosphatidylethanolamine biosynthesis

III Choline and Ethanolamine Transport in Hamster Heart

The involvement of a transport system for the uptake of choline and ethanolamine in the heart (44,96) and other tissues (62,110) is well-established. However, it is not clear if choline and ethanolamine are transported by the same or different mechanisms. The results from the preceding two sections and other related studies (44,96) suggest that these two compounds are transported by separate and distinct mechanisms in hamster heart. Since choline has no effect on the uptake of ethanolamine, it appears that the ethanolamine transport site has little or no affinity for choline. Hence, it is very unlikely that the ethanolamine site co-transports choline. The choline transport site may accomodate ethanolamine and hemicholinium-3 as competitive inhibitors, however it is unlikely that ethanolamine is co-transported with choline. This postulate is based on the fact that the intracellular pool size of ethanolamine was not significantly changed when hearts were perfused with various concentrations of ethanolamine (96) or a combination of ethanolamine and choline (111). As previously mentioned, the intracellular choline pool may be a regulatory factor in phosphatidylcholine biosynthesis in some tissues (44,65,66). Hence, the nonspecificity of the choline transport site, as demonstrated

in these studies (111,112), may facilitate the binding of ethanolamine and ethanolamine analogues (such as amino acids) which may provide control of choline uptake and consequently the rate of phosphatidylcholine biosynthesis in the heart.

IV Hamster Heart CTP:Phosphocholine Cytidylyltransferase

It is well-established that cytidylyltransferase catalyzes the rate-limiting step in phosphatidylcholine biosynthesis in mammalian tissues (10,103). The data presented in this thesis indicate that there are at least three forms of cytidylyltransferase in hamster heart cytosol. The H- and L-forms of heart cytidylyltransferase are of similar molecular weights to those reported for rat liver (21) and rat lung (113) cytosols. However, this is the first report of a S-form (small molecular weight form) of the enzyme. This form does not seem to be a proteolytic product of either the H- or L-forms, since similar Sepharose 6B chromatography profiles were obtained when phenylmethylsulfonylfluoride was added to the heart homogenate.

Since the S-form of cytidylyltransferase is present under all experimental conditions tested, it appears to be a true cytosolic enzyme form. However, the origin and functional significance of S-form remains unknown. It is possible that heart cytidylyltransferase originates from a different structural gene than liver and lung cytidylyltransferases. If this were the case, the existence of S-form can be explained. Alternatively, cytidylyltransferase from these tissues may be the product of the same structural gene. The S-form of the enzyme may be the nascent protein, which subsequently undergoes spontaneous aggregation to L- and H-forms in liver and lung but not in the heart. Another explanation is

that L-form of cytidylyltransferase is the nascent protein, which is then post-translationally modified in cardiac tissues yielding the S-form of the enzyme. Information on the origin and the functional relationship between the various enzyme forms may be achieved through the purification of cytidylyltransferase and the determination of the amino acid sequence.

Although cytidylyltransferase is an ambiquitous enzyme (18) the relationship between the cytosolic and microsomal cytidylyltransferases has not been clearly established. The results from this thesis suggest that cytosolic H-form is very similar to microsomal cytidylyltransferase. Both enzyme forms display similar pH optima, Mg^{+2} requirements, lipid insensitivity and K_m's for CTP and phosphocholine. Moreover, the turbidity of the fractions containing H-form from Sepharose 6B chromatography (Fig.10) suggests the presence of lipid. The nature of this lipid associated with H-form and the stoichiometry of its association with H-form is not known. Despite the remarkable similarities between these enzyme forms, an unambiguous comparison cannot be made until cytidylyltransferase is solubilized, isolated and characterized from the microsomal fraction.

The enzyme form of cytidylyltransferase which controls the rate of phosphatidylcholine biosynthesis in mammalian tissues is still a matter of much controversy (103). Kinetic analysis from this study suggests that the control of phosphatidylcholine biosynthesis in the heart is directly associated with the enzyme form and the intracellular supply of substrates. The H-form and microsomal form of cytidylyltransferase have a true K_m for CTP of 1.0 mM and for phosphocholine of 0.44 mM. The L-form of the enzyme is kinetically identical to the S-form with a

true K_m for CTP of 400 mM and for phosphocholine of 80 mM. Since intracellular CTP concentration is 15 nmol/g heart (72) and phosphocholine concentration is 250 nmol/g heart (44) the various forms of cytidylyltransferase may be regulated by changes in the pool size of these substrates. From the kinetic analysis, it appears that the catalytic activities of both H-form (and microsomal form) and L-form (and S-form) are highly dependent on intracellular CTP concentrations. Small changes in the pool size of CTP will alter enzyme activity dramatically and consequently the rate of phosphatidylcholine biosynthesis. However, the intracellular phosphocholine pool may provide a higher degree of regulation to the L-form (and S-form) of the enzyme than the H-form (and microsomal form) of cytidylyltransferase.

Recent reports (76,83,103) have suggested that the subcellular location of cytidylyltransferase from rat liver is regulated by protein phosphorylation. These investigators found that cAMP analogues cause a decrease in the cytidylyltransferase activity associated with the microsomes (76). Moreover, the time-dependent activation of cytidylyltransferase in cytosol was reduced in the presence of the protein kinase substrates ATP and magnesium or the phosphoprotein phosphatase inhibitor sodium fluoride (83). In an attempt to determine if heart cytidylyltransferase was affected by protein phosphatases which may alter enzyme activity and distribution in the cytosol, heart homogenates were prepared in the presence of 20 mM sodium fluoride. The elution profiles from Sepharose 6B chromatography after this treatment were similar to those depicted in Fig. 10 (data not shown). Therefore it appears that dephosphorylation of cytidylyltransferase has no effect on the distribution of this enzyme in the heart. However, evidence for the

phosphorylation of cytidylyltransferase and the functional significance of such covalent modification can only be obtained by investigations into the incorporation of [^{32}P] phosphate with a purified cytidylyltransferase in vitro.

It is ovbious that studies on the control of phosphatidylcholine biosynthesis by CTP:phosphocholine cytidylyltransferase have been hampered by a lack of purified enzyme. The results of this investigation have demonstrated that highly enriched preparations of L- and S-forms of cytidylyltransferase can be achieved through storage of cytosol and dissociation of H-form with SDS (Table XI). Although electrophoretic purity of these preparations was not established, the reproducibility of this dissociation instills a certain level of confidence to the procedure described. The purification scheme outlined in this thesis may be subsequently refined and improved to achieve greater yields of purified cytidylyltransferase. Antibodies against a purified enzyme could then be obtained and used for immunotitration experiments. Cytidylyltransferase activity is known to change under a variety of conditions (10,103). These changes have been explained by modulation of enzyme activity (24,84,85) or translocation of enzyme from one subcellular compartment to another (79,80,81). Immunotitration experiments would enable us to delineate which process is occurring in the cell in response to different physiological or pathological conditions.

V Control of Phosphatidylcholine Biosynthesis in Hamster Heart

It is clear that the mechanisms for the control of phosphatidylcholine biosynthesis in the mammalian heart are complex processes which may also be interrelated. Based on the results of the foregoing studies

and the work of others, it appears that phosphatidylcholine biosynthesis in the heart is subjected to at least four levels of control. The first two tiers of control, namely by exogenous substrate levels or analogues and by the modulation of CTP:phosphocholine cytidylyltransferase have been extensively discussed. This section of the thesis will focus on the control of phosphatidylcholine formation in cardiac tissues by other mechanisms.

The intracellular levels of CTP or the overall energy status of the cell may affect phosphatidylcholine biosynthesis. Two forms of high energy triphosphate nucleotides, ATP and CTP are required for the CDP-choline pathway (11). Choy (72) demonstrated that a 34% decrease in CTP concentration was observed in myopathic hearts. ATP concentration in this same tissue was also reduced by 33% (114). However, only the formation of CDP-choline, but not phosphocholine, was altered in the hearts of the myopathic animals (72). Since ATP and CTP are the nucleotides required for the formation of phosphocholine and CDP-choline, respectively, it can be concluded that only CTP, but not ATP, has a direct effect in the regulation of phosphatidylcholine formation in the myopathic hamster heart.

A final level of control may occur in the fatty-acyl composition of phosphatidylcholine in the heart. Recently a comparison of the molecular species of 1,2-diacylglycerol and phosphatidylcholine was investigated (115). These investigators found substantial differences in acyl content between these two lipids. Since 1,2-diacylglycerol is the immediate precursor for new phosphatidylcholine formation (44) the control of the molecular species of phosphatidylcholine is due to the selectivity of phosphocholine transferase or the resynthesis process

described by Lands (50) or a combination of both. Arthur and Choy have demonstrated that phosphocholine transferase had some specificity toward the selection of diacylglycerol groups (115). However, the authors also suggest that extensive remodelling of phosphatidylcholine occurs after the initial formation of the phospholipid. It appears that most of the newly synthesized phosphatidylcholine in hamster heart must undergo resynthesis by deacylation-reacylation. Hence, it may be concluded that phosphocholine transferase has only limited ability to select the required molecular species of 1,2-diacylglycerol for phosphatidylcholine biosynthesis in the mammalian heart.

VI Future Directions

The control of phosphatidylcholine biosynthesis is clearly a complex process. The present study has provided new information into how this process is controlled in the hamster heart. However, this study has also generated many more questions than were originally conceived. For example, what other metabolites will affect the uptake of choline? What are the mechanisms which control the cellular levels of these metabolites? Is this type of control important under normal or abnormal physiological conditions? Since the control of CTP:phosphocholine cytidylyltransferase activity is an area of considerable controversy, the purification of this enzyme is of paramount importance. The purified enzyme will be required to determine if cytidylyltransferase is covalently modified. In addition, antibodies obtained to the purified enzyme could then be used to determine if cytidylyltransferase is translocated in the cell. Questions relating to the various forms of cytidylyltransferase have also emerged from this study. What is the significance of S-form and how is it related (if at

all) to L- and H-forms? Which enzyme form(s) control the rate of phosphatidylcholine biosynthesis? These and many other questions remain unanswered. Clearly, the complete understanding of the control of phosphatidylcholine biosynthesis in mammalian tissues awaits future investigations.

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