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PHOSPHATIDYLCHOLINE BIOSYNTHESIS IN HYPERCHOLESTEROLEMIC RATS

by

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ABSTRACT

The regulation of the rate of phosphatidylcholine biosynthesis was investigated in the present study. Rats fed a high cholesterol/cholate diet had elevated levels of plasma cholesterol and phospholipids and this suggested that the rate of phosphatidylcholine biosynthesis might be altered during hypercholesterolemia. The activities of the enzymes of phosphatidylcholine biosynthesis were measured and the activities of phosphocholine cytidylyltransferase in cytosol and microsomes were increased 2- to 3-fold in the livers from hypercholesterolemic rats when compared to controls. The activities of choline kinase, phosphocholine-transferase and phosphatidylethanolamine methyltransferases, however, were unchanged. The stimulation of the cytidylyltransferase was not due to an adaptive increase in the quantity of enzyme and results suggested that a phospholipid modulator was responsible for the activation of the enzyme.

The rate of phosphatidylcholine biosynthesis in the liver was measured after intraportal injection of labelled choline. The estimated rate of synthesis was 2- to 3-fold higher in the hypercholesterolemic rats and this correlated with the 2- to 3-fold stimulation of phospho-choline cytidylyltransferase activity. The results clearly showed that the cytidylyltransferase activity in the liver was rate-limiting in the de novo synthesis of phosphatidylcholine during hypercholesterolemia and strongly indicated that the increase in plasma phospholipids during hypercholesterolemia was, at least in part, the result of increased synthesis of phosphatidylcholine in the liver.

TABLE OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AdoHcy	S-adenosylhomocysteine
AdoMet	S-adenosylmethionine
ATP	adenosine triphosphate
Ci	curie
CDP-choline	cytidine-diphosphocholine
CDP	cytidine diphosphate
CL	Cardiolipin
CMP	cytidine monophosphate
cpm	counts per minute
CTP	cytidine triphosphate
DG	diacylglycerol
dpm	disintegrations per minute
EDTA	ethylenediaminetetracetic acid
ev	electron volts
g	grams
GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
hr	hour
IgG	immunoglobulin G
Ki	inhibition constant
Km	Michaelis-Menten constant
l	liter
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
m	meter

M	molar
Mg^{++}	magnesium ion
min	minutes
Mn^{++}	manganese ion
mol	mole
N	normal solution
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
POPOP	1,4 Bis(2-(5-phenyloxazolyl))Benzene
PPO	2,5-diphenyloxazole
PS	phosphatidylserine
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
Rf	ratio of the distance travelled by a compound to that travelled by the solvent front
sec	second
SDS	sodium dodecylsulphate
SM	sphingomyelin
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)amino ethane
UMP	uridine 5'-monophosphate
UTP	uridine 5'-triphosphate
UV	ultraviolet

V	volume
V_{max}	maximum velocity
w	weight

Standard Prefixes

K	Kilo - 10^{3}
C	centi - 10^{-2}
m	milli - 10^{-3}
μ	micro - 10^{-6}
n	nano - 10^{-9}

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DEDICATION

To My Parents, Benjamin and Josephine

"In the beginning God created..."

Genesis 1:1

INTRODUCTION

I. Biological Roles of Phosphatidylcholine

Phospholipids are found in all living organisms and they are major components of cellular membranes and blood lipoproteins in animals. In biological membranes, phospholipids are arranged in a bilayer where the polar head groups face the aqueous environment on the outside of the bilayer while the fatty acyl chains face inward to form a hydrophobic matrix. The membrane is not merely a static structure of the cell but performs many vital functions such as ion transport and endo- and exocytosis. Although proteins found in membranes are responsible for many membrane functions, evidence suggests that phospholipids may play a role in some cellular processes such as membrane fusion (1). The importance of phospholipids in the structure and function of lipoproteins is unclear at the present, but it is very possible that phospholipids and apoproteins may coat the surface of the lipoproteins and render the core of triglycerides and cholesterol esters water soluble.

Phosphatidylcholine is the major class of phospholipids found in animal tissues. A glycerol molecule forms the backbone of this lipid and fatty acids are esterified to carbons 1 and 2 while phosphocholine is esterified to carbon 3 (Fig. 1). Phosphatidylcholine performs several specialized

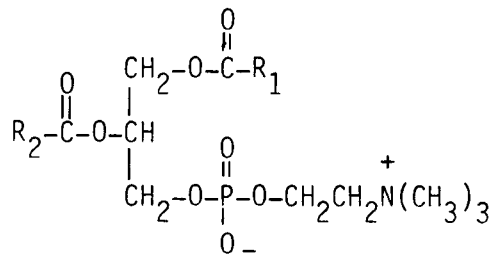


Fig. 1. Structure of Phosphatidylcholine. In most species, R_1 is saturated and R_2 is unsaturated.

functions in the body. In lung, the major component of surfactant is dipalmitoylphosphatidylcholine which facilitates the expansion and contraction of the lungs by lowering the surface tension in the alveoli (2). Phosphatidylcholine is also an important component of bile which aids in the solubilization, digestion and absorption of dietary triglycerides.

In the blood, PC plays an important role in the metabolism of lipoprotein cholesterol. Lecithin cholesterol acyltransferase (LCAT) esterifies cholesterol by transferring a fatty acid chain from PC. This reaction is essential for clearance of free cholesterol from cell membranes (3).

The regulation of PC biosynthesis is not well understood, although the two major biosynthetic pathways were elucidated in the 1950's and early 60's. However, in the past 20 years, the soluble enzymes in the pathways were isolated and characterized and the membrane bound enzymes were successfully solubilized. The following sections discuss some of the properties of the biosynthetic enzymes and the regulation of PC biosynthesis in lung and liver.

II. Pathways for the Synthesis of Phosphatidylcholine

The major pathway for PC biosynthesis was discovered by Kennedy and Weiss in the 1950's (4). Their work extended from the earlier studies of Kornberg (5) who reported that ^{32}P- and ^{14}C-labelled phosphocholine was converted by liver enzymes to a lipid, presumably PC and proposed the following scheme for PC synthesis:

phosphatidic acid + phosphorylcholine → lecithin + Pi

The reaction as shown should be reversible and ^{32}P should be readily incorporated into phospholipids *in vitro*. Work in Kennedy's laboratory, however, failed to demonstrate such a reaction and in the course of further

investigations a new type of cytidine containing coenzyme was discovered (4). The cytidine-diphosphocholine (CDP-choline) and cytidine-diphosphoethanolamine (CDP-ethanolamine) coenzymes were found to play an essential role in the de novo biosynthesis of PC and PE (4).

A second pathway was discovered around 1960 by Greenberg and co-workers (6) who were mainly interested in choline biosynthesis. They found that when ^{14}C-ethanolamine was given to rats, the label was incorporated into PC but not into phosphocholine. Extensive experiments failed to demonstrate choline formation from free ethanolamine and when [Me-^{14}C]methionine was used, radioactivity was found in mono- and dimethylPE as well as in PC. The turnover rates of the methylated PEs were very much faster than PC and this suggested that the mono- and dimethylated derivatives were intermediates in the synthesis of PC. These and other observations supported the conclusion that PE is methylated to form PC, with S-adenosylmethionine serving as the donor of all the methyl groups.

The de novo pathway is the major route for PC biosynthesis in the liver, lung, and other tissues, whereas the N-methylation pathway, which is responsible for about 20% of hepatic PC synthesis in rats (7) and humans (8), is of significance only in liver (9). The enzymes in both of these pathways have been studied and the known physical and enzymological properties are given below. The concentrations of substrates and intermediates of both pathways are given in Table 1.

1. The De Novo Pathway

The de novo pathway converts choline to PC by three separate reactions with phosphocholine and CDP-choline as intermediates (Fig. 2). Three enzymes catalyzed these reactions and choline which is water-soluble is eventually converted into a lipid-soluble molecule.

Table 1

Concentrations of Substrates and Intermediates of the Pathways for Phosphatidylcholine Biosynthesis in Rat Liver

De Novo Pathway

Infante (70)

Substrate	Concentration* (μmol·g wet liver wt.$^{-1}$)
ATP	1.95 ± 0.06
Choline	0.23 ± 0.10
ADP	1.4 ± 0.20
Phosphocholine	1.1 ± 0.03
Mg^{++}	0.7
CTP	0.083 ± 0.005
CDP-Choline	0.051 ± 0.003
PPi	0.0062 ± 0.0003

Phosphatidylethanolamine N-Methylation Pathway

Katyal and Lombardi (96)

Substrate	Concentration* (nmol·g liver wt.$^{-1}$)
Phosphatidylethanolamine	11 x 10^3
Phosphatidyl N-monomethyl-ethanolamine	12
Phosphatidyl N-dimethyl-ethanolamine	18
Phosphatidylcholine	18 x 10^3

* Concentration was calculated with the assumption that the liver of a 100 g rat weighs 5 g.

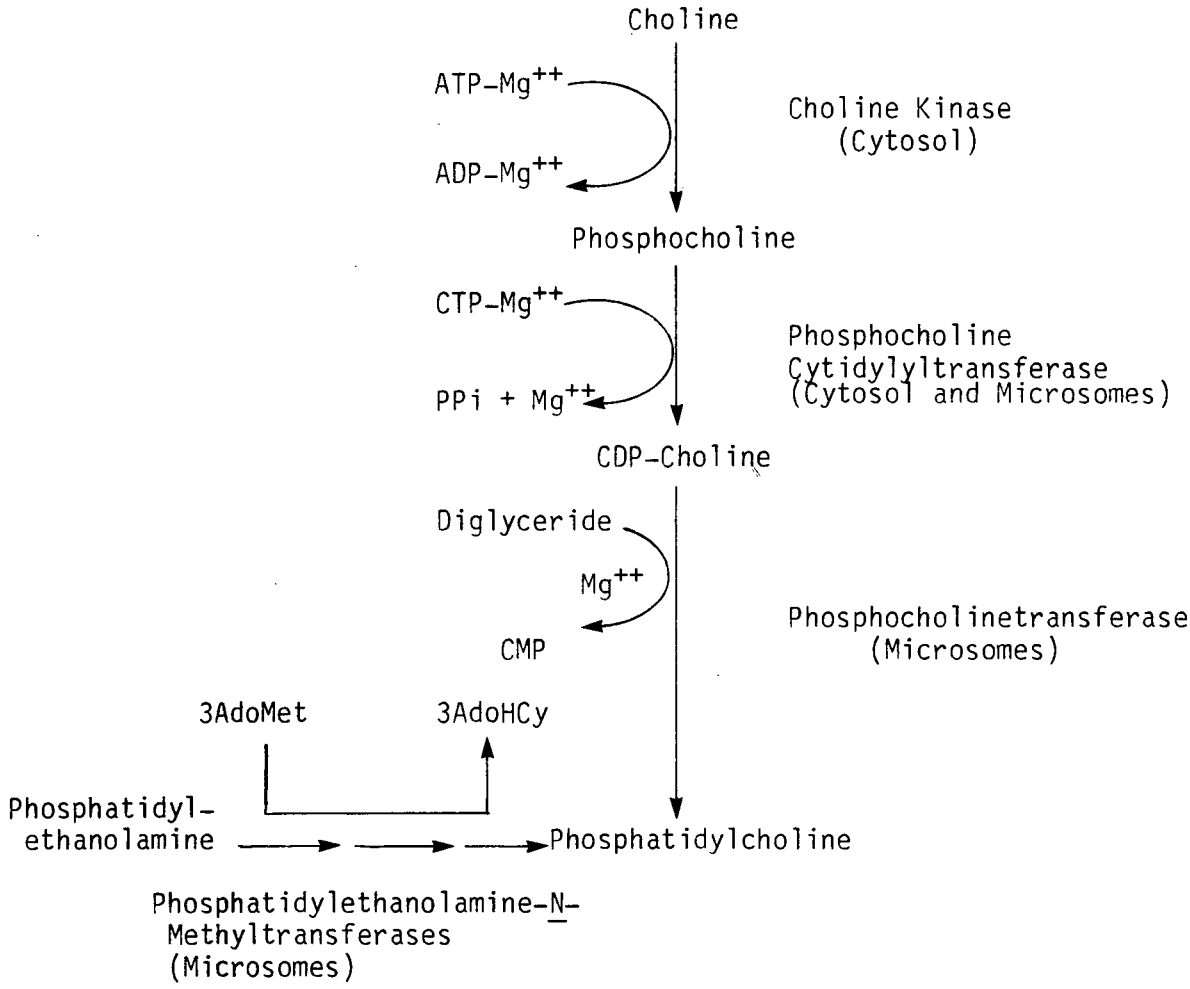


Fig. 2. Pathways for the Biosynthesis of Phosphatidylcholine

(a) Choline Kinase

The first enzyme of the pathway is choline kinase. This cytosolic enzyme was first described in yeast by Wittenberg and Kornberg (10) who established the stoichiometry of the reaction and demonstrated an absolute requirement of the enzyme for magnesium ions. Kornberg's early studies also showed that the kinase was most active between pH 8.0 and 9.0, and that it was able to catalyze the phosphorylation of monoethyl- and diethylethanolamine, monomethyl- and dimethylethanolamine and ethanolamine. However, substrate binding data indicated that the affinity of the enzyme for choline was several orders of magnitude higher than for ethanolamine. Phosphorylation of choline was demonstrated in liver, brain, kidney and intestinal mucosa of various animals (10).

The first evidence which indicated that the ethanolamine and choline phosphorylations might be catalyzed by separate kinases stemmed from the work of Sung and Johnstone (11). They found that Ehrlich ascites cell extracts converted choline and ethanolamine into their phosphorylated derivatives but the choline phosphorylating activity was much more stable during storage at -10°C. Cysteine and calcium ions inhibited ethanolamine phosphorylation but did not affect choline phosphorylation. Furthermore, various activators of choline phosphorylation inhibited ethanolamine phosphorylation. The results suggested that separate enzymes were involved but physical separation of the two was not achieved.

The choline and ethanolamine phosphorylating activities were partially separated by Weinhold and Rethy (12). By using DEAE-cellulose chromatography, they were able to purify the enzymes 31-fold and dissociated ethanolamine kinase into two distinct forms. Ethanolamine kinase I was much smaller than ethanolamine kinase II which had choline kinase activity. The

properties of the kinases are summarized in Table 2. Ethanolamine kinase I and kinase II have distinct characteristics and aside from the different Km's for ATP and ethanolamine, ethanolamine kinase II was strongly inhibited by choline (Ki = 0.03 mM) which competes with ATP, whereas kinase I was not affected. The authors suggested that the levels of ATP and choline could possibly regulate the rate of PE biosynthesis. The choline kinase activity was inhibited by high concentrations of ethanolamine which competes with choline (Ki = 4.5 mM) and ATP (Ki = 9.8 mM) for the enzyme.

The controversy of whether choline kinase and ethanolamine kinase II were one or two separate enzymes remained unresolved for some time. Brophy and Vance (13) reported in 1976 that the kinases from rat liver were co-purified 500-fold by affinity chromatography and the results supported the single enzyme hypothesis. However, in a later study (14), ethanolamine kinase and choline kinase were separated by gel electrophoresis at pH 8.7 from a highly purified preparation and ethanolamine kinase was resolved into two components which were distinct from choline kinase. Furthermore, when fresh cytosol was subjected to disc gel electrophoresis, four isozymes of choline kinase and ethanolamine kinase were found (14) and the function of these multiple forms of the enzymes remains unclear at present.

(b) Phosphocholine Cytidylyltransferase

Phosphocholine cytidylyltransferase catalyzes the formation of CDP-choline from phosphocholine and CTP and is the second enzyme of the de novo pathway. In early studies (15), Kennedy and his co-workers found that a large amount of ATP was required to support the synthesis of PC from phosphocholine. In later experiments, however, this reaction was shown to specifically require CTP which was a contaminant in the ATP preparation

Table 2

Properties of 31-Fold Purified Ethanolamine and Choline Kinase from Rat Liver

	EKI	EKII	CK
Weinhold and Rethy (12)			
Molecular Weight	36,000	166,000 (EKII and CK co-purified)	
pH Optimum	7.5-8.5	8.0-9.5	8.0
Substrates		Km for Substrate (mM)	
i) ATP	14.3	0.5	3.7
ii) Ethanolamine	0.4	1.7	-
iii) Choline	-	-	0.03
Competitive Inhibitors (Substrate)		Ki, Inhibition Constant (mM)	
(i) Ethanolamine (choline)	-	-	4.5
ii) Ethanolamine (ATP)	-	-	9.8
iii) Choline (ATP)	-	0.03	-

EK - ethanolamine kinase
CK - choline kinase

used in earlier studies. Shortly after Kennedy elucidated the role of CDP-choline in PC synthesis, he and co-workers described the enzymatic synthesis of CDP-choline in guinea pig liver (16). Their early work showed that this enzyme required magnesium or manganese ions for activity and utilized CTP. The pyrophosphorolysis of CDP-choline was also readily catalyzed by this enzyme.

The cytidylyltransferase could also utilize dCTP in the synthesis of dCDP-choline as was first shown by Kennedy, Borkenhagen, and Smith (17). This was confirmed by Schneider and Behki (18) and subsequent studies by Schneider (19) revealed some other interesting results. He found that the cytidylyltransferase activity preferentially partitioned into the cytosol when the liver was homogenized in saline, whereas most of the activity was found in the particulate fraction when distilled water was used. The enzyme activity partitioned almost equally between the soluble and particulate fractions when the liver was homogenized in sucrose. He also found that storage of the 100,000 x g supernatant obtained from 20% liver homogenate at 0°C resulted in a 5-fold stimulation of cytidylyltransferase activity. These results later facilitated the development of a purification procedure for the cytidylyltransferase (22).

Further ground work was established by Fiscus and Schneider (20) when they reported that the activity of the cytidylyltransferase was stimulated by phospholipids. The enzyme was delipidated by extraction with cold acetone and butanol, and this resulted in loss of almost all activity. Addition of phospholipids resulted in a 25-fold stimulation of enzyme activity and with this delipidated form of the enzyme, they found that LPC and LPE were potent activators, whereas purified egg PC showed little stimulative activity. The authors suggested a positive feedback mechanism in the regulation of PC synthesis.

Early attempts to purify this enzyme were unsuccessful because severe loss of enzyme activity occurred during ion exchange chromatography (20) and Sephadex G-200 chromatography (21). The enzyme was finally purified to homogeneity by Choy et al. (22) by Sepharose 6B chromatography. The authors also reported the presence of a high molecular weight (H-form) and low molecular weight (L-form) form of the cytidylyltransferase in rat liver cytosol.

The existence of the two forms in lung had been reported earlier by Stern, Kovac and Weinhold (23) and subsequent studies from the same laboratory showed that PG, CL and PI were potent activators of the enzyme (24). However, the effect of PG on the enzyme was unique since this phospholipid also aggregated the enzyme to the high molecular weight form. The physical and kinetic properties of the enzyme in lung and liver, as well as the phospholipid modulators of the lung, liver and intestinal cytidylyltransferase, are summarized in Tables 3 and 4.

The effects of various phospholipids on the activity of the cytidylyltransferase in rat liver were investigated by Choy and Vance (25). They showed that the delipidated form of the cytidylyltransferase, first used by Fiscus and Schneider (20), was stimulated by all classes of phospholipids. In contrast, the L-form, H-form and enzyme in cytosol were inhibited by certain phospholipids. The delipidated form of the enzyme may have been desensitized during acetone-butanol extraction and was probably not representative of native cytidylyltransferase.

Of the different classes of phospholipids tested, LPE was the most potent activator of the enzyme but unlike PG this lipid did not aggregate the enzyme. LPC, however, was a potent inhibitor of the enzyme. Phospholipids have detergent properties and the requirement of the

Table 3

Physical and Kinetic Properties of Phosphocholine Cytidylyltransferase from Rat Lung and Liver

	LUNG Stern et al. (23)	LIVER Choy et al. (22)
Molecular Weight		
Low Molecular Weight Form (lipid-requiring form)	1.9×10^5	2.0×10^5
Molecular Weight Range		
High Molecular Weight Form (non-lipid requiring form)	$5\text{-}50 \times 10^6$	$0.5\text{-}13 \times 10^6$
pH Optimum	6.0 - 7.0	7.0

	Km for Substrates (mM)		
Reactants	Lung Cytosol	L-Form	H-Form
i) phosphocholine	1.0	0.167	0.180
ii) CTP	4.0	0.208	0.296
iii) Mg^{++}	12.0	-	-
iv) CDP-choline	-	0.210	0.640
v) Inorganic pyrophosphate	-	0.004	0.007

Table 4

Modulators of the Activity of Phosphocholine Cytidylyltransferase from Rat Lung, Liver, Intestine and Brain

	LUNG Feldman et.al.(24)	LIVER Choy et.al. (25,26)	INTESTINE O'Doherty et.al(27)	CHICKEN BRAIN Chojnacki et.al(28)
Aggregators	PG	PG,DG	-	-
Activators	PG, PS, PI, CL	PG, LPE, PI, PS	1-sn and 3-sn LPC (C_{16} and C_{18} more effective than species with shorter chains)	1-stearoyl LPC
Inhibitors		Neutral Lipids, LPC		

cytidylyltransferse was studied by using a variety of detergents. Almost all of the detergents tested inhibited the activity of the enzyme from lung (23) and liver (25) while none stimulated the enzyme. The inhibition in lung was shown to be relieved, at least in part, by the addition of phospholipids, suggesting that the detergents inhibited activity by removing phospholipids from the enzyme. The fact that the detergents themselves do not stimulate the enzyme suggested that the activation by PG and LPE involve specific interactions between the lipids and enzyme.

The aggregation of the cytidylyltransferase in liver was further studied. Choy et al. (26) showed that the concentration of PG in rat liver cytosol was insufficient to affect the cytidylyltransferase and a survey of various classes of lipids showed that diacylglycerol was the only lipid from rat liver that aggregated the enzyme.

Cytidylyltransferase from crypt and villus cells was studied by O'Doherty, Smith, and Kuksis (27). They found that acetone and butanol extracted enzyme from intestine was stimulated by 3-sn-LPC and showed specificity for the fatty acid side chain. LPC with C_{16} and C_{18} fatty acids were several times more effective in stimulating enzyme activity than the lyso-lipids with shorter fatty acids. However, LPE as well as the serine and inositol phosphoglycerides were not stimulatory in contrast to the findings in liver and lung. The authors suggested that the stimulation by LPC may be related to its detergent characteristics since 1-sn-LPC was as effective as the naturally occurring 3-sn analog. This idea, however, was not supported by studies in lung and liver which showed that detergents do not stimulate the cytidylyltransferase.

(c) Phosphocholinetransferase

Phosphocholinetransferase is the last enzyme of the de novo pathway and it is associated with the endoplasmic reticulum (29). The characterization of this enzyme had been hindered by two main factors. The transferase is tightly bound to membrane and attempts to purify the enzyme were unsuccessful (30). A further complication stems from the physical properties of the substrate and products: DG and PC are both lipid soluble, whereas CDP-choline and CMP are water soluble. The enzyme kinetics for a reaction which involves two immiscible phases is not yet understood. However, the specificity of the enzyme for the fatty acid groups on the DG has been extensively investigated by various workers.

Kennedy and Weiss (4) were the first to describe phosphocholine-transferase in rat liver microsomes. They found that the particulate fraction converted CDP-choline to PC and this activity was stimulated 10- to 20-fold by the addition of Tween 20 and DG. The transferase specifically utilized CDP-choline and magnesium or manganese ions were required for activity whereas calcium ions inhibited phosphocholinetransferase even in the presence of magnesium ions. In a later study, Kennedy, Borkenhagen and Smith (17) found that dCDP-choline was as active as CDP-choline in the synthesis of PC but this finding was later refuted by Schneider and Behki (18) who found that CDP-choline had a 10-fold higher binding constant than dCDP-choline for the formation of PC.

The selectivity of phosphocholinetransferase for DG in mouse lung was studied by Sarzala and Van Golde (31). They found that the enzyme showed a distinct preference for 1-unsaturated DG over disaturated species. The membrane-bound DG substrates were prepared by treatment of lung microsomes with phospholipase C. In the presence of CMP, phosphocholinetransferase

catalyzed the formation of CDP-choline and in this reaction PC containing an unsaturated fatty acid was also preferentially utilized while dipalmitoyl-PC was not used preferentially. The results suggested that the de novo pathway primarily functions in the production of unsaturated PCs and the remodelling pathways (discussed later) may be mainly responsible for the production of disaturated species in lung.

The substrate-selectivity of phosphocholine transferase in rat liver microsomes was also studied with endogenous substrates (32). The enzyme appeared to utilize 1-myristoyl-PC most rapidly followed by the 1-palmitoyl- and 1-stearoyl-PC. The fatty acids on the C-2 position were not characterized, and in adipocytes Coleman and Bell (33) showed that the optimal acyl chain length of DG for phosphoethanolaminetransferase was longer than that for phosphocholinetransferase.

The specificity of phosphocholinetransferase and phosphoethanolaminetransferase in rat liver were subsequently studied more rigorously by Holub (34). With DG's with known C-1 and C-2 fatty acids as substrates, he found that the phosphocholinetransferase showed a marked preference for the 1-palmitoyl over the 1-stearoyl DG and this was observed whether the fatty acid on carbon 2 was a mono- (oleoyl), di- (linoleoyl), tetra- (arachidonoyl), or hexaene (docohexaenoyl) species. But the enzyme also showed selectivity for the fatty acids on C-2, although to a lesser degree. The 1-palmitoyl-2-arachidonoyl species was only slightly preferred 1.5 fold over the 1-stearoyl species, while the 1-palmitoyl-2-oleoyl, 2-linoleoyl and 2-docohexaenoyl DG's were preferred 3-fold over their 1-stearoyl counterparts. Thus, the phosphocholine transferase appeared to be mainly selective for fatty acid at the C-1 position. Interestingly, phosphocholinetransferase utilized 1-oleoyl 2-stearoyl and 1-oleoyl

2-palmitoyl glycerols equally well (35). This suggested that the influence of saturated fatty acids on the selectivity of the enzyme depended on the location of the fatty acid and that the C-1 position appeared to be most important for specificity.

On the other hand, ethanolaminephosphotransferase showed a distinct preference for 1-saturated 2-docohexaenoyl species over the mono-, di- and tetraene DGs, but the enzyme also showed specificity for the C-1 fatty acid. In direct contrast to the phosphocholinetransferase, phosphoethanolaminetransferase showed a preference for the 1-stearoyl over the 1-palmitoyl species when arachidonic acid was at the C-2 position (34).

The specificity of the transferases in a solubilized preparation were studied by Morimoto and Kanoh (35). By using chemically synthesized 1-acyl 2-oleoylglycerols having 12 to 18 carbons, they found that phosphocholinetransferase have similar Vmax when fatty acids with 12 to 17 carbons were used. However, a marked difference was observed with the 1-stearoyl species which had a Vmax that was only 60% of the 1-palmitoyl, 2-oleoyl glycerol. The phosphoethanolaminetransferase, in contrast, was most active with dioleoyl, 1-heptadecanoyl and 1-stearoyl 2-oleoylglycerols. The 1-palmitoyl species was 40% as active as the 1-stearoyl DG and 1-acyl 2-oleoylglycerol species shorter than C_{16} were only 10% as active. These results agree very well with Holub's study and both results are summarized in Table 5. Analysis of fatty acids from rat liver phospholipids showed that the C-1 position of PC was more enriched for palmitate than stearate when compared to PE and suggested that the specificities of phosphocholinetransferase and phosphoethanolaminetransferase may indeed be operating *in vivo* (36).

Table 5

Specificities of Phosphocholinetransferase and Phosphoethanolamine-transferase from Rat Liver for Various Species of Diacylglycerols

	Phosphocholinetransferase	Phosphoethanolamine-transferase
Morimoto and Kanoh (35)		
Carbon 1	V_{max} $C_{12:0}$ to $C_{17:0}$ greater than V_{max} $C_{18:0}$	V_{max} $C_{17:0}$, $C_{18:0}$ and $C_{18:1}$ greater than V_{max} $C_{16:0}$ which in turn is greater than V_{max} $C_{12:0}$ to $C_{15:0}$
Holub (34)		
Carbon 1	$C_{16:0}$ preferred over $C_{18:0}$	$C_{18:0}$ preferred over $C_{16:0}$ only in 2-arachidonoyl species
Carbon 2	No preference between unsaturated fatty	Marked preference for 2-docosahexaenoyl species over all others tested.

Although the kinetics for a biphasic system are not understood, attempts were made to characterize the kinetic properties of the phosphocholine- and phosphoethanolaminetransferases from rat adipocytes and rat liver. The Km's of the transferases for the various substrates are shown in Tables 6 and 7. Coleman and Bell (37) found that both transferases from fat cells had similar dependencies on $MgCl_2$ and pH, and both activities were inhibited by $CaCl_2$, detergents and dithiothreitol. Furthermore, CDP-ethanolamine and CDP competitively inhibited phosphocholinetransferase. CDP-choline appeared to be a non-competitive inhibitor of phosphoethanolamine transferase while the inhibition by CDP appeared to be of a mixed type (Table 6). The transferases were differentially inhibited by heating, trypsin digestion, $MnCl_2$ and palmitoyl CoA, and the authors suggested that phosphocholinetransferase and phosphoethanolaminetransferase are separate enzymes.

The transferases were solubilized from rat liver microsomes by Kanoh and Ohno (32). The solubilized preparation which was 4- to 5-fold purified contained both transferase activities and their properties were studied (Tables 7 and 8). Interestingly, phosphocholinetransferase required phospholipids for activity while phosphoethanolamine activity was inhibited by phospholipids. The partially purified preparation was treated with Triton X-100 and then applied to a sucrose density gradient and centrifuged. The phosphocholinetransferase was separated into a magnesium and a manganese-requiring component. Phosphoethanolaminetransferase activity on the other hand, formed a single band which co-migrated with the manganese requiring component of the phosphocholinetransferase. These results, along with the kinetic properties of the transferases, strongly indicate that phosphocholinetransferase and phosphoethanolaminetransferase are two distinct enzymes.

Table 6

Properties of Phosphocholinetransferase and Phosphoethanolamine-transferase from Rat Adipocyte Microsomes

	Phosphocholine-transferase	Phosphoethanolamine-transferase
Coleman and Bell (37)		
pH Optimum	8.5 to 9.3	8.5 to 9.3
Substrate	Apparent Km (mM)	
CDP-choline	0.239	-
CDP-ethanolamine	-	0.018
1,2-dioleoyl-sn-glycerol	0.051	0.012
Inhibitors	Ki, Inhibition Constants (mM) (Type of Inhibition)	
CDP-choline	-	1.62 (non-competitive)
CDP-ethanolamine	0.23 (competitive)	-
CDP	0.360 (competitive)	(mixed type)
Other Inhibitors		$CaCl_2$ Organic Solvents Triton X-100 Tween X-20 Dithiothreitol Palmitoyl CoA

Table 7

Properties of Partially Purified Phosphocholinetransferase and Phosphoethanolaminetransferase from Rat Liver Microsomes

Kanoh and Ohno (32)	Phosphocholine-transferase	Phosphoethanolamine-transferase
pH Optimum	8.0-8.5	8.0-8.5
Substrate	Apparent Km (mM)	
i) Diacylglycerol	0.081	0.063
ii) CDP-choline	0.036	-
iii) CDP-ethanolamine	-	0.022
Other Properties	1. Requires phospholipids for activity	Inhibited by phospholipids
	2. Consists of two physically distinct components: a mg^{++} requiring and a mn^{++} requiring activities	One activity dependent on Mn^{++}

Table 8

Modulators of Phosphocholinetransferase and Phosphoethanolamine-transferase from Rat Liver Microsomes

	Phosphocholine-transferase	Phosphoethanolamine-transferase
Kanoh and Ohno (32)		
Competitive Inhibitor (Substrate)	Ki, Inhibition Constant (mM)	
i) CDP-choline (CDP-ethanolamine)	-	0.35
ii) CDP-ethanolamine (CDP-choline)	0.35	-
Parthasarathy et al. (38)		
Drug Inhibitors (30 mM)	Percent Inhibition of Enzyme Activity	
Centrophenoxine	99	
Dimethylethanolamine	69	
Dimethylaminoethyl p-chlorophenoxyacetate	77	

2. Phosphatidylethanolamine N-Methylation Pathway

The enzymes in this pathway catalyze the N-methylation of PE to form PC with S-adenosylmethionine serving as donor of methyl groups. The difficulties in characterizing the methyltransferases were similar to those encountered with the phosphocholinetransferase. The methyltransferases are membrane bound and secondly, the reactants PE and its N-methylated derivatives, as well as the product PC, are water insoluble while S-adenosylmethionine and S-adenosylhomocysteine, are water-soluble. But in spite of these obvious difficulties, successful attempts have been made in characterizing the enzymes.

The initial characterization of the methyltransferases from rat liver was carried out by Bremer and Greenberg (39). They found that enzyme activities were optimal at very alkaline conditions (pH 10). The methyltransferases were inhibited by sulfhydryl reagents while S-adenosylethionine was a competitive inhibitor.

Studies with N. crassa, Agrobacterium tumefaciens, rat liver and bovine adrenal medulla indicated that two distinct methyltransferases were involved in the pathway. This suggestion was made as early as 1946 by Horowitz (40) who studied two mutants of N. crassa which were defective in PC synthesis. N. crassa does not have the enzymes for de novo synthesis of PC and these mutants each carry a mutation on separate genes (41). Strain 34,486 was unable to synthesize phosphatidylmonomethylethanolamine but was able to utilize this intermediate when supplied, while strain 47,904 had a partial block between phosphatidylmonomethylethanolamine and PC. The result of this mutation was an accumulation of the monomethyl intermediate in the cells and medium. Furthermore, this strain was also unable to utilize dimethylaminoethanol as readily as strain 34,846 when grown on agar

and, Horowitz suggested that the same enzyme catalyzed the methylation of the mono- and dimethyl intermediates. Subsequent studies of Scarborough and Nyc (42) showed that strain 34,486 had subnormal PE methyltransferase activity and strain 47,904 was unable to convert either phosphatidylmonomethyl- or phosphatidyldimethylethanolamine to PC. These *in vitro* studies also support the hypothesis that the last two methylations in the formation of PC are catalyzed by one enzyme. The properties of the methyltransferases from *N. crassa* are summarized in Table 9.

Phosphatidylcholine is the most abundant phospholipid in many plants and animals but is rarely found in bacteria. *Agrobacterium*, however, produces PC and other methylated derivatives of PE using methionine as the donor of methyl groups (43), while *Clostridium butyricum* only produces phosphatidylmonomethylethanolamine and no PC (44). The methyltransferase system in *Agrobacterium tumefaciens* was studied by Kaneshiro and Law (45). They found a soluble methyltransferase which only catalyzed the methylation of PE with *S*-adenosylmethionine as donor. This enzyme was purified 40-fold and did not show any cofactor requirement in contrast to the rat liver enzymes (46). Furthermore, the soluble methyltransferase was not inhibited by sulfhydryl reagents but was strongly inhibited by *S*-adenosylhomocysteine and exogenously supplied PE was utilized by the enzyme (Table 10). However, as in rat liver, the particulate fraction from this bacteria catalyzed all three methylation reactions.

The methyltransferases from rat liver microsomes were further studied by Rehbinder and Greenberg (46). The enzyme was solubilized from microsomes by deoxycholate or by sonication and the removal of phospholipid substrate made the enzyme completely dependent upon added substrates. Like the enzyme in its native state, the solubilized preparation could not

methylate exogenously supplied PE. Kinetic studies showed that the amounts of phosphatidyldimethylethanolamine and PC produced *in vitro* were dependent upon the ratios of the concentration of phosphatidylmonomethylethanolamine, *S*-adenosylmethionine and the enzyme. When the ratio of phosphatidylmonomethylethanolamine to *S*-adenosylmethionine was 10, the product was predominantly the dimethyl intermediate while PC was the major product when the ratio was 1.0. The results suggested to the authors that the last two methylation steps were catalyzed by the same active site, i.e. the same enzyme.

Recently, two methyltransferases from bovine adrenal medulla were studied by Hirata *et al.* (47). Both enzymes were localized in the microsomal fraction. The enzyme that catalyzes the first methylation could only be solubilized by detergents while washing the microsomes with EDTA removed an enzyme that catalyzes the conversion of phosphatidylmonomethylethanolamine to PC. The results from various studies strongly support the involvement of two enzymes in the pathway.

The methyltransferases have been successfully solubilized and partially purified. Morgan (48) reported the solubilization of the enzyme from dog lung microsomes and partial purification was achieved by ammonium sulfate precipitation and DEAE-cellulose chromatography. In rat liver, Schneider and Vance (49) solubilized the methyltransferases by sonic disruption of the microsomes in the presence of 0.2% Triton X-100 and the enzyme was purified by a combination of octyl-Sepharose and Sepharose 6B chromatography. The partially purified enzymes were characterized and the results are summarized in Table 14, along with the properties of the methyltransferases from *N. crassa* (Table 9), *Agrobacterium tumefaciens* (Table 10), bovine adrenal medulla (Table 11), dog lung (Table 12) and rat

liver (Tables 13 and 14). More recently, Tanaka *et al.* (50) reported the solubilization of the methyltransferases from mouse liver by treatment of microsomes with deoxycholate and Triton X-100. Interestingly, the solubilized enzyme was strongly stimulated by PE and its methylated derivatives as well as PC. The authors suggested that PC, and to a lesser degree, PE, activated the enzymes by maintaining it in an active conformation.

The methylation of PE in brain has been investigated and earlier experiments *in vivo* (51) indicated that this pathway was absent. Subsequent studies, however, showed that the *N*-methylation reactions occur in brain (52,53). Recently, Mozzi and Porcellati (54) have investigated *N*-methylation in rat brain homogenate and found that the activities of the methyltransferases were very much lower in brain than in liver.

The methyltransferases in liver have a preference for unsaturated PE's (34), while in lung, Morgan suggested that the enzymes preferred more saturated species (55). However, in contrast, experiments with rat lung (130) and rabbit lung (131) showed that the methylation of PE resulted primarily in the formation of unsaturated PC.

The methyltransferase from *Agrobacterium* was quite different from the rat liver enzyme. The bacterial system possesses a soluble PE methyltransferase, which is absent in rat liver, as well as the three particle bound methyltransferase activities. The bacterial system required exogenous substrate while the liver enzymes can only utilize endogenous PE. Furthermore, the soluble methyltransferase, unlike the rat liver

Table 9

Properties of Phosphatidylethanolamine Methyltransferase from N. crassa Microsomes

Scarborough and Nyc (56)	Phosphatidylmonomethylethanolamine Methyltransferase
pH Optimum	8.0
Substrate	Apparent Km (mM)
S-adenosylmethionine	0.013
Phosphatidylmonomethyl-ethanolamine	Results Variable

Table 10

Properties of Phosphatidylethanolamine Methyltransferase from Agrobacterium tumefaciens

Kaneshiro and Law (45)	Phosphatidylethanolamine Methyltransferase (40-fold purified preparation)
pH optima	8.4 and 9.6
Substrates	Apparent Km (mM)
S-adenosylmethionine	0.02
Phosphatidylethanolamine	kinetic plots non-linear
Other Properties	i) No requirement for divalent cation
	ii) Utilized exogenous phosphatidyl-ethanolamine
	iii) Enzyme found in soluble fraction
	iv) Insensitive to sulfhydryl inhibitors
	v) S-adenosylhomocysteine is a potent inhibitor
	vi) Unable to methylate sulfhydryl groups

Table 11

Properties of Phosphatidylethanolamine Methyltransferase from Bovine Adrenal Medulla

Hirata et al. (47)	Phosphatidylethanolamine Methyltranferase	Phosphatidylmonomethyl-ethanolamine Methyl-transferase
pH Optimum	6.5	10
Reactants	Apparent Km (mM)	
S-Adenosylmethionine	0.0014	0.1
Mg^{++}	0.4	-
Competitive Inhibitor	Ki, Inhibition Constant (mM)	
i) S-Adenosylhomo-cysteine	0.0016	
Other Properties		
i) Mg^{++}	required	not required
ii) Substrate	utilized exogenous phospha-tidylethanolamine	

Table 12

Properties of Partially Purified Phosphatidylethanolamine Methyltransferases from Dog Lung

Morgan (48)	Methyltransferases
pH Optimum	8.0-9.0
Substrate Specificity	Saturated phosphatidylethanolamine preferred over unsaturated species.
Sulfhydryl Inhibitors	$HgCl_2$ P-Chloromercuribenzoate N-ethylmaleimide
Other Properties	i) Requires Mg^{++} for maximum Activity ii) Requires cysteine for stability

Table 13

Properties of Phosphatidylethanolamine

Methyltransferases from Rat Liver

Bremer and Greenberg (39,57)	N-Methyltransferases
pH Optimum	10
Competitive Inhibitor of S-Adenosylmethionine	S-adenosylethionine
Sulfhydryl Inhibitors (10 mM)	Percent Enzyme Activity Remaining
$HgCl_2$	44
p-Chloromercuribenzoate	14
p-Chloromercuriphenylsulphonate	33
Iodoacetic Acid	90
Other Properties	i) Methylates sulfhydryl compounds
	ii) Does not utilize exogenous phosphatidylethanolamine
	iii) Slightly stimulated by cholate
	iv) Does not require Mg^{++}

Table 14

Properties of Solubilized Phosphatidylethanolamine

Methyltransferases from Rat Liver

Schneider and Vance (49)	Methyltransferase Activities (5-fold purified)	
	PME→PDE	PDE→PC
Substrate	Apparent Km (mM)	
Phosphatidylmonomethyl-ethanolamine	0.08	-
Phosphatidyldimethyl-ethanolamine	-	0.45
S-adenosylmethionine	0.022	0.016
Competitive Inhibitors (Substrate (mM)	Ki Inhibition Constants (mM)	
S-adenosylhomocysteine (S-adenosylmethionine)	0.0049	0.0067
	Percent Enzyme Activity	
Phosphatidylcholine at 0.36 μM	56	38

Other Properties — All Methyltransferases

i) pH optimum at 9.5
ii) Molecular weight of 2.0×10^5
iii) Utilized exogenous phosphatidylethanolamine
iv) Does not require Mg^{++}
v) Required sulfhydryl reagents for activity
vi) Activities inhibited by Triton X-100

PME - phosphatidylmonomethylethanolamine
PDE - phosphatidyldimethylethanolamine

enzyme, was insensitive to sulfhydryl reagents and was not capable of methylating foreign sulfhydryl compounds. The evidence indicates that the soluble bacterial enzyme is not the same as the enzyme found in rat liver.

3. Other Pathways for Phosphatidylcholine Biosynthesis

Phosphatidylcholine in lung and liver is synthesized mainly by the de novo and the N-methylation pathways. However, these two pathways which preferentially synthesize 1-saturated-2-unsaturated PCs (see previous sections) do not fulfill the requirements of the lung for disaturated PC's. Two "remodelling" pathways in lung have been demonstrated which convert 1-saturated, 2-unsaturated PC's into disaturated species. Another pathway for PC biosynthesis involved the Ca^{++}-dependent exchange of phospholipid headgroups with free nitrogen bases. The remodelling pathways and base exchange reactions are briefly discussed below.

The substrates for the remodelling reactions are 1-acyl-LPC which are formed by the action of phospholipases. One pathway, first described in rat liver by Marinetti (58), required two lysolecithins but not fatty acids or CoA. A saturated fatty acid from LPC is transacylated to the second position of another molecule to form a disaturated PC. This reaction was studied by Abe and Akino (59,60) and they found higher activities in lung than in other organs. However, the relative importance of this pathway is in dispute since others (61) have found it to be of minor importance.

The acylation of LPC by acyl-CoA was first demonstrated by Lands (62) and was subsequently studied in lung by Webster (63). Saturated fatty acids from acyl-CoAs are used to acylate 1-saturated PC to form disaturated species.

In mammalian brain, choline and ethanolamine can be incorporated into phospholipids directly by base exchange (64,65). This reaction requires Ca^{++} but does not utilize energy and does not result in net synthesis of phospholipids. Recent studies suggest that base exchange is a very fast reaction and may be important in vivo (66). In liver, base exchange appears to be unimportant for the synthesis of PC. Experiments have shown that the rate of de novo biosynthesis of PC is at least 20 times faster than base exchange (67).

III. Regulation of the Rate of Phosphatidylcholine Biosynthesis

The control of the rate of the de novo and the N-methylation pathways in liver and to a lesser extent in lung, is briefly discussed below. The rate-limiting enzyme of each pathway as well as the effects of various hormones and chemicals on the rate of PC biosythesis are also discussed.

1. De Novo Pathway

The regulation of PC biosynthesis in rat liver during development was studied by Weinhold and co-workers (68). They found that the concentration of phosholipids in fetal liver increased after birth and attained adult values 22 days later. Studies with liver slices showed that the incorporation of ^{32}P into fetal liver was low but reached adult values 5 days after birth. In a subsequent study (69), the activities of the enzymes in the de novo pathway were measured and the developmental pattern of cytidylyltransferase activity was the only one found to closely parallel the pattern of labelled choline incorporation into tissue slices. The developmental studies suggested that the cytidylyltransferase may play a rate limiting and regulatory role in the pathway. This idea was further reinforced by results from studies with rat hepatocytes. Sundler and Akesson (7) found that by increasing the concentration of choline in the

culture medium, they were able to saturate the capacity of the de novo pathway. The synthesis of PC was stimulated several fold at low concentrations of choline. At higher concentrations, however, choline and phosphocholine accumulated in the cells while CDP-choline concentration as well as PC synthesis remained unaltered.

The belief that the cytidylyltransferase was the only rate-limiting enzyme of the pathway is not shared by all workers. By a mathematical analysis of the pathway based on "the logic derived from theoretical principles of metabolic regulation", Infante (70) showed theoretically that both choline kinase and phosphocholine cytidylyltransferase were potentially rate-limiting. Furthermore, a quantitative determination of the disequilibrium ratio for the reactions catalyzed by both enzymes showed that the choline kinase step was forty times "more rate-limiting" than the cytidylyltransferase step. Evidence that the activity of choline kinase can change the flux of molecules through the pathway in vivo was subsequently presented by Infante and Kinsella (71). In rats deprived of essential fatty acids, the pool size of the $C_{18:2}$ and $C_{20:4}$ PC were decreased while the level of $C_{16:1}$ and $C_{18:1}$ PC's were elevated (72). The authors calculated the rate of PC biosynthesis, by a method which was not clearly explained, and found it to be 3.8 times higher than normal. The activities of choline kinase and the cytidylyltransferase were measured in normal and deficient rats. The activity of choline kinase was increased 3.5-fold while the cytidylyltransferase activity was unchanged in the deficient animals. The authors concluded that choline kinase must regulate the rate of de novo PC biosynthesis at least during essential fatty acid deficiency.

Studies by Schneider and Vance (73) showed that the activities of choline kinase and phosphocholine transferase were unchanged during choline deficiency but the cytidylyltransferase activity was reduced about half. Although the rate of PC biosynthesis was not measured in choline deficient rats, the results further indicate that the cytidylyltransferase may play a regulatory role in the pathway. Further work by Choy et al. (74) showed that the amount of immunotitratable enzyme in normal and choline deficient rats was the same. The results suggested that the decrease in cytidylyltransferase activity was not due to an adaptive decline in enzyme quantity. The various results presented above indicate that the phosphocholine cytidylyltransferase, and perhaps choline kinase, regulate the rate of de novo PC synthesis. The results presented in this thesis show that the activity of the cytidylyltransferase regulates the rate of PC biosynthesis in the livers from hypercholesterolemic rats.

The effects of hormones, n-alkanes, ethanol and phenobarbital on the rate of biosynthesis have been investigated. In propylthiouracil-induced hypothyroid chicks, the specific activity of phosphocholinetransferase in the liver declined to about 10% of controls after 15 days of treatment and the transferase activity was increased to normal values 2 days after administration of L-thyroxine (75). Other hormones, however, inhibited phosphocholinetransferase activity. Administration of estradiol to castrated and normal rats decreased the specific activity of the transferase in both groups but stimulated PE methyltransferase activity (76). Prostaglandins also affect PC synthesis in rat liver. Prostaglandin E_1, administered intraperitoneally for 4 weeks stimulated the incorporation of ^{32}P into liver PC (77).

Non-physiological agents also affect PC synthesis. In an in vitro system consisting of rat liver cytosol and/or microsomes, several n-alkanes, such as n-hexane and n-octane, stimulated the incorporation of labelled choline into PC (78) and the added organic solvents were found to stimulate the reaction catalyzed by the cytidylyltransferase. Feeding of rats with 5% ethanol for two weeks stimulated phosphocholinetransferase activity (79).

The literature on the effect of phenobarbital on PC biosynthesis is somewhat confusing. Earlier studies (80,81) showed that chronic administration of the anaesthetic in rats stimulated the incorporation of ^{32}P into hepatic PC's and this was associated with proliferation of endoplasmic reticulum. Results from a recent study (82), however, indicated that the increase in PC was due to a decrease in breakdown rather than an increase in synthesis. The amount of glycerophosphorylcholine which is one of the breakdown products of PC was decreased by 50% in the treated animals. The effects of development, various diets, hormones and other substances on liver PC biosynthesis are summarized in Table 15.

The interest in the production of surfactant has stimulated research into the regulation of PC biosynthesis in lung. Developmental studies of Weinhold (68) showed that the amount of PC in 20-day old rat fetuses were 50% of adult values while newborns had as much lung phospholipids as adults. A subsequent study (83) showed that the incorporation of labelled choline into lung slices from 19-day old fetuses was low but increased to adult values in 22-day old fetuses. A different pattern, however, was observed for the incorporation of radioactivity into water-soluble precursors. Incorporation into phosphocholine was high in 19-day old fetuses but decreased in older fetuses. The specific radioactivity of

Table 15

The Effect of Development, Diet, Hormones and Other Compounds on the Biosynthetic Enzymes and the Incorporation of Labelled Choline into Phosphatidylcholine in Rat Liver and Lung

Stimuli	Effect on the Incorporation of ^{32}P or labelled choline into phosphatidylcholine	Effect on the Activities of the Biosynthetic Enzymes
Development of Rat Lung		
Weinhold et al. (69)		
perinatal period	Increased after birth	Only the activity of phosphocholine cytidylyltransferase followed the changes observed with incorporation of labelled choline
Liver		
Essential Fatty Acid Deficiency Kinsella and Infante (71)	Calculated 3.8-fold increase	Activity of choline kinase was increased 3.5-fold
Choline Deficiency Schneider and Vance (73)		Activity of phosphocholine cytidylyltransferase was halved
Estradiol Treatment of Male Rats Young (76)		Phosphocholinetransferase activity was decreased
Administration of Prostaglandin E Horiuchi (77)	Increased	
Addition of n-Alkanes to an In Vitro System Takagi (78)	2 to 3-fold increase	Formation of CDP-choline was stimulated
Ethanol Feeding Uthus et al. (79)		Activity of Phosphocholinetransferase was increased

phosphocholine, however, were the same in fetal and adult lungs. The results suggested that the increase in labelled choline incorporation into lung PC between day 19 and 22 of gestation was due to a stimulation of the enzymatic reactions between phosphocholine and PC. Thus, the cytidylyltransferase during development was investigated (23). Cytidylyltransferase activity in fetal lung was about a quarter of the adult activity when assayed under normal condition. However, addition of phospholipids stimulated the fetal enzyme to values comparable to that found in adult lung. Two forms of the cytidylyltransferase were found in cytosol: a high molecular weight form which does not require addition of phospholipid for maximum activity and a low molecular weight form which required addition of phospholipids. The fetal enzyme existed predominantly in the low molecular weight form while the high molecular weight form was predominant in adult lung. The developmental pattern of choline incorporation into PC in lung slices correlated with the pattern of cytidylyltransferasee activity. However, the rise in enzyme activity could not totally account for the more rapid incorporation: the rate of choline incorporation in 1-day old fetuses increased to adult values while the cytidylyltransferase activity remained at 40% of adult values.

The synthesis of PC in lung during the early post-natal period was also studied by Chida et al. (84). The incorporation of labelled choline into PC as well as the activities of the enzyme of the de novo pathway were measured in minced lung from rats at various stages of development. They found that the developmental pattern of cytidylyltransferase activity was different from the other two enzymes. The activities of choline kinase and phosphocholinetransferase were high during the prenatal and newborn period while the activity of the cytidylyltransferase in the same period was low.

But, more significantly, these workers found that the developmental pattern of choline incorporation was similar to patterns of cytidylyltransferase activity and both reached maximum values five days after birth. These results further support the idea that the cytidylyltransferase catalyzed the rate-limiting step of the pathway.

The conversion of the cytidylyltransferase from the low molecular weight form in fetal lung to the high molecular weight form in adult lung was investigated (24). The stimulation of the enzyme by various phospholipids were tested and compared to the stimulation by total lung lipid and only PG was as active in stimulating the enzyme. In contrast, PS and PI were only half as effective as total lung lipid in activating the enzyme. The effect of PG on the cytidylyltransferase is also unique because only this lipid aggregated the enzyme. The workers speculated that the activity of the cytidylyltransferase, and therefore the rate of PC synthesis, may be coupled with the level of PG in lung. Interestingly, the level of glycerol phosphatide increased coincidentally during the later stages of lung development.

The production of lung surfactant was stimulated by various hormones (85). For studies with fetal lung from various animals, glucocorticoid and estrogen administration increased the amount of PC in lung by at least 2-fold and incorporation of labelled choline into PC in lung slices was also increased to the same extent. Phosphocholine cytidylyltransferase activity measured <u>in vitro</u> was also increased when experimental animals were treated with hormones but the increase was not as extensive as the elevation in lung PC and the incorporation of labelled choline.

As mentioned earlier, the activity of phosphocholine cytidylyltransferase was stimulated by PG and, significantly, the synthesis of this lipid was also stimulated by glucocorticoids and estrogen. The activities of the cytidylyltransferase from control and hormone treated animals were similar when assayed in the presence of PG suggesting that the elevated levels of this lipid may be responsible for the stimulation of the cytidylyltransferase. The evidence indicates that PG may play a regulatory role in the control of PC synthesis in the lung.

Results from studies in lung and liver strongly support the hypothesis that the cytidylyltransferase is regulatory and rate limiting in the de novo synthesis of PC. The concentrations of intracellular phosphocholine and CDP-choline are consistent with this hypothesis. The concentration of CDP-choline is twenty times lower than the concentration of phosphocholine (Table 1) in rat liver and the availability of CDP-choline appears to be the rate limiting factor in PC synthesis

2. Phosphatidylethanolamine N-Methylation Pathway

The regulation of the rate of conversion of PE to PC has not been investigated as extensively as the regulation of de novo PC biosynthesis. However, studies in rats and yeast have yielded some important insights.

Early studies of Greenberg (39) suggested that two enzymes are involved in the pathway and that PE methyltransferase, the first enzyme of the pathway, appear to be rate limiting. These observations have now been confirmed by other workers.

The activities of the methyltransferases were affected by hormones and diet. Administration of estradiol to normal and castrated rats increased PE methyltransferase activity (76). In dietary studies, incorporation of

methyl labelled S-adenosylmethionine *in vivo* into phospholipids was stimulated in rats fed a choline deficient diet (86) and the activity of PE methyltransferase was increased in the microsomes (73,87).

Regulation of N-methyltransferases has been studied in *Saccharomyces cerevisiae* (88). The presence of N-methylethanolamine, N,N-dimethylethanolamine or choline in the growth medium resulted in reduced activities of all the methyltransferase activities involved in the formation of that particular phosphatidyl ester. The activity of the last methyltransferase for instance, was 90% lower in cells grown with sufficient choline than in cells grown in choline deficient medium. Cells grown in medium supplemented with any of the three methylated aminoethanols also resulted in increased cellular levels of the corresponding phosphatidyl esters and diminished amounts of precursor phosphatidyl esters.

The mechanisms by which the methyltransferase activities are regulated in yeast and rats remain to be investigated.

IV. Mechanisms of Regulation of the Rate of Phosphatidylcholine Biosynthesis

Studies in rat lung, liver and in various other systems strongly indicated that phosphocholine cytidylyltransferase is rate limiting as well as regulatory in the control of *de novo* PC biosynthesis. Two mechanisms by which the activity of the cytidylyltransferase may be regulated have emerged from these studies. As described in a previous section, PG appear to be important in regulating PC biosynthesis during lung development by modulating the activity of the cytidylyltransferase. A second mechanism was elucidated by Vance *et al.* in studies with HeLa cells. Infection of the cells with Polio virus stimulated the incorporation of labelled choline into PC and the reaction catalyzed by phosphocholine cytidylyltransferase, which is rate limiting in HeLa cells, was found to be stimulated (89). A

subsequent study by Choy *et al.* (90) showed that an increase in cytosolic CTP concentration in Polio virus-infected cells was responsible for the stimulation of *in vivo* activity of the cytidylyltransferase which in turn resulted in a higher rate of PC biosynthesis. The concentration of CTP in cytosol under normal circumstances appear to be below the Km of the enzyme for CTP and a 2- to 3-fold increase in cytosolic CTP would result in a proportional increase in enzyme activity. The control of PC biosynthesis by changes in the levels of the biosynthetic enzymes has not been demonstrated. The amount of cytidylyltransferase has been shown to be unchanged during choline deficiency, where enzyme activity is decreased. The amount of immunoprecipitable enzyme in liver cytosols from choline deficient rats were unchanged even though the activity of the cytidylyltransferase was half the control value (73). Thus, the presence of a lipid modulator or alteration of *in vivo* levels of substrate appear to be two mechanisms by which the rate of PC biosynthesis may be regulated.

V. Hepatic Phosphatidylcholine Biosynthesis and Hypercholesterolemia

A relationship between the level of plasma cholesterol and phospholipids has been observed in humans and in various animals. In normal persons, the ratio of serum cholesterol to serum phopspholipids is essentially constant. Peters and Man (91) found that lipid phosphorus increased in a linear fashion with cholesterol in serum when serum cholesterol concentration exceeded 100 mg/100 ml. A similar relationship exists in swine and rats. Cholesterol feeding of miniature swine (92) resulted in a 4-fold increase in plasma cholesterol and an almost 3-fold increase in plasma phospholipids. The hypercholesterolemia was also associated with hyperlipoproteinemia and the experimental animals subsequently developed atherosclerosis. In rats fed a diet with high

levels of cholesterol, cholate and propylthiouracil, plasma cholesterol was very dramatically increased while plasma phospholipid was also elevated (93).

The relationship between plasma cholate, phospholipids, and cholesterol in rats was studied by Friedman and Byers (94). They found that after biliary obstruction, the level of plasma cholate was increased and this was followed by a rise in plasma phospholipid which in turn was followed by an increase in plasma cholesterol. Intravenous infusion of cholate resulted in increased levels of plasma phospholipids and cholesterol while injection of phospholipids led to an elevation of plasma cholesterol only. The results suggested that during biliary obstruction, elevated levels of cholate, rather than cholesterol, was responsible for the increase in plasma phospholipids and this may also be the case during diet-induced hypercholesterolemia.

The increase in plasma phospholipids during cholesterol feeding could be explained in a number of ways. First, there could be decreased degradation of plasma phospholipids or, secondly, phospholipid synthesis could be stimulated in the liver which is a major site of lipoprotein synthesis. Is the rate of PC synthesis stimulated in the livers from hypercholesterolemic animals? And if it is, which enzymes are involved? What are the regulatory mechanisms? The hepatic synthesis of PC in normal and hypercholesterolemic rats was investigated and the results are reported here.

The level of plasma cholesterol in adult rats was difficult to manipulate. Thus, young rats whose plasma cholesterol levels could be more readily altered (95), were used to study the effects of cholesterol/cholate feeding on hepatic PC biosynthesis.

The results presented in this thesis consist of two major components. The first segment deals with the *in vitro* studies of the enzymes of PC biosynthesis, with particular emphasis on the cytidylyltransferase. The second part deals with the *in vivo* rate of PC biosynthesis in livers from normal and cholesterol/cholate fed rats.

MATERIALS AND METHODS

I. Animals, Enzymes, Chemicals and Radioactive Compounds

1. Animals

Animals were purchased from the University of British Columbia Animal Unit. Female Wistar rats, 18 days old, weighing 30-35 g, were used in all feeding experiments while older female rats weighing 150 to 250 g were used for preparing phosphocholine cytidylyltransferase and total rat liver phospholipids. New Zealand buck rabbits were used to raise antibodies against the cytidylyltransferase.

2. Enzymes, Chemicals and Radioactive Compounds

The following companies supplied the enzymes, chemicals, radioactive compounds and other materials listed below. All other chemicals used were of reagent grade.

Ralston Purina Company

Purina Rat Chow and Rabbit Chow

ICN Pharmaceuticals, Inc.

Special basal atherogenic diet, modified and choline deficient diet supplemented with 4% choline (used as control diet)

Matheson, Coleman, and Bell

Cholesterol and Cholate

Sigma Chemical Company

Bis(trimethylsilyl)-tri-fluoroacetamide (BSTFA), choline chloride, choline iodide, phosphocholine, Tris, ATP, CTP, CDP, CMP, GTP, GDP, GMP, UTP, UDP, UMP, NAD, NADH, NADP, NADPH, acetyl-CoA, stearoyl-CoA, malonyl-CoA, palmitoyl-CoA, standard even numbered fatty acids, egg PC, PE, PS, synthetic palmitoyl-LPE, bovine serum albumin, alkaline phosphatase, phospholipase c from B. cereus and Cl. perfringens, trypsin and trypsin inhibitor, glycerol dehydrogenase, choline kinase, pyrophosphatase.

Serdary Research Laboratories

CDP-choline, synthetic palmitoyl-LPC, pig liver PC, pig brain PE, pig liver PI, pig brain SM, pig liver LPE, synthetic palmitoyl- and oleoyl-LPE.

Difco Laboratories

Complete and incomplete Freund's adjuvant.

Pharmacia

Sepharose 6B.

Mallincrodt Company

Chromar plates, 7 GF, 20 cm x 20 cm precoated glass plates, 0.25 mm layer thickness

Macherey-Nagel

Precoated TLC plates, Sil G-25, 0.25 mm layer without gypsum.

E. Merck Laboratories Inc.

Precoated TLC plates, Silica Gel 60 (without fluorescence indicator), 0.25 mm layer thickness.

Amersham/Searle

[Me-^{3}H]choline chloride

New England Nuclear Canada

[1,2-^{14}C]choline chloride, [Me-^{14}C]cytidine diphosphocholine, [1,2-^{14}C]ethanolamine hydrochloride, S-[Me-^{3}H]adenosyl-L-methionine

ICN, Chemical and Radioisotope Division

[5-^{3}H]cytidine 5'-triphosphate, tetrasodium salt.

II. Preparative Procedures

1. Isolation of Total Rat Liver Phospholipid

The method of Folch et al. (97) was used. Approximately 20 g of liver obtained from 2 female rats, each weighing about 220 g, were homogenized in 400 ml of CH_3OH in a Thomas tissue grinder, with Teflon pestle. In a 3 l flask, 800 ml of $CHCl_3$ were added to the homogenate and the mixture was agitated extensively. The extract was then filtered and the filtrate was collected in a 1 l separating funnel. To the organic phase, 250 ml of 0.145 M NaCl were added and the two-phase solution was vigorously extracted. The phases were allowed to separate and the lower organic phase was drained into a preweighed 1 l round bottom flask. The solvent was evaporated under reduced pressure and the dry lipid residue was washed 3 times with 50 ml of ice-cold acetone. The acetone wash, which contained mostly neutral lipids, was discarded and the residue was dissolved in chloroform at a concentration of 20 to 30 mg/ml. This extraction procedure yielded 27 mg of phospholipid per g liver.

2. Preparation of Partially Purified Phosphocholine Cytidylyltransferase

The enzyme was enriched from fresh 20% (w/v) rat liver cytosol by passage of 5 ml of cytosol through a 200 ml Sepharose 6B column. The fractions in the included volume were assayed for enzyme activity in the presence of 5 mg/ml rat liver phospholipid. Ten of the fractions that contained the highest enzyme activity were pooled and concentrated to 2 ml by ultrafiltration with an Amicon XM-100 membrane filter.

In later experiments where a much more active enzyme preparation was required the enzyme was first precipitated from cytosol by $(NH_4)_2SO_4$ (25 to 50% fraction; w/v). The precipitated enzyme was redissolved in 5 ml

of column buffer and chromatographed as described above. The four fractions that contained the most activity were pooled and used immediately or stored in small aliquots at -70°C.

3. Delipidation of Phosphocholine Cytidylyltransferase by Extraction with Acetone and Butanol

The extraction procedure was first described by Fiscus and Schneider (20). The acetone and butanol used in the procedure were cooled in an ethanol-dry ice bath before use. Briefly, 50 ml of freshly prepared rat liver cytosol were first extracted with 4 volumes of cold acetone and the precipitated proteins were collected by centrifugation. The extraction procedure was repeated but cold butanol was used and the protein pellet from the final extraction was purged of butanol in a lyophilizer. By this procedure, 14 mg of precipitate was obtained per ml cytosol while Fiscus and Schneider obtained 23 mg of precipitate per ml cytosol.

The enzyme was stored as a solid at -20°C and when required 50 mg of the precipitate was added to 1 ml of distilled water in a small test tube. The mixture was vigorously vortex mixed and any undissolved particles were sedimented by centrifugation. The brownish supernatant contained phosphocholine cytidylyltransferase which was active only in the presence of phospholipids. This preparation was used as the source of enzyme in some experiments.

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

Commercially available [Me-^{3}H]choline was converted to [Me-^{3}H]phosphocholine by the action of choline kinase. In each preparation, 0.24 µmol of [Me-^{3}H]choline (8.3 mCi/µmol) was treated with 0.2 units of choline kinase (1 unit will catalyze the phosphorylation of 1.0 µmole of choline per min at pH 8.5 at 25°C) in the presence of 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 10 mM ATP in a final volume of 0.25

ml. The reaction was incubated at 37°C for 1 h, and then boiled for two min. The mixture was spotted on a 2.5 cm lane on a Brinkman Sil G-25 TLC plate. A small amount of the reaction mixture was spotted on an adjacent lane and the plate was developed in CH_3OH/0.6% NaCl/25% NH_4OH (50/50/5; v/v/v). The mobility of the radioactive choline and phosphocholine was determined by measuring the amount of radioactivity in 1 cm sections of the lane that had a small amount of radioactivity. The Rf's for choline and phosphocholine are 0.10 and 0.33, respectively. The [Me-^{3}H]phosphocholine was eluted from the silica with 10 ml of distilled water and the yield of the procedure varied between 70 and 90%.

5. Preparation of [1,2-ethanolamine-^{14}C]Phosphatidylethanolamine and Labelled Phosphatidylethanolamine-Phospholipid Vesicles.

Radioactively-labelled PE was made by incubating 75 x 10^6 freshly isolated hepatocytes (98), maintained in monolayer culture, with 0.013 mmol of [1,2-^{14}C]ethanolamine (3.85 mCi/mmol) for 24 h at 37°C. The cells were subsequently harvested and extracted by the Bligh and Dyer procedure (99). Labelled PE was isolated by thin-layer chromatography with $CHCl_3/CH_3OH/H_2O$ (70/30/4; v/v/v) as solvent. Of the 50 µmCi of radioactivity added, 4.9% of the label was incorporated into PE and 2.5% into PC. The labelled PE had a specific radioactivity of 1.75 µCi/mmol.

Radioactive PE was suspended in solution as phosphatidylethanolamine-phospholipid mixed vesicles which were prepared with 0.5 mg of [1,2-ethanolamine-^{14}C]phosphatidylethanolamine (1.75 µmCi/mmol) and 0.5 mg of total rat liver phospholipid in 10 ml of saline. The mixture was sonicated at 0°C, two times with a Quigley-Rochester Sonic Dismembrator for 1 min at a setting of 80. The sonicated vesicles were used within 2 hr after preparation.

6. Preparation of Buffers

A systematic study of a variety of buffers has been reported (100) and procedures for preparing buffers have also been published (101). Tris-HCl was used for buffering solutions between pH 7.0 and 9.0 while Tris succinate was used between pH 6.0 and 7.0. Buffers were prepared starting with a concentrated solution of Tris and the solution was titrated with 1 N HCl or 0.2 M succinic acid. When the desired pH was obtained, the buffer solution was diluted to the appropriate concentration with distilled water and the pH of the final buffer solution was measured and adjusted accordingly if necessary.

III. General Procedures

1. Measurement of Protein

Protein content was determined by the procedure of Lowry et al. (102). Approximately 60 µg of protein (10 µl of cytosol and 5 µl microsomes) were added to 0.66 N NaOH to make up a final volume of 1.5 ml and samples that contained lipid were first incubated at 37°C overnight before use.

The following stock solutions were prepared:

Solution A

100 ml of 13% (w/v) Na_2CO_3

3 ml of 4% (w/v) $NaKC_4H_4O_6\ 4H_2O$

3 ml of 2% (w/v) Cu_2SO_4

To the sample in 0.66 N NaOH, 1.5 ml of solution A was added and mixed. After 10 min, 0.5 ml of 2 N phenol reagent solution was added and the mixture was again mixed. Absorbance of the solution at 625 nm was measured 30 to 60 min later. A standard curve was generated by using 10 to 100 µg of fatty acid free bovine serum albumin and buffer, distilled water or saline was used as blank.

2. Measurement of Phosphorus in Phospholipids

A rapid colorimetric method that does not involve acid digestion of phospholipid was used (103). To the dry lipid samples contained in test tubes, 0.4 ml of chloroform and 0.1 ml of chromogenic solution were added. The chromogenic solution contained molybdate, mercury, sulfuric and hydrochloric acids. The tubes were placed in boiling water for 80 sec and then cooled at room temperature for 30 min. The chromogen was diluted with 5 ml of $CHCl_3$, the mixture agitated, and the absorbance of the $CHCl_3$ at 710 nm was measured immediately. A stock solution of 0.237 mg per ml $CHCl_3$ (equivalent to 10 µg lipid phosphorus per ml) of dipalmitoyl-PC was used to generate a standard curve which was linear from 1 to 10 µg of lipid phosphorus.

3. Liquid Scintillation Counting

Aqueous radioactive samples were counted in 10 ml of ACS. Samples in 0.1 N NaOH were first acidified with 0.1 ml of glacial acetic acid. With lipid samples, the solvent was first evaporated before scintillation counting in 10 ml of a toluene based scintillant containing PPO (4 g/l and POPOP (50 mg/l).

Radioactive samples on TLC plates were scraped with the silica into scintillation vials and 1 ml of water was added to deactivate the silica. The samples were counted in 10 ml of ACS with recovery ranging from 95% to 100% for water soluble samples and 78% to 83% for lipid samples.

All liquid scintillation counting was done in an ISOCAP/300 counter and counting efficiencies were determined by either channels ratio or external standard ratio. Each set of samples was counted with quenched standard of either ^{3}H-hexadecane in ACS or ^{14}C-toluene in toluene, PPO, POPOP

4. Thin-Layer Chromatography

(a) Solvents for Water-Soluble Compounds

Various solvent systems were used to separate choline, phosphocholine, CDP-choline and betaine (167). Machery-Nagel Sil G-25 or chromar plates were used. When only one solvent system was used, samples and standards were applied as a thin band, 2.5 cm long and 2 cm above the bottom edge of the plate. In two dimensional chromatography, one sample was applied as a small spot at the bottom right hand corner of the plate and 2 cm from each edge. The plate was developed in the first solvent from bottom to top, and after air drying overnight, the plate was developed from right to left with the second system.

In the *in vivo* studies where the amount of radioactivity incorporated into water-soluble pools was measured, 0.20 mg of choline, 0.75 mg of phosphocholine, 0.30 mg of CDP-choline and/or 0.30 mg of betaine were chromatographed with the samples. The carriers increased the recovery and resolution and also facilitated visualization of each compound. When chromar plates were used, the amount of CDP-choline carrier was reduced to 0.10 mg. CDP-choline was visualized under U.V. while the other compounds were visualized by iodine staining.

i) Single Dimensional Systems

CH_3OH/0.6% NaCl/27% NH_4OH (50/50/5; v/v/v)

Choline Rf 0.10, phosphocholine Rf 0.33, betaine and CDP-choline Rf 0.66.

This system was mainly used in the phosphocholine cytidylyltransferase assay as a means to separate radioactive CDP-choline from labelled phosphocholine. This system was also used to separate labelled phosphocholine from choline during the preparation of [*Me*-^{3}H]phosphocholine.

ii) Two Dimensional Systems

System I: As in III.4.a.i

System II: CH_3COCH_3/CH_3OH/concentrated HCl (10/90/4/; v/v/v)

Choline Rf 0.31, phosphocholine Rf 0.39, CDP-choline Rf 0.21, betaine Rf 0.49.

All four compounds were completely resolved by this two dimensional system.

(b) Solvents for Lipids

Single dimensional TLC was routinely used to separate the major classes of phospholipids. A more complete resolution of the different classes was achieved by two dimensional TLC (104,105). Silica gel 60 plates from Merck were heat activated at 110°C for 1 h before use and samples were spotted on TLC plates according to the procedure described above (Section III.4.a.)

i) Single Dimensional Systems

System I (neutral): $CHCl_3/CH_3OH/H_2O$ (70/30/4; v/v/v)

System II (acid): $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (100/60/16/8, v/v/v/v).

The mobilities of the different phospholipids are given in Table 16.

ii) Two Dimensional Systems

System III : Basic $CHCl_3/CH_3OH/40\%\ CH_3NH_2$ (13/6/1.5; v/v/v)

Acid $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ (10/4/2/3/1; v/v/v/v)

System IV: Basic $CHCl_3/CH_3OH/25\%\ NH_4OH/H_2O$ (90/54/5.7/5.3; v/v/v/v)

Acid $CHCL_3/CH_3OH/CH_3COOH/H_2O$ (60/30/8/2.85; v/v/v/v)

With system III, after developing in the basic solvent, the plate was air-dried and acidified with HCl fumes before developing the plate in the acid system. The mobilities of phospholipids for both systems are given in Table 16.

5. Column Chromatography

All preparations of cytosol were fractionated in a 200 ml Sepharose 6B column which had been equilibrated with 20 mM Tris, pH 7.0 buffer containing 100 mM NaCl. Enzyme grade sucrose was dissolved in each sample at a concentration of 0.2 g/ml and the dense solution was carefully layered on top of the gel bed. The column flow rate was about 50 ml/h and 8 ml fractions were collected. The column was washed with buffer equivalent to five times the bed volume of the column after each run.

IV. Enzyme Assays

1. Choline Kinase

Choline kinase was assayed according to the procedure of Weinhold and Rethy (12). The assay cocktail consisted of the following:

150 µl 0.1 M $MgCl_2$

100 µl 1.0 M Tris-HCl pH 8.0

20 µl 25 mM [Me-^{3}H]choline chloride, 1 µCi/µmole

150 µl 100 mM ATP

100 µl enzyme

480 µl distilled H_2O

Table 16

Mobilities of Phospholipids on Thin-Layer Plates Developed in Various Solvent Systems

	Solvent Systems					
	I	II	III		IV	
	Neutral	Acid	Basic	Acid	Basic	Acid
Phospholipids	Mobilities (Rf)					
PC	0.22	0.28	0.57	0.31	0.54	0.35
PE	0.53	0.75	0.69	0.64	0.58	0.83
PS		smeared	0.27	0.56	0.39	0.50
PI		0.43			0.37	
LPC	0.08	0.09	0.32	0.08	0.22	0.07
LPE	0.26	0.43	0.59	0.22	0.39	0.32
SM				0.39	0.17	0.12

The 1 ml reaction was incubated at 37°C for 20 min and the tube immersed in boiling water for 2 min. The labelled phosphocholine produced was bound to a 3 x 0.5 cm column of AG1-X10 and labelled choline was washed from the column with 10 ml of water. The radioactive phosphocholine was eluted with 1 ml of 0.1 N NaOH and radioactivity was measured by scintillation counting.

The rate of the reaction was linear for at least 25 min with up to 1.4 mg of protein from both control and cholesterol/cholate-fed rat liver cytosol (Fig. 3). In routine measurements, choline kinase was assayed with 1.0 to 1.4 mg of protein for 10 min at 37°C and under these conditions, 21% of the substrate was converted to phosphocholine.

2. Phosphocholine Cytidylyltransferase

The assay used for measuring the cytidylyltransferase in cytosols and microsomes was the same (69) and consisted of the following components:

10 µl 10 or 40 mM CTP

10 µl 10 mM phosphocholine, 20 µCi/µmole

10 µl 1.0 M Tris-succinate, pH 6.0

10-40 µl enzyme

The final volume of the assay was made up to 0.1 ml with distilled water. When the enzyme was to be assayed in the presence of phospholipids, an appropriate amount was first added to the assay tubes and the solvent was evaporated under a stream of nitrogen. The enzyme and every other component of the assay except for the labelled phosphocholine were added and the phospholipid was emulsified into solution by agitation on a Vortex mixer. The mixture was incubated at 37°C for 3 min before starting the reaction with phosphocholine.

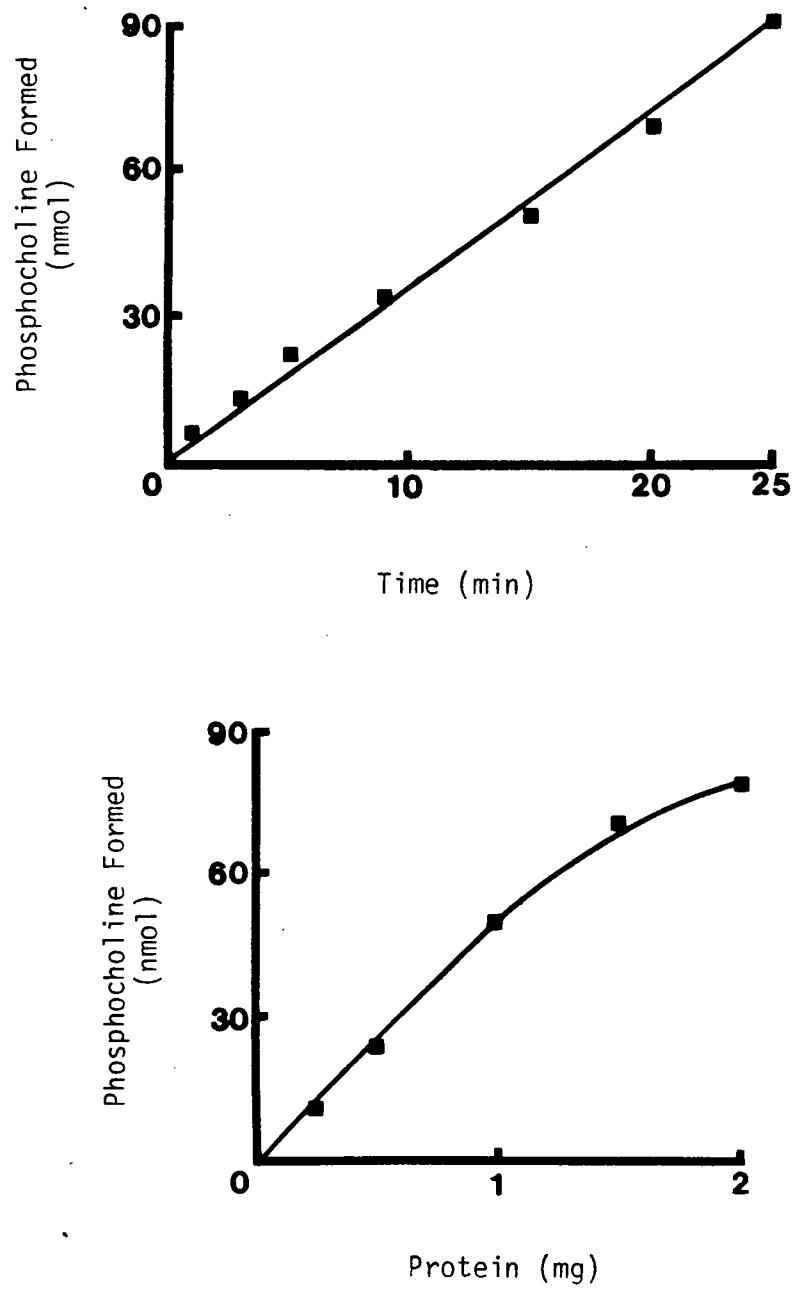


Figure 3. Choline Kinase Activity as a Function of Time and Protein. The enzyme was assayed according to the procedure given in the text. In the upper graph, 1.0 mg of protein was used in the assay and in the lower figure, the reaction was incubated at 37°C for 20 min. The figures show the results obtained with control cytosol. A similar result was obtained when experimental cytosol was used.

The reaction was incubated at 37°C for the appropriate time and the tubes immersed in boiling water for 2 min. The protein was pelleted by centrifugation and 60 µl of the supernatant along with CDP-choline carrier was subjected to TLC (see Section III.4.a.i). The amount of labelled CDP-choline produced was determined by liquid scintillation counting (see Section III.3). In routine assays, less than 5% of the substrate was converted.

The concentration of CTP in the assay varied depending upon the state of the enzyme. The purified and partially purified enzymes were measured in the presence of 1 mM CTP (23) whereas 1 or 4 mM CTP was used when the cytidylyltransferase was measured in cytosol.

(a) Cytosolic Activity

The rate of the reaction was linear with up to 260 µg of protein and up to 10 min incubation time for both control and cholesterol/cholate-fed rat liver cytosols (Fig. 4). Thus, the measurement of cytosolic cytidylyltransferase activity was carried out with 150 µg of protein for 10 min.

(b) Microsomal Activity

The activity of the cytidylyltransferase was linear with up to 240 µg of protein and up to 5 min incubation time (Fig. 5). Thus, 120 µg of protein and 3 min of incubation were routinely used in measuring enzyme activity in liver microsomes from control and cholesterol/cholate-fed rats.

3. Phosphocholinetransferase

Phosphocholinetransferase activity was measured according to the procedure of Vance and Burke (106). The reaction mixture contained the following:

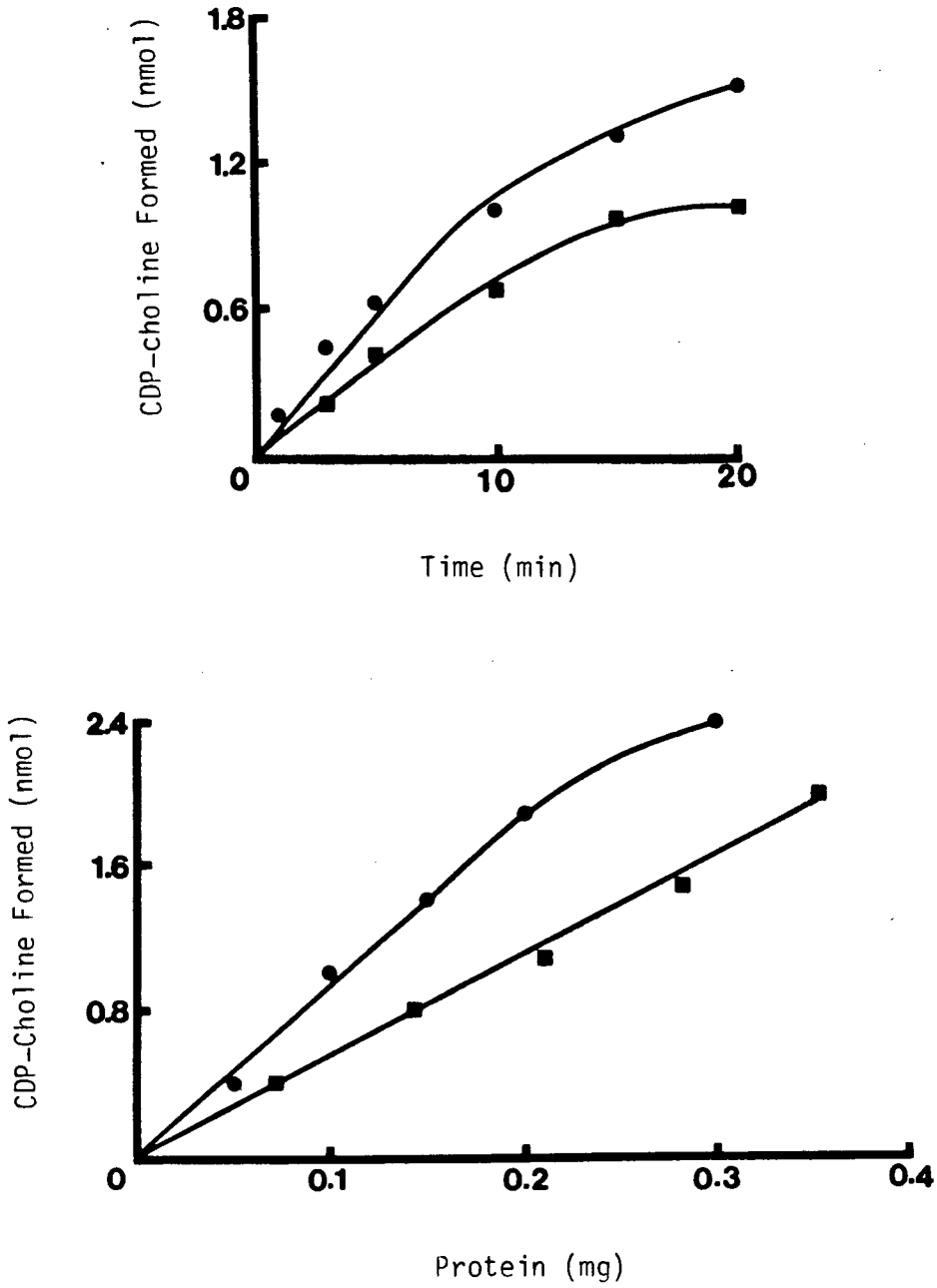


Figure 4. Activity of Cytosolic Phosphocholine Cytidylyltransferase With Time and Various Amounts of Protein. The cytidylyltransferase was assayed according to the procedure given in the text. In the upper figure, 100 µg of cytosolic protein was used and in the lower graph, the reaction was incubated for 10 min at 37°C for both control (■) and experimental (●) cytosols. Under the same conditions, control cytosol converted half as much phosphocholine to CDP-choline as the experimental cytosol.

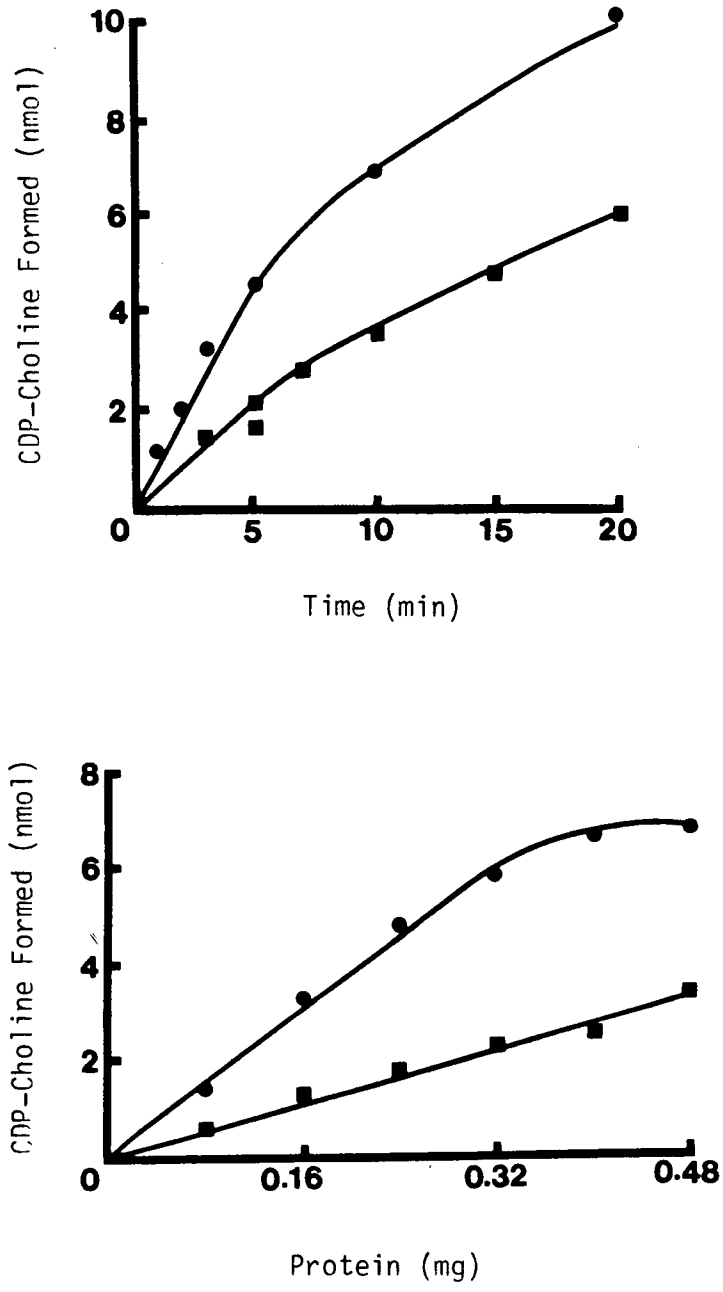


Figure 5. Activity of Microsomal Phosphocholine Cytidylyltransferase with Time and Various Amounts of Protein. The conditions used in the assay were as described in the text. In the upper graph, 240 µg of protein were used and in the lower figure, the reactions were incubated for 5 min at 37°C. The data presented were from experiments with microsomes from control (■) and cholesterol/cholate-fed rats (●).

20 µl 20 mM [Me-^{14}C]CDP choline (0.3 µCi/µmol)

40 µl 1 M Tris-HCl, pH 8.5

75 µl 0.1 M $MgCl_2$

20 µl microsomes

200 µl sonicated DG (1 mg/ml)

65 µl H_2O

The reaction mixture was preincubated for 5 min at 37°C and the assay was started by addition of labelled CDP-choline. Two ml of $CHCl_3/CH_3OH$ (1/1) was added to stop the reaction, followed by 1 ml of 0.9 NaCl and 1 ml of $CHCl_3$. The mixture was extracted and the organic phase was recovered and washed twice with 3 ml of theoretical upper phase. Finally, the bottom phase was quantitatively transferred into a scintillation vial, the solvents were evaporated under a stream of dry air and radioactivity was measured by scintillation counting.

The DG preparation contained 2 mg of DG, 60 µl of 1% Tween 20 and 1.94 ml of H_2O. The mixture was sonicated with a Quigley-Rochester Sonic Dismembrator, at a setting of 80, for two 3-min periods. The optimal ratio of Tween 20 to diglyceride was not determined.

The rate of the reaction was linear up to 80 µg of microsomal protein and up to 12 min of incubation (Fig. 6). The amount of DG used was optimal for the rate of the reaction (Fig. 7) and about 5% of the substrate was converted to PC.

4. Phosphatidylethanolamine Methyltransferase

This enzyme was assayed by the procedure of Schneider and Vance (73). The reaction contained:

50 µl 100 mM Tris-HCl, pH 9.2

50 µl microsomes

15 µl 0.6 mM S-[Me-^{3}H]adenosylmethionine, 100 µCi/µmole

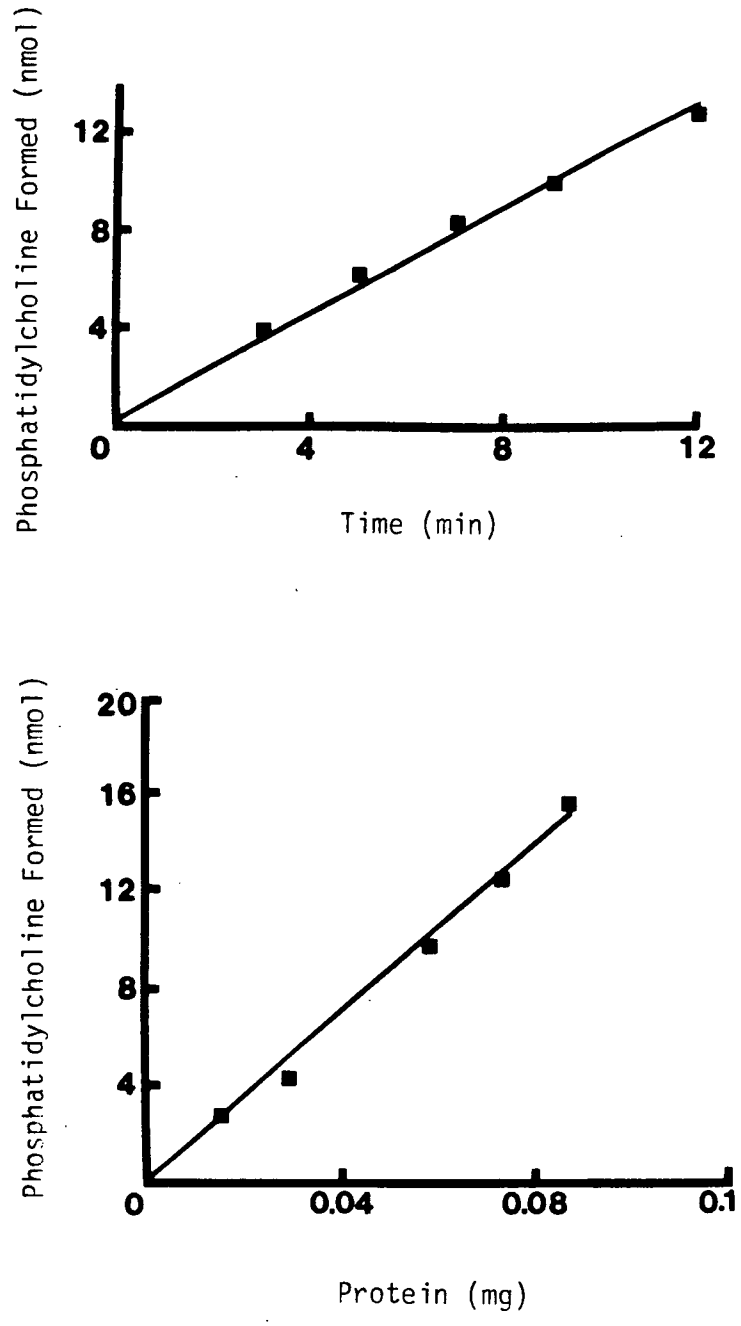


Figure 6. Activity of Phosphocholinetransferase with Time and Various Amounts of Proteins. The conditions used in the assays were described in the text. The results were obtained with control microsomes. In the top figure, 58 µg of microsomal protein were used and in the bottom graph the reactions were incubated for 10 min at 37°C. Similar results were obtained when microsomes from cholesterol/-cholate-fed rats were used.

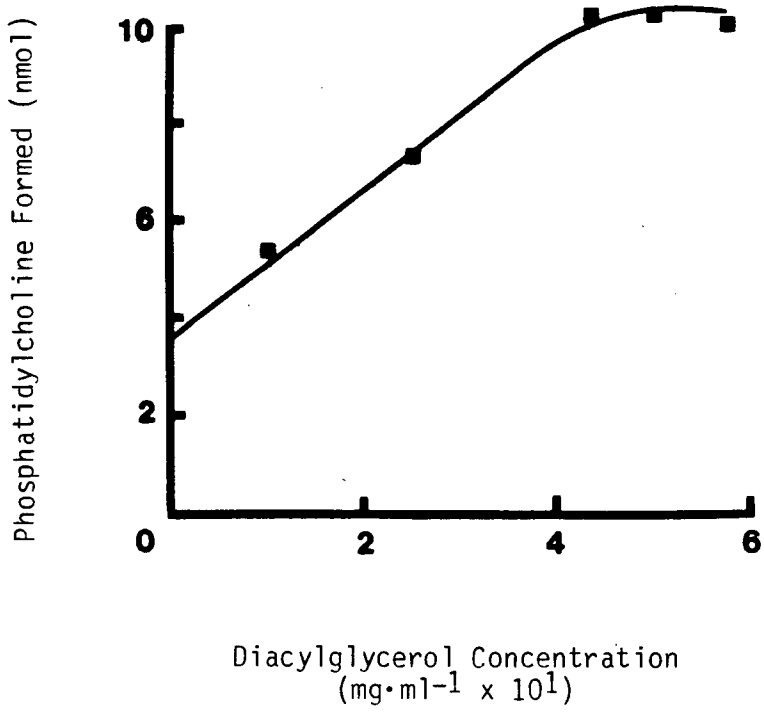


Figure 7. Activity of Phosphocholinetransferase in the Presence of Various Amounts of Diacylglycerols. The assay conditions are described in the text. The assay contained 58 µg of control microsomal protein and activity of the transferase was maximal when 175 µg of diacylglycerol were added to the reaction. Thus, in routine assays 200 µg of diacylglycerol were used.

The assay was incubated for 40 min and stopped with 2.1 ml of 0.1 N HCl. The mixture was extracted with 2 ml of butanol and 0.25 ml was used for scintillation counting. The rate of the reaction was linear up to 30 min. Since a significant proportion of the total incorporated radioactivity was associated with the monomethyl and dimethyl derivatives of PE, activity of the methyltransferase was therefore expressed as nanomoles of methyl groups transferred rather than nanomoles of PC formed.

V. Methods Used in In Vitro Studies

1. Care and Feeding of Experimental Animals

Female Wistar rats (18 day) weighing 30 to 35 g were maintained on Purina rat chow and tap water ad libitum in a light- and temperature-controlled room. Three days later, the diet of half the animals was altered to one containing 2% cholate, 5% cholesterol and 93% purina rat chow. The cholesterol and cholate content of this diet was the same as that found in other experimental diets (93) and the rats were maintained on their respective diets for six or nine days. This same protocol was used when other experimental diets were tested.

New Zealand buck rabbits weighing about 2 kg were maintained on Purina rabbit chow and tap water ad libitum.

2. Preparation of Experimental Diets

Purina rat chow pellets were ground into powder form by a Christie and Norris eight inch laboratory mill. The experimental diets were prepared by slowly adding the appropriate amount of cholesterol, cholate or cholesterol and cholate to the powdered rat chow in a food mixer set a medium speed. For example, 2 g of cholic acid and 5 g of cholesterol were added to 93 g of powdered chow. The experimental chows were mixed for another 15 min until a homogenous powder was obtained. The powder foods

were made solid by compressing in a food pelleter or alternatively, the chows were moistened with distilled water and dispensed into small food jars. The chows were compressed in the jars and dried overnight in a lyophilizer.

3. Measurement of Total Plasma Cholesterol and Phospholipid

A colorimetric method for measuring total plasma cholesterol developed by Zak was used (107). Briefly, 0.10 ml of plasma was added to 10 ml of ethanol. The mixture was centrifuged and 2 ml of the supernatant was reacted with 2.0 ml of colour reagent which contained ferric chloride, phosphoric acid and sulfuric acid. Thirty to sixty minutes later, the absorbance of the mixture at 550 nm was measured. Standards were used to generate a curve ranging between 0 and 1,000 mg cholesterol per 100 ml.

Plasma phospholipids were extracted from 0.20 ml of Plasma with 3 ml of $CHCl_3/CH_3OH$ (2/1, v/v) and precipitated proteins were removed by filtration through glass wool. Phospholipids were measured by estimating lipid phosphorus (see Section III.2).

4. Blood Sampling, Preparation of Cytosol and Microsomes and Perfusion of Rat Liver

Rats were decapitated and blood collected in a heparinized tube. The red and white blood cells were sedimented by centrifugation (2,000 x g) at 4°C and the plasma lipids were analyzed. The rat livers were quickly excised and placed in ice-cold 0.145 M NaCl. Previous studies have shown that the activity of the cytidylyltransferase in cytosol increases when stored at 4°C (22). Thus as a precautionary measure, a control rat was sacrificed first followed by an experimental rat and this sequence was repeated. Subsequently, 4 livers from control animals were homogenized in 4 volumes of ice-cold 0.145 M NaCl in a glass homogenizer with teflon

pestle and the experimental livers were treated identically. The homogenates were centrifuged at 10,000 x g for 30 min, and the supernatants were removed and centrifuged at 100,000 x g for 90 min. The high speed supernatant, excluding the upper layer of white lipid, was taken as cytosol and the microsomal pellet was resuspended by homogenization in 0.145 M NaCl (5 mg protein/ml). Both were immediately stored at -70°C.

The livers obtained by the above procedure contain a significant amount of blood. Since plasma lipids and proteins could affect the activities of the enzymes of PC biosynthesis, blood was washed out of the liver by perfusion in a few control experiments. The rats were killed by cervical dislocation and the livers were perfused *in situ* through the portal vein with 50 ml of ice-cold 0.145 NaCl. The perfused livers, which had a light pink appearance, were subsequently treated as normally isolated livers.

5: Measurement of Diacylglycerol in Cytosol

The concentration of DG in cytosol was measured according to the procedure of Choy *et al.* (26). The measurement basically involves the isolation and subsequent conversion of diglyceride to glycerol. The amount of glycerol was then measured enzymatically by glycerol dehydrogenase. This enzyme converted glycerol to dihydroxyacetone and at the same time produced NADH from NAD+. The amount of NADH produced was determined by measuring the absorbance at 340 nm.

Lipids were extracted from 12 ml of cytosol by $CHCl_3/CH_3OH$ (2/1) and the solvents in the extract were evaporated under reduced pressure. The residue was dissolved in $CHCl_3$ and passed through an alumina column. The neutral lipids were eluted with $CHCl_3/CH_3OH$ (95/5; v/v) and diglyceride was isolated by thin-layer chromatography with $C_6H_6/CHCl_3/CH_3OH$ (80/15/5; v/v/v) as solvent. The DG was eluted

from the silica and hydrolyzed in potassium hydroxide. Free fatty acids were absorbed by albumin and it was removed by ultrafiltration. The amount of glycerol was measured by glycerol dehydrogenase and the assay contained hydrazine which converted dihydroxyacetone to the hydrazone and facilitated complete conversion of the substrate. Various amounts of diacylglycerols which were hydrolyzed and assayed in the same manner were used as standards.

6. Ultrafiltration and Phospholipase C Treatment of Cytosols

The stimulation of the phosphocholine cytidylyltransferase activity in hypercholesterolemic rat liver cytosol could have been caused by the presence of a low molecular weight modulator. This possibility was examined by ultrafiltration. Cytosols were diluted and then subjected to ultrafiltration through an Amicon XM-100A membrane filter. Only molecules with molecular weight larger than 100,000 were retained and the diluted cytosols were concentrated back to their original volume. Cytidylyltransferase activities were subsequently measured in the ultrafiltrated cytosols.

The cytidylyltransferase is strongly stimulated by phospholipids and the presence of a lipid activator could explain the stimulation of the enzyme in experimental cytosol. If this were the case, removal of phospholipids from both cytosols would abolish the difference in enzyme activity. Cytosols containing 1 mM $CaCl_2$ and Tris-succinate pH 7.0 were treated with 4 units per ml of phospholipase C from Cl. Welchii and the reaction was stopped with 3 mM final concentration of EGTA. Aliquots of the treated cytosol were subsequently assayed for cytidylyltransferase activity.

7. Purification of Phosphocholine Cytidylyltransferase and Assessment of Purity

The procedure for the purification of the cytidylyltransferase was that of Choy et al. (22). The cytidylyltransferase exists as two distinct species in cytosol: a high molecular weight (H-form) and a low molecular weight (L-form). The purification scheme took advantage of the fact that the cytidylyltransferase which exists predominantly as the L-form in fresh cytosol aggregates into the H-form upon incubation at 4°C for several days. In the first step of purification, the H-form was separated from low molecular weight proteins by gel filtration. Subsequently, the H-form was dissociated to the L-form by sodium dodecyl sulfate and purified L-form was isolated by a second gel filtration step which eliminated any high molecular weight contaminants.

In a typical purification, rat liver homogenate (20% w/v) was prepared in isotonic saline and centrifuged at 100,000 x g for 60 min. One hundred ml of the supernatant (cytosol) was stored at 4°C for 5 days, after which precipitated materials were removed by centrifugation at 10,000 x g for 10 min. The cytosol was adjusted to 20% ammonium sulfate saturation and the precipitate was removed by centrifugation (10,000 x g for 10 min). The supernatant was readjusted to 25% ammonium sulfate saturation and the precipitate formed after 2 h of storage at 4°C was collected by centrifugation (10,000 x g for 10 min) and resuspended in 10 ml of column buffer (see Section III.5). Half of this sample was applied to a Sepharose 6B column (2.5 x 80 cm) which had been equilibrated with the same buffer. Enzyme activity was detected in the fractions eluted with the void volume and active fractions were combined. The chromatography step was repeated with the second half of the ammonium sulfate fraction. Sodium dodecyl

sulfate (1%) was added to the pooled fractions to a final concentration of 0.05% and the sample was stored at 4°C for 2 h. Part of this solution (4 ml) was applied to a Sepharose 6B column (2.5 x 80 cm) equilibrated with column buffer containing 0.005% SDS and the cholinephosphate cytidylyltransferase activity was separated into two distinct fractions. The second fraction of enzyme activity (which required lipid for maximum activity) was collected and concentrated by ultrafiltration over an Amicon PM 30 membrane. A 1,000-fold purification of the enzyme was achieved with an overall yield of 10%.

The purity of the preparation was determined by nondenaturing polyacrylamide gel (5%) electrophoresis at pH 7.5 (108). A single band was observed when the gel was stained for protein. When duplicate gels were electrophoresed and one stained for protein and the other sliced and assayed, 17% of the cytidylyltransferase activity was recovered and it coincided with the protein band. Since each gel (0.55 x 8 cm) was loaded with 50 μg of protein and no other protein bands were observed, the enzyme appears to be pure. Choline kinase, ethanolaminephosphate cytidylyltransferase, and cholinephosphotransferase activities were not detected in purified preparations.

The purified enzyme was found to be unstable and the presence of dithiothreitol, CTP-Mg^{2+}, or bovine serum albumin did not significantly stabilize the enzyme. When the enzyme was frozen and thawed once, more than 90% of the activity was lost.

8. Preparation of Antibodies Against the Purified Cytidylyltransferase and Immunotitration of the Enzyme from Cytosol

The cytidylyltransferase was purified according to the above procedure and 12 preparations were pooled, concentrated to 1 mg protein per ml and

dialyzed against 100 volumes of 0.145 M NaCl, 10 mM Tris HCl, pH 7.4. A 2 kg rabbit was immunized subcutaneously at two sites with a total of 0.5 mg enzyme per kg body wt, emulsified in Freund's complete adjuvant. This was followed by two injections of 0.25 mg enzyme per kg body weight, emulsified in Freund's incomplete adjuvant at 7 day intervals. Another rabbit was treated identically except that only buffer was used in the injections. A week after the last injection, 10 ml of blood was obtained from each rabbit and immunoglobulin G was prepared.

The clotted blood cells were sedimented at 1,000 x g for 5 min. The sera were allowed to stand overnight at 4°C and the formed clots were sedimented at 12,000 x g for 10 min. The pellets were discarded and one volume of ice-cold saturated $(NH_4)_2SO_4$) solution was added to each serum. After 30 min on ice, the protein precipitates were pelleted by centrifugation at 6,000 x g for 15 min and the pellets were washed 4 times with 5 ml of 40% $(NH_4)_2SO_4$). Finally, the pellets were dissolved in 10 ml of 10 mM phosphate buffer, pH 7.0 and dialyzed 2 times against 100 volumes of 10 mM phosphate buffer, pH 7.0 for 6 h at 4°C. The dialyzed immunoglobulin G preparations were diluted to give a final concentration of 7 mg of protein per ml.

The immunoglobulin G preparations were analyzed by cellulose acetate electrophoresis and had one band which corresponded to the immunoglobulin G band of whole serum. The specificity of the anti-serum was tested by double diffusion analysis in agar gel against cytosol or L-form and in both cases a single sharp precipitin line was observed. Normal rabbit serum analyzed similarly did not show any reaction. The purification of immunoglobulin G did not affect the antigen-antibody reaction nor the specificity as tested by double diffusion analysis.

Agar plates used for double diffusion analysis were prepared by pipetting 5 ml of hot 1% agar containing 20 mM Tris-HCl, pH 7.0, 0.1 M NaCl and 0.1% sodium azide into 7 cm petri dishes. After the agar had solidified, 0.4 cm wells were made with diffusion distances of 0.8 cm. The center well was filled with 50 µl of L-form and immune and control sera or immunoglobulin G were added to the peripheral wells. The immunoprecipitin lines were allowed to develop for 72 h at 4°C. The plates were washed with saline and water and stained for 15 min with 0.04% ponceau S in 2% trichloroacetic acid and 5% acetic acid. Destaining was carried out in 5% acetic acid for 24 h.

The isolated immunoglobulins were used to titrate the cytidylyltransferase from cytosol. In these experiments, 25 µl (about 0.3 mg protein) of normal and experimental cytosols were diluted with 75 µl of 0.20 M phosphate buffer, pH 8.0. Duplicate sets of incubations were made for each cytosol where 0 to 150 µl (1.1 mg) of control antibodies were added to one set of incubations and the same amount of anti-cytidylyltransferase antibodies were added to another set. The volumes of all the incubations were finally made up to 250 µl with 10 mM phosphate buffer, pH 8.0. The antigen-antibody complexes were allowed to form for 8 h at 4°C and the precipitate was sedimented at 4,200 x g for 30 min at 4°C. The tubes were handled very gently and 60 µl of the supernatant was used to measure cytidylyltransferase activity. The enzyme assay contained 1 mM CTP and it was incubated for 15 min at 37°C.

9. Treatment of Cytosols with Trypsin and Column Chromatography of Trypsin-inactivated Cytosols.

The cytosols were treated with 0.1 mg of trypsin per ml for 15 min at 37°C and proteolysis was stopped with 0.15 mg of trypsin inhibitor per ml.

The cytidylyltransferase activity was completely eliminated after this treatment and 40 µl of each of the inactivated cytosols were reconstituted with 20 µl of delipidated or partially purified enzyme. Enzyme activity was measured as previously described (see Section IV.2.a).

A trypsin-resistant activator was found and its properties were further studied by subjecting 4 ml of trypsin treated control and experimental cytosol to Sepharose 6B chromatography. The activator was assayed in the column fractions by measuring the activity of 20 µl of delipidated or partially purified enzyme in the presence of 40 µl of column eluate.

10. Procedure for the Large Scale Isolation of Cytosolic Lipids and the Fractionation of These lipids by Thin Layer Chromatography

A large amount of activator was required for further studies. Previous experiments showed that the activator had a high molecular weight and it was therefore precipitated from cytosol by 25% ammonium sulfate. The precipitate was sedimented by centrifugation and the pellet obtained was extracted 3 times with $CHCl_3/CH_3OH$ (2/1; v/v) under nitrogen. The extracts were pooled and concentrated to 1/10 the volume of the cytosol extracted.

The cytosolic lipids were fractionated by one dimensional and two dimensional thin-layer chromatography. Lipids were identified by iodine and eluted from the silica with either three 4 ml volumes of $CHCl_3/CH_3OH/25\ NH_4OH$ (50/50/2; v/v/v) or $CHCl_3/CH_3OH/H_2O$ (100/50/2; v/v/v).

11. Analysis of Lysophosphatidylethanolamine by Mass Spectrometry

Lysophosphatidylethanolamine was isolated by preparative two dimensional thin layer chromatography (solvent system IV, Section III.4.b.ii). The amount of lipid extracted from 30 ml of control and

experimental cytosol (2.0 to 3.0 mg) was each applied to three 2.5 cm lanes on a thin layer plate. After development in one solvent, the standard LPE was visualized by iodine and the silica in the corresponding areas on the sample lanes were scraped and eluted with 30 ml of $CHCl_3/CH_3OH$ (1/1). The eluted lipids were concentrated and rechromatographed as described above except that the plate was developed in the second solvent system.

Isolated LPE was silylated according to published procedures (109). The solvents in the preparations were evaporated under a stream of N_2 and 0.1 ml of dry pyridine and 0.1 ml of bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane were added. The mixtures were agitated and incubated at room temperature for 1 h. After the reaction solvents were evaporated and the derivatized products, either solid or disolved in $CHCl_3$, were analyzed in an AEI-902 Mass Spectrometer. Each sample was applied on a probe which was introduced directly through the vacuum lock. Insertion probe temperature was 150-180°C and the spectra was obtained at 70 ev.

12. Quantitation of Lysophosphatidylethanolamine by Ninhydrin

The amount of lipid in 1.5 ml of control and experimental cytosols (.87 and .165 mg, respectively) was chromatographed by TLC with $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (100/60/16/8; v/v/v/v). A suitable 2-dimensional TLC solvent system was not available at the time. The lipids were eluted from the silica into test tubes and the solvent was evaporated under N_2. The amount of LPE was measured by a procedure developed by Lea and Rhodes (110). Briefly, 1 ml of methylcellosolve and 1 ml of Stein and Moore ninhydrin reagent, which contains 0.80 g of $SnCl_2 \cdot 2\ H_2O$ in 500 ml of 0.2 M citrate buffer, pH 5.0 and 20 g of ninhydrin in 500 ml of methylcellosolve, were added to the samples. After mixing, the tubes, capped with marbles, were heated in boiling water for 20 min and 5 ml of

methylcellosolve were added to each tube after a brief period of cooling. The absorbance of the solutions at 575 nm were measured. Synthetic palmitoyl-LPE was treated identically as the samples and was used to generate a standard curve ranging up to 50 µg of LPE (4 µg lipid phosphorus).

13. Analysis of Fatty Acids on Lysophosphatidylethanolamine by Gas-Liquid Chromatography

Lysophosphatidylethanolamine was isolated from cytosolic lipids by two dimensional thin layer chromatography (see Section III.4.b.ii) in two separate experiments using different solvent systems. The LPE's from 0.2 mg of cytosolic lipids were eluted from the silica and reacted with 1 ml of 0.75 N HCl in methanol for 16 h at 80°C. The excess methanol HCl was evaporated under a stream of N_2 and the fatty acid methyl esters, dissolved in 10 µl of hexane, were analyzed with a Hewlett-Packard 7610A High Efficiency Gas Chromatograph. A few microlitres of the samples were injected into a column of 12% HI-EFF-2BF (ethylene glycol succinate) at 170°C. Methyl esters derived from synthetic palmitoyl-LPE and methyl esters of standard $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{20:0}$ were analyzed identically. The fatty acids from the samples were identified by comparing retention times of their methyl esters with the retention times of the standard fatty acid methyl esters.

VI. Methods Used in In Vivo Studies

1. Intraportal Injection of [Me-^{3}H]Choline and Freeze Clamping of Livers

The procedure used was similar to that described by Akesson *et al.* (111). A control rat was anaesthetized with ether and its abdomen was cut open and the portal vein was exposed. A 100 µl solution of saline containing 60 µM [*Me*-^{3}H]choline (50 µCi/100 µl, 8.3 Ci/mmol) was injected into the portal vein. After a predetermined time, not longer than

4 min, approximately 1 g of liver from the right side of the median lobe was excised and immediately frozen between two aluminum blocks which have been cooled in liquid nitrogen. The frozen tissue was stored in liquid N_2 and was extracted within a few h. A cholesterol/cholate-fed rat was treated identically and the sequence was repeated until all rats were killed. Excess liver tissues from all animals were excised and weighed immediately after killing.

2. Bligh and Dyer Extraction

The frozen tissues were extracted by the procedure developed by Bligh and Dyer (99). The frozen tissue (1 g) was homogenized with 3 ml of $CHCl_3/CH_3OH$ (1/2, v/v) in a Sorval Omni-mixer micro chamber. The tissue was homogenized at maximum speed for four 15 s intervals at 0°C and the homogenate was poured into a 30 ml glass centrifuge tube. The micro chamber was washed with 3.8 ml of $CHCl_3/CH_3OH/H_2O$ (1/2/0.8; v/v/v) which was pooled with the homogenate. The one phase extract was made into a two phase system by addition of 2 ml of $CHCl_3$ and distilled water. The mixture, which consisted of $CHCl_3/CH_3OH/H_2O$ (1/1/0.9) was vigorously mixed and the phases were separated by centrifugation at 6,000 x g for 20 min at 4°C. The total amount of radioactivity incorporated was calculated by adding the total amount of radioactivity incorporated into each of the phases. Radioactivity in each phase was determined by using 100 µl of upper and lower phases for scintillation counting. Previous experiments showed that over 95% of the radioactivity was incorporated into water soluble compounds in the upper phase. Thus to minimize contamination of radioactivity from upper phase, the lower organic phase was washed 2 times with 4 ml of theoretical upper phase and the washings were discarded. Theoretical upper phase was obtained from the top phase of an equilibrated mixture of $CHCl_3/CH_3OH/H_2O$ (1/1/0.9).

3. Separation of Choline, Phosphocholine, CDP-choline and Betaine

The water-soluble compounds in the upper phase were separated by thin layer chromatography. In earlier experiments, an aliquot containing 200,000 cpm was subjected to two dimensional thin layer chromatography (see Section III.4.a.ii) and all four compounds were well resolved. But because the amount of radioactivity incorporated into CDP-choline was insignificant (about 0.1 to 0.5 of total radioactivity), a single dimensional thin layer system was used in all subsequent experiments. This basic solvent system (see Section III.4.a.i) separated choline, phosphocholine and betaine but CDP-choline co-chromatographed with betaine.

4. Measurement of Phosphocholine Pool

The amount of phosphocholine in liver was measured enzymatically (112). An aliquot of upper phase containing about 100 nanomoles of phosphocholine was first extracted with acid-washed charcoal. Endogenous CTP and CDP-choline which interfere with the measurement were adsorbed to the charcoal and sedimented at 6,000 x g for 10 min. The supernatant was filtered through glass wool to remove any residual charcoal and the filtrate was concentrated to a final phosphocholine concentration of 80 nanomoles per ml.

The measurement of phosphocholine involved two enzymatic reactions. The first reaction converted phosphocholine to CDP-choline by enzyme phosphocholine cytidylyltransferase. The second reaction digested all the unreacted radioactive cytidine-5'-triphosphate to cytidine and inorganic phosphate by alkaline phosphatase. The labelled CDP-choline was then separated from labelled cytidine by paper chromatography and the radioactivity in cytidine diphosphocholine was measured by scintillation counting.

The reaction cocktails and conditions used for the two reactions are given below:

Reaction I: Cocktail

10 µl 1 M Tris-succinate, pH 7.0; 0.12 M $Mg(OAc)_2$

10 µl 0.8 µmol/min/ml of inorganic pyrophosphatase

5 µl [5-^{3}H]-CTP, 10 mM, 10 µCi/µmol

50 µl partially purified L-form from rat liver

25 µl sample containing 1-3 nmol of phosphocholine or standards containing 1-4 nmol of phosphocholine

The reaction, in a final volume of 100 µl, was carried out at 37°C for 45 min and stopped by immersion of the tubes in boiling water for 2 min.

The precipitated proteins were sedimented and 50 µl of the supernatant was used in the second reaction.

Reaction II: Cocktail

20 µl 5 mM CDP-choline; 1 M Tris-glycine, pH 10.5

30 µl 10 µmol/min/ml of alkaline phosphatase

50 µl supernatant from reaction I

The reaction was allowed to proceed at 37°C for 30 min and stopped by boiling for 2 min.

A 25 µl aliquot of reaction II was subjected to descending paper chromatography along with 100 µg of CDP-choline carrier. The aliquots were applied as spots not larger than 1 cm in diameter and a mixture of ethanol/1 M ammonium acetate, pH 7.1 (7/3; v/v) was used as solvent.

After development, the dried chromatograms were placed under U.V. and the dark CDP-choline spots were marked. The areas of the paper containing the spots were cut into thin strips and placed in a scintillation vial containing 1 ml of water. Subsequently, radioactivity was determined by

scintillation counting. A standard solution of phosphocholine was used to generate a linear curve from 0 to 4 nanomoles. The concentration of phosphocholine in each sample was determined twice where one measurement contained twice as much sample than the other.

5. Calculation of the Rate of Phosphatidylcholine Biosynthesis

The rate of PC biosynthesis was estimated by measuring the conversion of phosphocholine to PC. Evidence to date strongly indicates that the rate limiting step of the de novo pathway is the reaction catalyzed by phosphocholine cytidylyltransferase. Since the first and last reactions of the pathway are believed not to be rate limiting, the rate of conversion of phosphocholine to PC should be equivalent to the rate of PC biosynthesis.

Experimentally, [Me-^{3}H] choline was injected into the portal vein of control and experimental rats and the radioactivity incorporated into choline, phosphocholine, CDP-choline, betaine and PC were followed with time. Early experiments showed that the specific radioactivity of phosphocholine remained almost unchanged between 1 and 4 minutes after injection. Therefore, the rate of biosynthesis was measured between 1 and 2 minutes post-injection.

The amount of radioactivity incorporated per min per liver into PC between 1 and 2 min was calculated by subtracting the dpm at 1 min from the dpm at 2 min. Specific radioactivity of phosphocholine was calculated by dividing the average dpm per liver at 1 and 2 min by the average number of nanomoles of phosphocholine per liver at the same time points. Finally, the rate of PC biosynthesis per liver was calculated by dividing the amount of dpm incorporated into PC per min per liver by the specific radioactivity of phosphocholine. The estimated rate of PC biosythesis was expressed as nanomoles per min per liver.

VIII. Statistical Methods

A standard statistical test was used (113). For each set of data, the mean, standard deviation, T value, and degree of freedom were calculated as follows:

MEAN

$$X = \frac{\sum_{n=1}^{n=t} X_n}{t}$$

where X = mean

X_1 to X_t = individual value

t = total number of values

Standard Deviation

$$S = \sqrt{\frac{\sum_{n=1}^{n=t} (X_n - X)^2}{t - 1}}$$

where S = standard deviation

T Value

$$T = \frac{X - Y}{\sqrt{Z^2 \left(\frac{1}{t_x} + \frac{1}{t_y}\right)}}$$

where t_x, t_y = number of values in X and Y respectively

$$Z^2 = \frac{S_x^2 + S_y^2}{2}$$

if, and only if, the number of X values equal Y values, i.e. $t_x = t_y$

Degree of Freedom

$$DF = t_x + t_y - 2$$

where DF = degree of freedom

To determine if the mean of the X (control) population was equal to that of the Y (experimental) population, value α was obtained from a table by using the T value and DF. The α value is the probability that the mean of the control and experimental populations are the same.

In the analysis of all experimental results, the means of the control and experimental populations were considered to be different only if the α values calculated were less than 0.05.

RESULTS

I. Characterization of Phosphocholine Cytidylyltransferase: The Effect of pH, Phospholipids, Acyl-CoAs, Various Adenosine-containing Coenzymes and Nucleotides on Enzyme Activity

The results from earlier studies suggested that phosphocholine cytidylyltransferase may play an important regulatory role in the control of de novo phosphatidylcholine biosynthesis. Therefore, the effect of pH and various physiological compounds on the activity of the cytidylyltransferase was investigated.

Two distinct forms of the cytidylyltransferase have been described in rat lung (23) and rat liver (22). The activities of the two forms of the enzyme at various pH's were measured (Fig. 8). Activity of the low molecular weight form (L-form) was maximal at pH 6.5, whereas the high molecular weight form (H-form) was most active at pH 7.5. Furthermore, while the activity of the L-form falls dramatically at high and low pH, the H-form activity remained high under alkaline conditions but was severely inhibited under more acidic conditions.

Synthesis of PC requires DG and it is conceivable that the DG and fatty acid synthesis may be coordinately regulated with the synthesis of PC. Therefore, the effect on cytidylyltransferase activity of some important

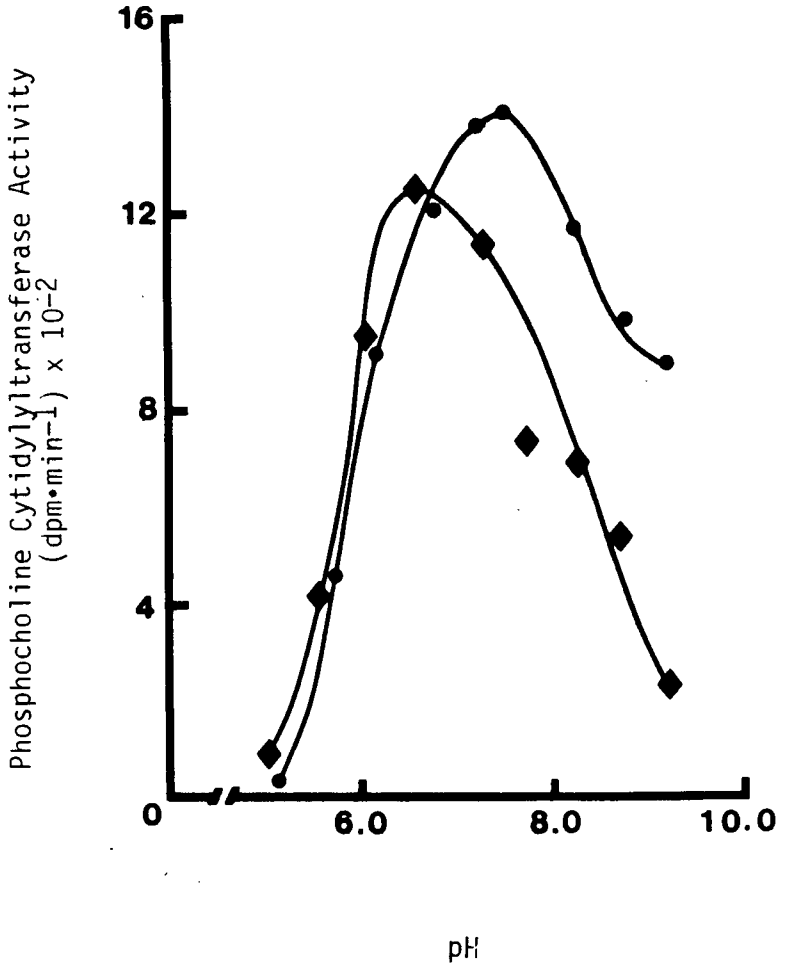


Figure 8. Activities of the High (●) and Low (◆) Molecular Weight Forms of Phosphocholine Cytidylyltransferase at Various Values of pH. The enzyme assay and the preparation of partially purified L-form was described in the Materials and Methods section. H-form was prepared by chromatography of aged cytosol (4 ml) on Sepharose 6B and the fractions near the void volume which contained the highest H-form activity were used. The curves have been matched for convenience of comparison. Tris-HCl buffer (50 mM) was used between pH 7.0 and 9.0 while Tris-succinate (50 mM) was used between pH 6.0 and 7.0.

intermediates of fatty acid and phosphatidic acid biosynthesis were investigated. The compounds tested did not exert a marked effect on the enzyme. Acetyl-CoA at 5 μM and malonyl-CoA at 50 μM had no effect while 50 μM palmitoyl-CoA and 50 μM stearoyl-CoA slightly stimulated enzyme activity by 14 and 35% respectively.

The synthesis of PC from choline and DG requires the expenditure of 1 ATP and 1 CTP molecule. Phosphatidylcholine biosynthesis must therefore be, in some manner, coupled to the energy level of the cell. With this in mind, the effects of various compounds involved in the energy metabolism of the cell were examined (Table 17). NAD and NADP inhibited enzyme activity to half the control value at 5 mM while NADH, and to a lesser extent NADPH, stimulated the enzyme. In contrast, effects of nucleotides did not fall into a simple logical pattern. Although UMP inhibited the cytidylyltransferase, GMP had no effect. Furthermore, all the nucleoside triphosphates tested inhibited enzyme activity and the most potent was ATP which inhibited 80% of enzyme activity at 10 mM. Interestingly, activity of the cytidylyltransferase in cytosol was also inhibited by high concentrations of CTP. The activity of the enzyme when assayed with 12 mM CTP was less than 50% of the enzyme activity when 4 mM CTP was used.

A preliminary study of the mode of inhibition by ATP and NAD with respect to CTP and phosphocholine was performed (Fig. 9 and 10) and results from kinetic experiments suggested that both ATP and NAD were non-competitive with respect to CTP. Further experiments are required before any conclusions can be made.

Table 17

Effect of Various Adenosine-Containing Coenzymes and Nucleotides on the Activity of Phosphocholine Cytidylyltransferase

	Relative Enzyme Activity (o/o control activity)		
	1 mM	5mM	10 mM
NAD	65	39	-
NADH	142	142	-
NADP	88	57	-
NADPH	128	112	-
ATP	-	-	20
GTP	129	110	69
UTP	109	86	75
GMP	114	-	105
UMP	78	69	56

The reaction contained 30 µl of partially purified cytidylyltransferase, 1 mM CTP and the appropriate concentration of the compound to be tested. The assay was incubated for 20 min at 37°C and all the other conditions were according to the procedure described in the Materials and Methods section.

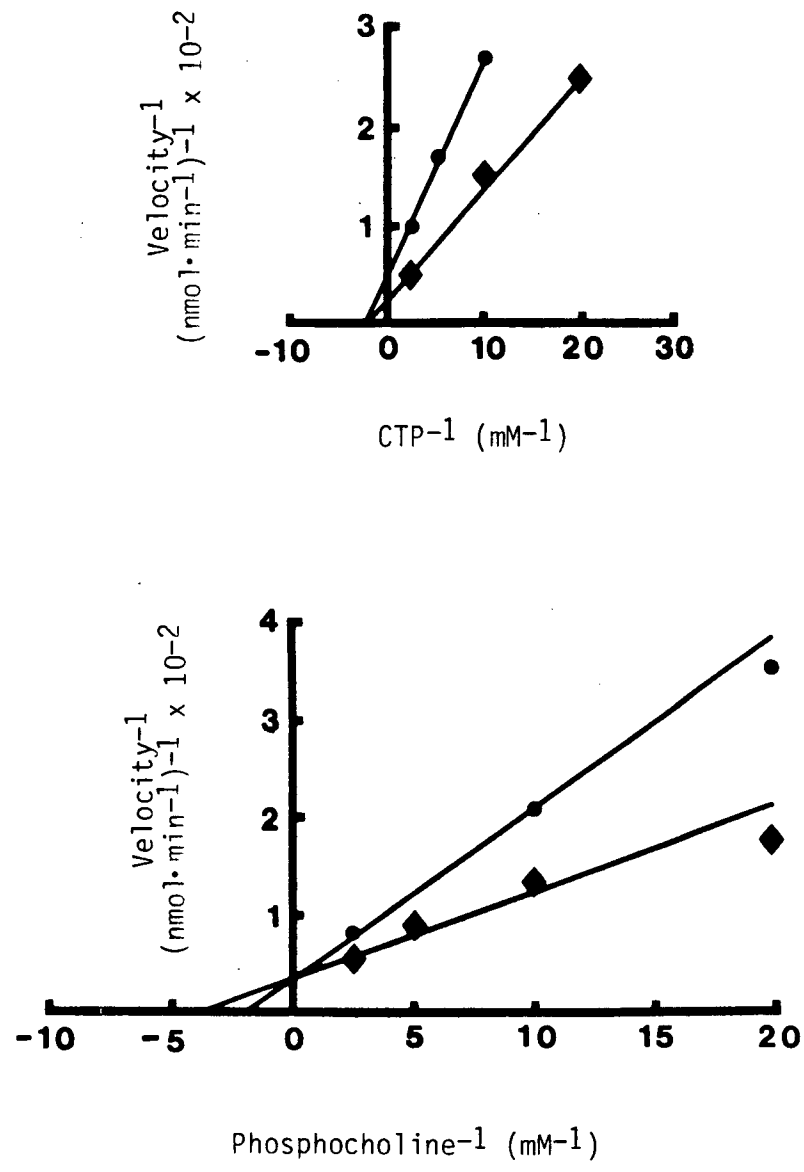


Figure 9. Double Reciprocal Plots of the Initial Velocity of Phosphocholine Cytidylyltransferase Versus Various Concentrations of CTP or Phosphocholine in the Presence of ATP. Reactions were carried out in the presence (●) and absence (◆) of 10 mM ATP, and in all experiments the concentration of only one substrate was varied. The assay contained 30 µl of partially purified enzyme and the concentration of the non-varied substrate was saturating.

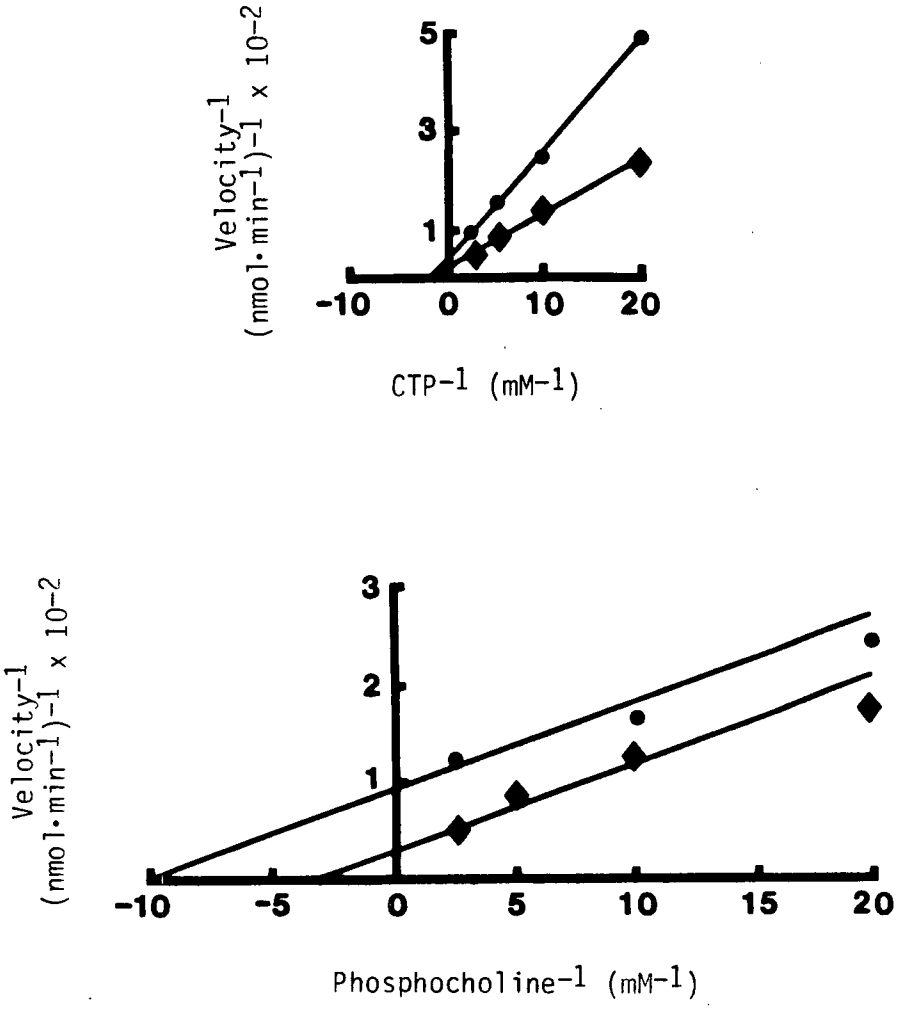


Figure 10. Double Reciprocal Plots of the Initial Velocity of Phosphocholine Cytidylyltransferase Versus Various Concentrations of CTP or Phosphocholine in the presence of NAD. The conditions used were similar to Fig. 9 except that reactions were carried out in the presence (●) and absence (◆) of 5 mM NAD.

In contrast, the modes of inhibition by ATP and NAD with respect to phosphocholine were quite different. ATP increased the Km of the enzyme for phosphocholine from a control value of 0.25 mM to 0.50 mM and competes with phosphocholine for the same site on the enzyme. The results indicated that ATP was a competitive inhibitor of the cytidylyltransferase with respect to phosphocholine. Results from experiments with NAD were more difficult to interpret since the velocity as well as the affinity of the enzyme for phosphocholine appeared to be diminished by the inhibitor. The parallel lines obtained from the double reciprocal plot suggested that NAD was an uncompetitive inhibitor of the enzyme with respect to phosphocholine.

II. Effects of Various Diets on the Levels of Plasma Lipids and Phosphocholine Cytidylyltransferase Activity

Phosphocholine cytidylyltransferase has been implicated as the regulatory enzyme of the pathway for PC biosynthesis. Further insights into the regulation of PC biosynthesis could be obtained from studies with an animal model whose rate of PC biosynthesis was significantly altered from normal. As discussed in the introduction, rats with diet-induced hypercholesterolemia had elevated levels of plasma phospholipids and this could be the result of elevated rates of phospholipid synthesis in the liver where plasma lipoproteins are synthesized. This idea was tested in young female rats whose plasma cholesterol levels could be easily manipulated (95).

The effect of feeding a commercially available Basal Atherogenic Diet was investigated and the results are shown in Table 18. The plasma cholesterol and phospholipids in the experimental group were increased over controls by 4- and 2-fold after 3 days and by 10- and 3-fold, respectively after 6 days of feeding. The activity of phosphocholine

cytidylyltransferase was also measured in both groups (Table 18) and was found to be elevated in the liver cytosols from rats fed the Basal Atherogenic Diet. Cytidylyltransferase activity was increased after 3 and 6 days of feeding by 3.5- and 2.5-fold, respectively.

The diets used in these experiments have different compositions aside from the 5.3% cholesterol and 2% cholate contained in the atherogenic diet. The control diet consisted of 20% lard and 56% sucrose, while the experimental diet contained 40% butterfat and 20.3% sucrose. It was possible that the stimulation of the cytidylyltransferase could have been caused by a difference in calorie uptake rather than by the intake of cholesterol and cholate.

In a subsequent study, experimental rats were fed a modified control diet (Purina Rat Chow) supplemented with either 2% cholate, 5% cholesterol, or 2% cholate and 5% cholesterol for different lengths of time (Table 19). The rats fed the diet containing cholate and cholesterol had a 6-fold elevation of plasma cholesterol, a 2-fold increase in plasma phospholipids and a 2-fold increase in cytosolic activity of liver phosphocholine cytidylyltransferase when compared to rats fed ordinary purina rat chow. Activity of the cytidylyltransferase was also stimulated, although to a much lesser degree, when rats were fed diets containing only cholesterol or cholate. Plasma cholesterol was increased while the level of plasma phospholipids remained unchanged in the rats fed the diet containing

Table 18

Plasma Lipids and Phosphocholine Cytidylyltransferase Activity in Liver Cytosols from Rats Fed a Control High Fat Diet and a Basal Atherogenic Diet (BAD)

	Day on Diet	Plasma Lipids (mg per 100 ml)		Phosphocholine cytidylyltransferase Activity (nmol/min/mg)
		Cholesterol	Phospholipids	
Control (2)	3	164	116	0.78
(3)	6	149 ± 10	157 ± 14	0.54 ± 0.05
BAD (2)	3	633	205	2.7
(3)	6	1514 ± 236	427 ± 98	1.5 ± 0.06

The conditions and animals used have been described in the Materials and Methods section. The numbers in brackets indicate the number of animals used and values are given as mean ± S.D.

Table 19

Effect of Diets Containing 2% Cholate, 5% Cholesterol or 2% Cholate and 5% Cholesterol on the Level of Plasma Lipids and the Cytosolic Activity of Hepatic Phosphocholine Cytidylyltransferase

Diet	Days on Diet	Plasma Lipids (mg per 100 ml)		Enzyme Activity
		Total Cholesterol	Phospholipids	
Control	6	100 (2)	91 (2)	0.46 ± 0.1 (10)
2% Cholate	3	162 (2)	77 (2)	0.89 (2)
	6	146 ± 14 (3)	85 ± 26 (3)	0.72 ± 0.33 (3)
5% Cholesterol	6	-	-	0.84 (2)
2% Cholate 5% Cholesterol	3	809 ± 144 (3)	191 ± 22 (3)	1.21 ± 0.34 (3)
	6	672 ± 98 (3)	197 ± 51 (3)	1.1 ± 0.19 (10)
	9	664 (2)		1.0 (2)

Control rats were fed purina rat chow while the experimental rats were fed a diet composed of purina rat chow supplemented with cholesterol, cholate or cholesterol and cholate. The values are expressed as means or mean ± S.D. while the numbers in brackets indicate the number of rats used.

only cholate. Since the only difference between the control and experimental diets was the content of cholesterol and cholate, the stimulation of the cytidylyltrasferase in experimental rats must be the result of intake of these lipids. The diet containing 2% cholate and 5% cholesterol induced the most marked effect and this diet was used in all succeeding experiments.

III. Cholesterol/Cholate Feeding: In Vitro Studies

1. The Effect of the Cholesterol/Cholate-rich Diet on Body Weight, Liver Weight and Plasma Lipid Levels

Rats fed the diet containing 5% cholesterol and 2% cholate for six days gained less weight ($P < 0.05$) than rats fed a normal diet (52 $\pm$ 6 and 67 $\pm$ 3 g respectively, mean $\pm$ S.D., with 24 rats per group) although the amount of food consumed per rat in each group was the same. However, the average weight of the livers from cholesterol/cholate-fed rats was only slightly less ($P < 0.05$) than control values, (3.1 $\pm$ 0.5 and 3.3 $\pm$ 0.2 g respectively). The protein content of cytosol and microsomes, as well as the recoveries of cytosols from homogenates, were similar in both groups. The cholesterol/cholate-fed rats had a 6-fold ($p < 0.05$) elevation of plasma cholesterol (631 $\pm$ 78 compared to 99 $\pm$ 18 mg/dl) and almost a 2-fold ($P < 0.05$) increase in plasma phospholipid (243 $\pm$ 31 compared to 132 $\pm$ 24 mg/dl). The effects of this diet on the enzymes of PC biosynthesis were subsequently investigated.

2. Effect of Diet on Enzymes of Phosphatidylcholine Biosynthesis

The cholesterol/cholic acid diet had a marked effect on the cytidylyltransferase from liver (Table 20). A 2 to 3-fold stimulation in this enzyme activity in cytosol and microsomes from experimental rats was observed whether enzyme activity was expressed per mg protein, per g liver or per liver. The activities of choline kinase, phosphocholinetransferase

Table 20

Specific Activities of Enzymes of Phosphatidylcholine Biosynthesis from Rat Liver

Enzyme	Cholesterol/Cholate Diet	Control Diet
	(nmol· min^{-1}· mg^{-1})[a]	
Choline Kinase	5.6 ± 0.3	4.5 ± 0.7
CTP:Phosphocholine cytidylyltransferase		
Cytosolic	1.5 ± 0.4*	0.49 ± 0.06
Microsomal	2.7 ± 0.3*	1.4 ± 0.1
Phosphocholinetransferase	24 ± 1	27 ± 4
Phosphatidylethanolamine methyltransferase	0.12 ± 0.01	0.13 ± 0.02

[a] Activities are ± S.D., n = 3

* $P < 0.05$

and phosphatidylethanolamine methyltransferase from liver, however, were similar to control values. Subsequent studies were directed toward understanding the activation of the cytidylyltransferase.

We investigated the distribution of the cytidylyltransferase between the H- and L-forms by chromatography of fresh cytosol on Sepharose 6B (Fig. 11) and showed that 25-30% of the total cytosolic enzyme from hypercholesterolemic rats was in the H-form, compared to 10% for control rats. Thus, the cholesterol/cholic acid diet resulted in an increased aggregation as well as a stimulation of the cytidylyltransferase in cytosol.

3. The Effect of the Cholesterol/Cholate-rich Diet on the aggregation of Phosphocholine Cytidylyltransferase and Diacylglycerol Concentration in Cytosol

Diacylglycerol promotes aggregation of the cytidylyltransferase in cytosol from rat liver (26). Thus, the concentration of DG in the cytosols were determined. The experimental rats had an elevated level ($P < 0.05$) of DG (4.25 and 4.23 compared to 1.66 and 1.41 nmoles/l in control rats as measured in the 2 sets of pooled liver cytosols from 4 control and 4 cholesterol/cholate fed rats). A previous experiment had shown that trypsin-treated cytosol from cholesterol-fed rats caused an increase in aggregation of the partially purified L-form to the H-form (26). Together, these results suggest that the increased DG in the cytosol from the experimentally fed rats was responsible for the increased concentration of H-form.

4. Studies on Phosphocholine Cytidylyltransferase in Cytosols from Normal and Cholesterol/Cholate-fed Rats

The livers isolated in the usual way (see Materials and Methods section) contain a significant amount of blood and since plasma phospholipids were increased in the hypercholesterolemic animals, it was

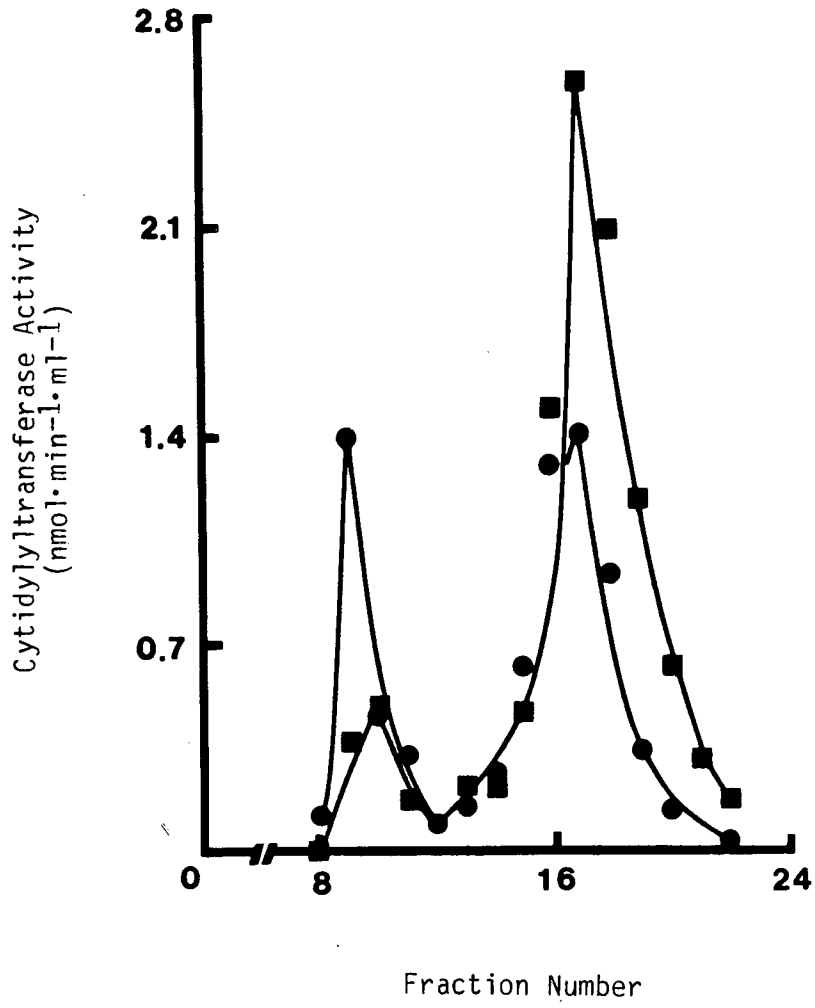


Fig. 11. Chromatography of Cytosols from Normal (■) and Cholesterol/Cholate-Fed (●) Rats. Four ml of freshly prepared cytosol were applied to a column of Sepharose 6B which had been equilibrated with 100 mM NaCl, 20 mM Tris-HCl, pH 7.0. Fractions (7 ml) were collected and assayed for cytidylyltransferase activity in the presence of 0.5 mg rat liver phospholipid per 0.1 ml of assay mixture. The data shown were the averaged results obtained from three separate experiments.

reasonable to think that plasma lipids from residual blood in the experimental livers could be responsible for stimulation of the cytidylyltransferase. In a few experiments with cytosols prepared from livers perfused with ice cold saline, the activity of the cytidylyltransferase was similar to the activity found in cytosols prepared from normally isolated livers. Thus plasma lipids do not appear to be involved in the stimulation of the enzyme.

Activity of the cytidylyltransferase was determined in cytosol which contained a large variety of other enzymes. It was conceivable that the higher activity of the cytidylyltransferase observed in cholesterol/cholate-fed rat liver cytosol, as measured by the amount of CDP-choline formed in the reaction mixture, was really due to a slower rate of breakdown of CDP-choline in the experimental cytosol. To test this possibility, the conditions used in the cytidylyltransferase assay were duplicated, except that none of the usual substrates were used. Instead, 0.65 nmol of C-labelled CDP-choline, the amount normally produced in the assay, were added to the reaction cocktail. The labelled CDP-choline was not degraded in the presence of the cytosols but 50% of the label was degraded when microsomes were used (Table 21). The unrecovered CDP-choline was most likely used by cholinephosphotransferase for PC synthesis. The recovery of radioactivity in PC, phosphocholine and choline were not determined however.

There are a number of possible explanations for the stimulation of the cytidylyltransferase in experimental cytosol and some of the possibilities involve the presence or absence of an enzyme modulator, covalent modification of the enzyme or an increase in the amount of enzyme molecules. These three possibilities were explored in the succeeding experiments. The presence or absence of a soluble low molecular weight modulator was first tested. Low molecular weight molecules were removed

Table 21

Degradation of ^{14}C-labelled CDP-choline by Cytosol and Microsomes from Normal and Cholesterol/Cholate-Fed Rats

	dpm Remaining in CDP-Choline	
	Control Samples	Experimental Samples
Boiled Cytosol	36,808	34,664
Cytosol	36,443	37,565
Boiled Microsomes	38,580	39,417
Microsomes	20,052	21,765

The conditions used in this experiment are given in the text.

from cytosols and the activities of the cytidylyltransferase before and after ultrafiltration were measured. If the specific activities of the enzyme in control and experimental cytosols after ultrafiltration were similar, then the stimulation of enzyme activity could be due to the presence of a small soluble modulator in either of the cytosols. Specific activities of the enzyme in both cytosols increased by about 2-fold after ultrafiltration and this was in part due to the loss of 30% of total cytosolic protein; however, the ultrafiltrated cytosol from experimental animals still had 2 times more cytidylyltransferase activity than control samples.

When control and experimental cytosols were kept at 4°C, the activity of the cytidylyltransferase was markedly stimulated. After three days, enzyme activity was maximally stimulated and the difference in cytidylyltransferase activities in control and experimental cytosols was essentially eliminated (Fig. 12). The 2-fold difference in enzyme activity observed in fresh cytosol was reduced to a 10% difference after 3 days of incubation at 4°C. Previous studies have shown that the stimulation of cytidylyltransferase activity during storage of cytosol at 4°C was probably due to a rise in LPE (25). The increase in LPE could be accounted for by a small amount of phospholipase A which was present in cytosol. In the present experiment, the stimulation of cytidylyltransferase activities in control and experimental cytosols was most probably due to an increase in the concentration of LPE in the cytosols. The difference in the activities of the cytidylyltransferase in control and experimental cytosols observed in fresh cytosol was masked by the five fold stimulation in cytidylyltransferase activity.

Since phosphoplipids are potent activators of the cytidylyltransferase, cytosols from the two groups of rats were assayed for enzyme activity in the presence of various amounts of phospholipid (Fig. 13). At saturating

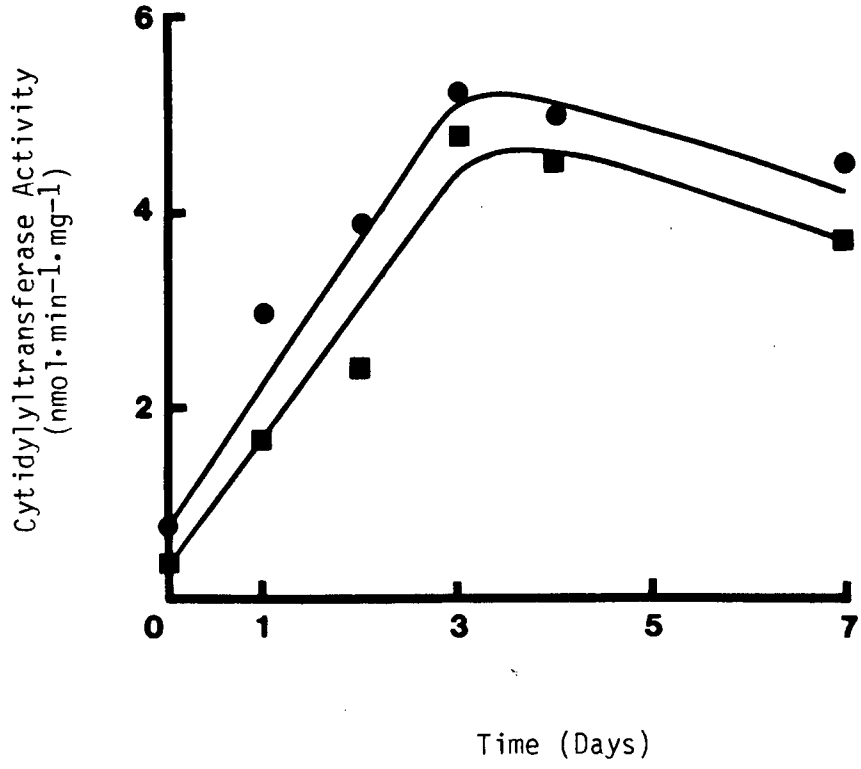


Figure 12. Activation of Phosphocholine Cytidylyltransferase Activity in Control (■) and Experimental (●) Cytosols by incubation at 4°C.

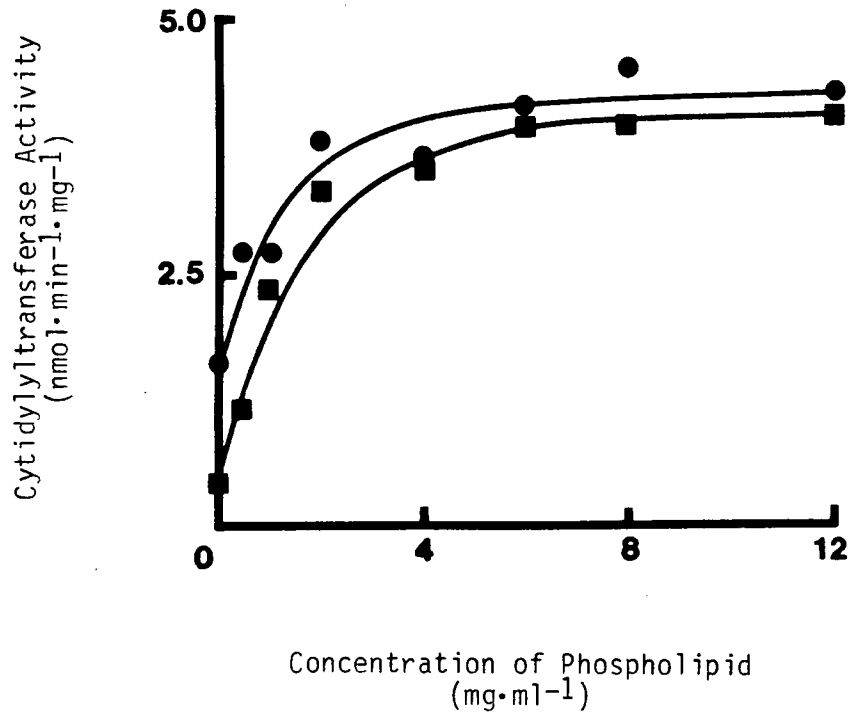


Figure 13. Activation of Phosphocholine Cytidylyltransferase in Cytosol from Control (■) and Cholesterol/Cholate-Fed (●) Rats. The enzyme in cytosol (10 µl) was assayed in the presence of various amounts of phospholipid from rat liver.

levels of phospholipid, the difference in cytosolic enzyme activities was eliminated. This result implied that the amount of enzyme in the two cytosols was the same and that an activator of the cytidylyltransferase was present in the cytosol from cholesterol/cholic acid-fed rats. Further studies were directed toward these two possibilities.

5. Immunotitration of Phosphocholine Cytidylyltransferase

The cytidylyltransferase in cytosol was titrated with immunoglobulin from normal and immunized rabbits. Some nonspecific inhibition of enzyme activity was observed when control gamma globulin G was incubated with cytosol. This inhibition was probably due to the binding of phospholipid in the cytosol by the gamma globulin since the inhibition was relieved by the addition of phospholipids to the assay tubes (Fig. 14). Hence, 1.6 mg of phospholipids was added to each assay tube for the immunotitration experiments.

The cytidylyltransferase was incubated with gamma globulin at different pH for 8 h at 4°C (Fig. 15). The antigen-antibody complex was sedimented by centrifugation at 5,000 x g for 30 min. The inhibition of the cytidylyltransferase activity was constant between pH 7.5 and 9.0 which indicates that the antibody-antigen complex was not formed by non-specific electrostatic interactions.

The amount of enzyme present in control and cholesterol/cholic acid-fed rat liver cytosols was measured by immunotitration. Cytosolic protein (0.35 mg) was incubated with various amounts of immunoglobulins from normal and immunized rabbits. After centrifugation the supernatant was carefully removed and assayed for cytidylyltransferase activity (Fig. 16). The amount of antibodies required to precipitate half of the enzyme activity was the same for control and cholesterol/cholic acid-fed rat liver cytosol

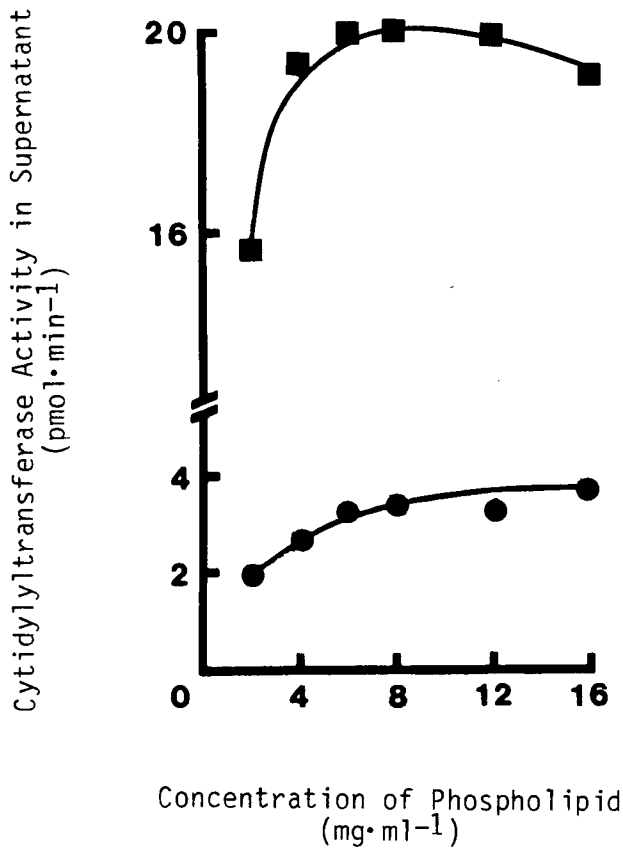


Figure 14. Stimulation by Rat Liver Phospholipids of Phosphocholine Cytidylyltransferase which Remained in the Supernatant after Immunoprecipitation. The incubation contained 0.6 mg of cytosolic protein and 2.2 mg of control (■) or anti-cytidylyltransferase (●) antibodies. Other conditions were described in the Materials and Methods section.

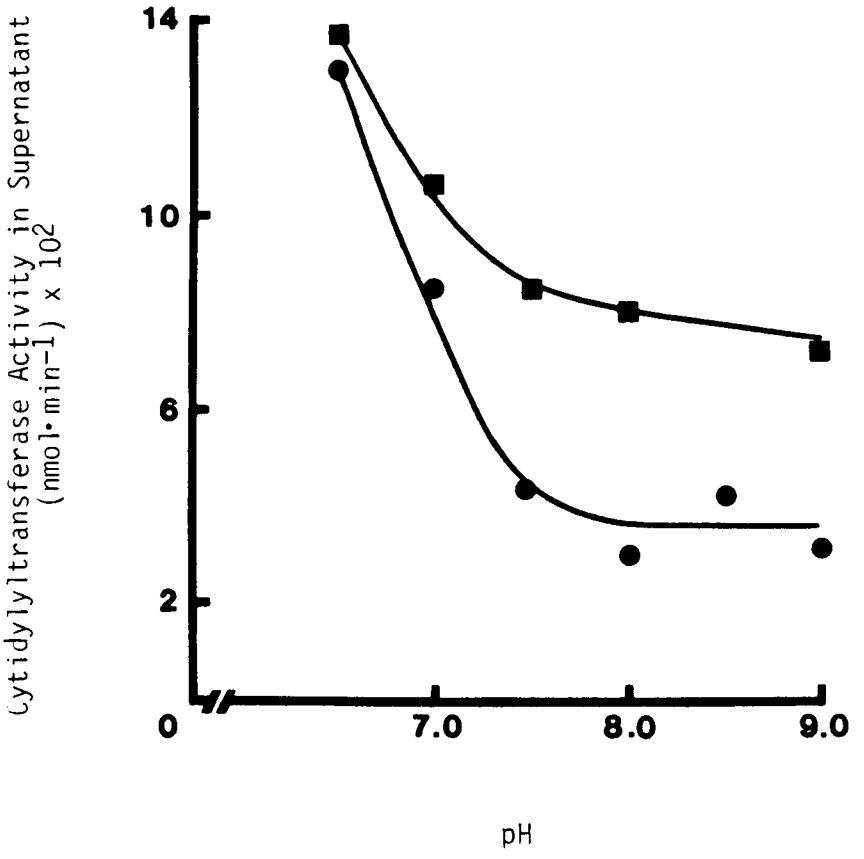


Figure 15. Precipitation of Phosphocholine Cytidylyltransferase by Control (■) and Anti-Cytidylyltransferase (●) Antibodies at Various pH. The enzyme in 0.42 mg of cytosolic protein was reacted with 1.0 mg of antibodies and the amount of enzyme remaining in the supernatant was assayed for activity. All other conditions were described in the Materials and Methods section.

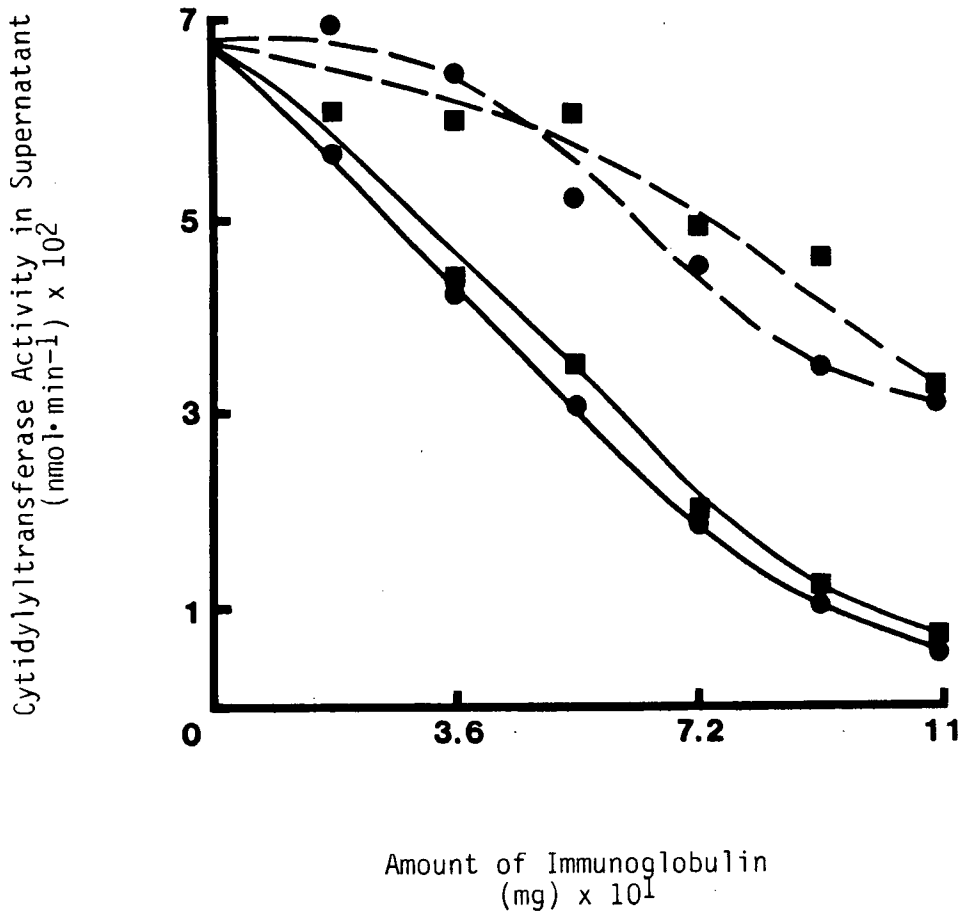


Figure 16. Immunotitration of Phosphocholine Cytidylyltransferase in Cytosols from Control (■) and Cholesterol/Cholate-Fed (●) Rats with Control (▬ ▬) and Anti-Cytidylyltransferase (▬▬) antibody. Each cytosol was incubated with various amounts of immunoglobulins from normal and immunized rabbits. After 8 h incubation at 4°C in 60 mM sodium phosphate buffer, pH 8.0, the mixture was centrifuged at 5,000 x g for 30 min and the supernatant was assayed for cytidylyltransferase in the presence of 1.6 mg of phospholipid.

(Fig. 17). It appears that the two cytosols contain the same amount of enzyme. The amount of enzyme found in liver cytosols from rats fed the basal atherogenic diet was titrated in the same way with another preparation of antibodies and a similar result was obtained.

6. Characterization of the Modulator of Phosphocholine Cytidylyltransferase

The immunotitration experiments showed that the stimulation of the cytidylyltransferase was not due to an increase in enzyme protein and the experiments which involved the activation of the cytidylyltransferase by phospholipids suggested that a phospholipid modulator was responsible for the stimulation of the enzyme. If this were indeed the case, then elimination of phospholipids from the cytosols followed by reactivation with an identical preparation of phospholipids should abolish the difference in enzyme activities. Experimentally, phospholipids in the control and experimental cytosols were digested with phospholipase C and the cytidylyltransferase activities in both cytosols were completely lost after 1 h at 37°C (Fig. 18). The activity of the cytidylyltransferase in experimental cytosol appear to be more sensitive to phospholipase than enzyme activity in control cytosol. The attempts to reactivate the phospholipase treated enzymes, however, were unsuccessful and the identity of the enzyme modulator remained unresolved.

If the modulator were a phospholipid, then proteolytic treatment of the cytosols should not damage the modulator and its effects should still be observed upon readdition of partially purified L-form. Rat liver cytosol was incubated with trypsin which eliminated all endogenous cytidylyltransferase activity. After trypsin inhibitor was added, the same amount of delipidated form or partially purified L-form of the enzyme was added to the two cytosols and twice as much activity was detected with the

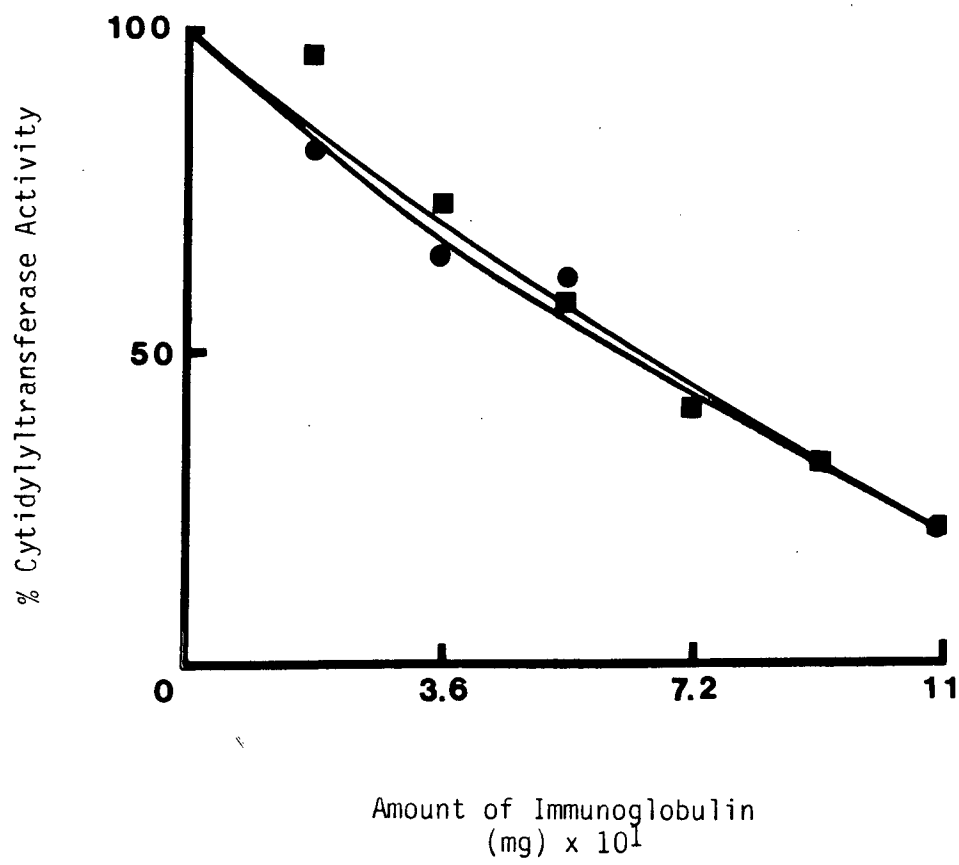


Figure 17. Immunochemical Quantitation of Cytosolic Phosphocholine Cytidylyltransferase from Normal (■) and Cholesterol/Cholate-Fed (●) rats. See legend to Figure 16.

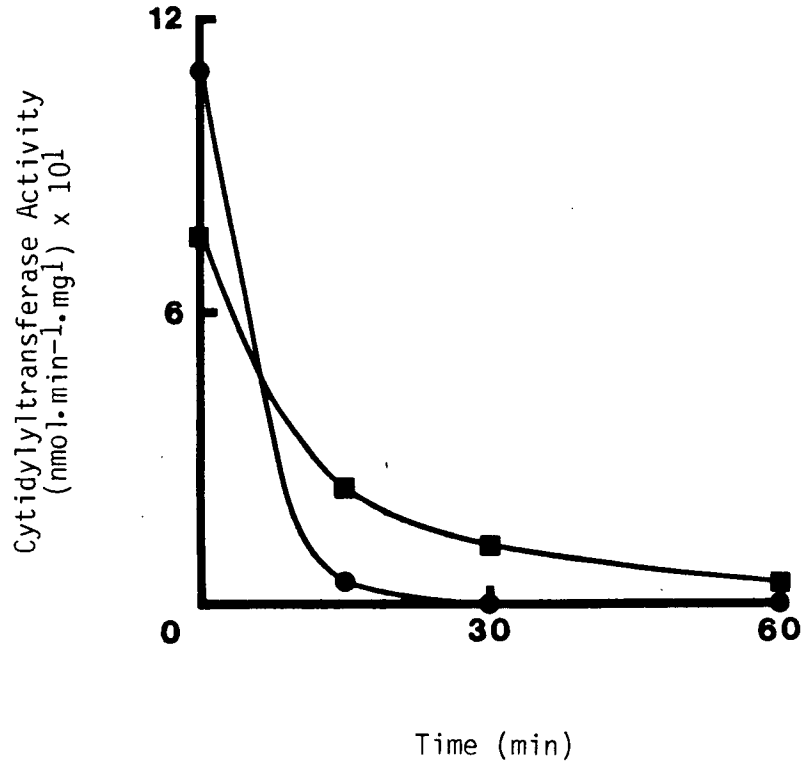


Figure 18. Effect of Phospholipase C (Cl. welchii) Digestion on the Activity of Phosphocholine Cytidylyltransferase in Cytosols from Normal (■) and Cholesterol/Cholate-Fed (●) Rats. Control and experimental cytosols (1 ml) were incubated with 10 mM $CaCl_2$ and 2 units (μmol/min) of phospholipase C at 37°C. At various times, aliquots of the cytosols were removed and phospholipase C activity was inhibited by 30 mM EGTA. The aliquots were kept at 4°C until the cytidylyltransferase activity was measured.

cytosol from the experimental rats. The specific activities of the added enzymes though varied from one preparation to another. This experiment indicated the presence of a trypsin-resistant activator.

The inactivated cytosols were chromatographed on Sepharose 6B and the fractions assayed in the presence of partially purified L-form. An activator eluted near the void volume and the cytosol from cholesterol/cholic acid-fed rats contained twice as much as the control cytosol (Fig. 19). When the fractions that contained the activator were extracted with $CHCl_3/CH_3OH$ (2/1), all the activator was recovered in the organic phase (Table 22). The results strongly suggested that the activator in cytosol is a lipid.

A larger amount of cytosolic lipid was prepared from a 25% $(NH_4)_2SO_4$ extract of trypsin-inactivated cytosol and greater than 90% of cytosolic phospholipid was recovered by this procedure. The lipid extract from cholesterol/cholate-fed rats had more phospholipid ($P < 0.05$) than the lipid extract from control rats [110 $\pm$ 53 (S.D.) and 56 $\pm$ 20 (S.D.) µg/ml of cytosol as measured in 6 different batches of cytosols] and was more effective in stimulation of the cytidylyltransferase (Fig. 20). The classes of phospholipids present in the cytosols were determined in two experiments and the composition was similar in both groups (Table 23).

In order to determine which phospholipid in cytosol was responsible for the activation of the cytidylyltransferase, a portion of each extract was fractionated by thin layer chromatography on silica gel. Lipids were eluted and areas on the plate with no detectable lipid were also extracted. The lipids were added to assay tubes, the solvent evaporated and activity of partially purified L-form was determined (Table 24). In the experiment using a neutral solvent system (I), lipid from only one area

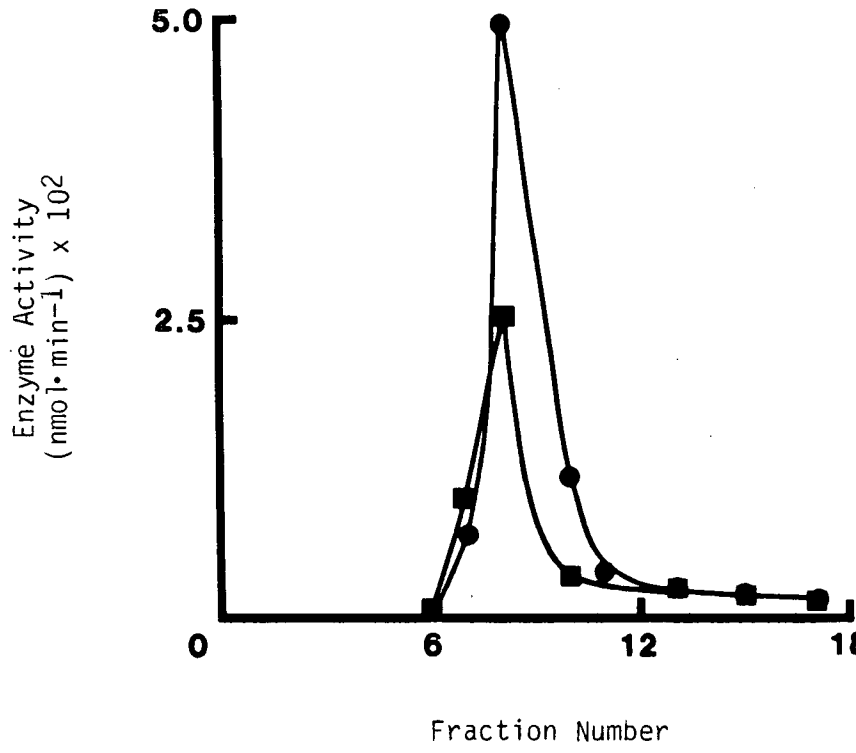


Figure 19. Chromatography of Trypsin-Treated Cytosol from Control (■) and Cholesterol/Cholate-Fed (●) Rats. Four ml cytosol (which had been incubated with 0.1 mg trypsin/ml for 15 min at 37°C) from control and cholesterol-fed rats was applied to a column of Sepharose 6B and 7 ml fractions were collected. Partially purified L-form of the cytidylyltransferase was assayed in the presence of 40 µl of each fraction.

Table 22

Stimulation of Delipidated Cytidylyltransferase by the Water-Soluble and Lipid-Soluble Components of Column Fractions Which Contained the Activator

	Cytidylyltransferase (nmol/min)
Column Fractions from Control Cytosol	
Water-Soluble Fraction	0
Lipid-Soluble Fraction	0.021
Water-Soluble and Lipid-Soluble Fractions	0.023
Column Fractions from Experimental Cytosol	
Water-Soluble Fraction	0
Lipid-Soluble Fraction	0.074
Water-Soluble and Lipid-Soluble Fractions	0.050

The amount of water- and lipid-soluble materials obtained from column fractions were used to stimulate 20 µl of delipidated enzyme.

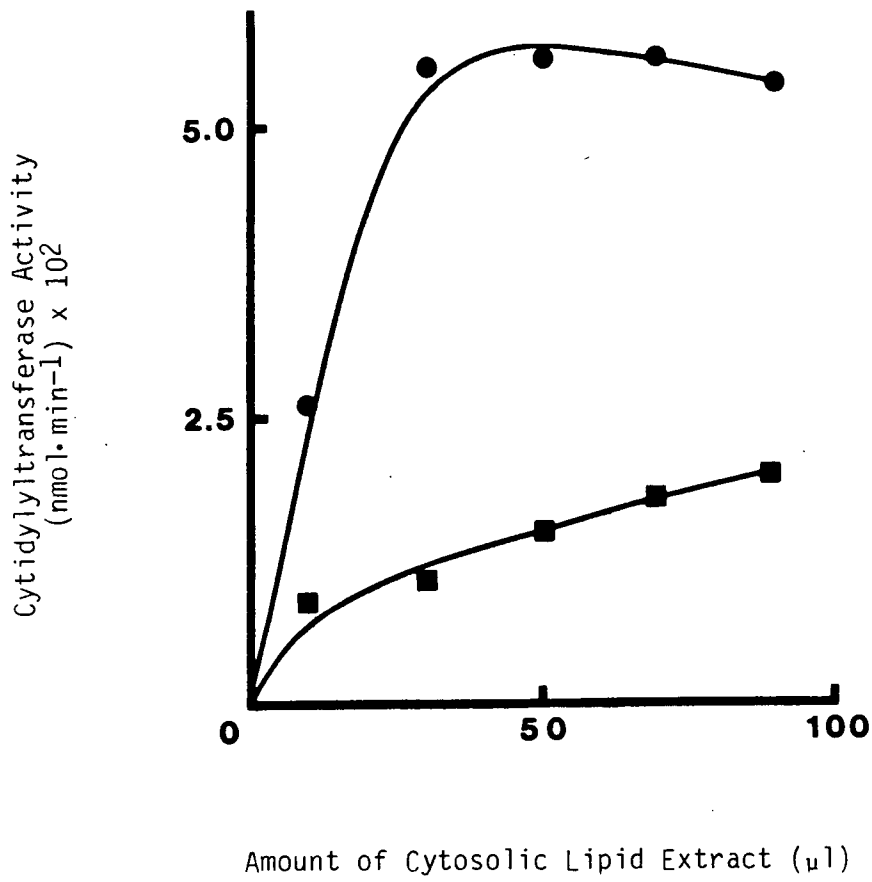


Figure 20. Stimulation of Partially Purified Cytidylyltransferase by Lipid Extracts from Control (■) and Cholesterol/Cholate-Fed (●) Rat Liver Cytosol. The lipid was extracted from cytosol as described in the Materials and Methods section. Lipids from equivalent volumes of the cytosols were added to assay tubes, the organic solvent evaporated and the activity of the enzyme was determined. The concentrations of phospholipid in the extracts were 0.43 and 0.93 mg/ml for the control and cholesterol/cholate-fed group, respectively.

Table 23

Composition of Phospholipids in Cytosols from

Normal and Hypercholesterolemic Rats

	Amount of Phospholipid (μg ml cytosol^{-1})	
	Controls	Experimental
Phosphatidylcholine	18 (63)	73 (74)
Phosphatidylethanolamine	6 (20)	13 (13)
Phosphatidylserine	2 (7)	6 (6)
Lysophosphatidylethanolamine	2.8 (10)	6.9 (7)

Lipids from control and experimental cytosols were fractionated by 2-dimensional thin-layer chromatography (solvent system IV). Numbers in brackets indicate the mole percent of the phospholipid with respect to the total amount

Table 24

Stimulation of Partially Purified L-form by Cytosolic Phospholipids from Normal and Hypercholesterolemic Rats

	Solvent System I $CHCl_3/CH_3OH/H_2O$ (70/30/4)			Solvent System II $CHl_3/CH_3OH/CH_3COOH/H_2O$ (100/60/16/8)		
	Enzyme Activity (nmol/min) X10[1]					
	RF	Control	Experimental	Rf	Control	Experimental
No Addition	-	0.33	0.33	-	0.10	0.10
PC	0.27	0.16	0.27	0.32	0.28	0.58
PE	0.59	0.32	0.34	0.77	0.12	0.22
LPC	0.13	0.32	0.34	0.10	-	-
LPE	0.31	0.72	0.88	0.41	0.20	0.27
SP	0.13					
Origin	0-0.05	0.22	0.26	-	-	-
Unknowns						
1	0.18	0.30	0.27	0.45	0.13	0.17
2	0.21	0.11	0.19	0.50	0.13	0.21
3	0.36	0.35	0.38	0.66	0.13	0.13
4	0.81	0.12	0.11	-	-	-
Spaces						
A	0.35-0.51	0.13	0.10	0-0.29	0.07	0.15
B	0.57	0.28	0.28	0.39-0.48	0.11	0.14
C	-	-	-	0.51-0.63	0.08	0.10
D	-	-	-	0.68-0.74	0.12	0.19
E	-	-	-	0.79-1.0	0.03	0.06

Lipids from 2 ml of control and 1 ml of experimental cytosols were chromatographed with solvent system I while the lipids from 1.5 ml of each cytosol were used in another experiment with solvent system II. The details were described in the Materials and Methods section. Unknowns refer to unidentified bands which were detected by iodine staining while the spaces refer to areas on the plate which did not contain any iodine-positive material.

on the plate stimulated the partially purified enzyme and this lipid chromatographed with LPE. In this experiment lipids from 2 ml of control and 1 ml of experimental cytosols were used and when this difference was accounted for, the lipid activator from experimental cytosol was 2 times more active than control in stimulating the added enzyme (1.43 and 0.72 nmol/min for the experimental and control lipids, respectively). This experiment was repeated with another set of cytosols except that lipids from an equivalent amount of normal and experimental cytosols were used and an acidic solvent system (II) was employed. The lipids that chromatographed with PC and LPE were most active stimulators of the enzyme. Previous studies showed that PC does not activate the cytidylyltransferase (25) and the stimulation observed in this experiment could be due to contamination with LPE. Phosphatidylethanolamine and an unknown lipid band from experimental cytosol were more active in stimulating the enzyme than the corresponding lipid bands from control cytosols and there were no readily obvious explanations for these results. The recovery from silica of the ability of the cytosolic lipids to stimulate the cytidylyltransferase was determined (Table 25) and 70% or more was lost whether the neutral or acidic solvent system was used. The low recoveries made it difficult to interpret the results given in Table 24.

Results from a previous study suggested that LPE might be a physiologically important modulator of the cytidylyltransferase (25). Thus, the stimulation of enzyme activity by palmitoyl-LPE before and after spotting on and elution from silica was tested. Standard palmitoyl-LPE did not significantly stimulate cytidylyltransferase activity. However, the same lipid which had been eluted from silica was very much more potent in stimulating the enzyme (Fig. 21). This surprising result could be

Table 25

Recovery of the Ability of Cytosolic Lipids to Stimulate Cytidylyltransferase Activity After Thin-Layer Chromatography on Silicic Acid

	Stimulation of Cytidylyltransferase Activity ($nmol \cdot min^{-1}$)	
	Applied	Recovered (percent recovered)
Solvent System I		
Control	0.17	0.04 (23%)
Experimental	0.98	0.06 (6%)
Solvent System II		
Control	0.14	0.04 (31%)
Experimental	0.40	0.12 (30%)

Recovery was calculated by the sum of the ability of all the lipid components detected by iodine staining to activate the cytidylyltransferase. The ability to stimulate enzyme activity was determined from the difference in cytidylyltransferase activities in the presence and absence of lipid. The other details of the experiments were given in Table 24.

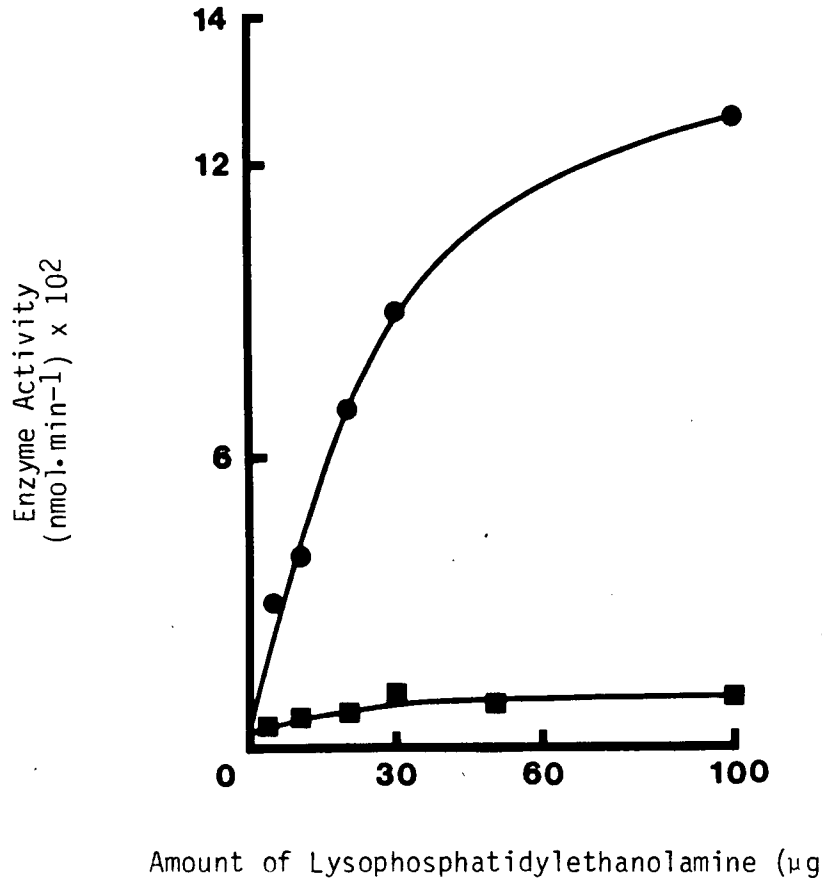


Figure 21. Stimulation of Phosphocholine Cytidylyltransferase Activity by Standard Palmitoyl-Lysophosphatidylethanolamine and by the Same Lipid which Had Been Applied on and Eluted from Silica. Various amounts of palmitoyl-LPE were used to stimulate cytidylyltransferase activity. The same amounts of this lipid were applied on a TLC plate and immediately extracted from silica with $CHCl_3/CH_3OH$ (2/1). The eluted lipid (●) and stock LPE (■) were subsequently used to stimulate enzyme activity and cytidylyltransferase activity was measured as described in the Materials and Methods section.

explained in a number of ways but the most likely possibility appears to be degradation of LPE during the spotting and elution procedure. This idea was supported by results from an experiment where eluted and stock palmitoyl-LPE were analyzed by TLC. The stock lipid contained a major component (Rf 0.16); presumably LPE, and a minor component (Rf 0.34) (Table 26). In contrast, LPE which had been eluted from silica resolved into 5 components. Two bands chromatographed in the same area as LPE and three other components, which had high mobilities, were also detected (Table 26). It appears that a portion of LPE was degraded during elution with $CHCl_3/CH_3OH$ (2/1) and a degradation product may be responsible for stimulation of cytidylyltransferase activity.

The identity of the activator remains unresolved at the present time. There is no convincing evidence which indicates that LPE is the activator and in fact, the difference in the effect of elution from silica on the stimulation of cytidylyltransferase activity by cytosolic lipids and LPE suggest that the activator is not a lysolipid. Furthermore, it is apparent that silicic acid chromatography is not suitable for use in further experiments and various other means of fractionation need to be employed before the identity of the activator can be clearly established.

7. Analysis of Palmitoyl-lysophosphatidylethanolamine by Mass Spectrometry

In the course of identifying the activator of the cytidylyltransferase, mass spectrometry was used to determine whether the activator and LPE had similar chemical structures.

Phospholipids had been previously analyzed by field desorption (114) and electron ionization (110) mass spectrometry. By field desorption mass spectrometry, palmitoyl- as well as oleoyl-LPE show fragments which appear to correspond to phosphoethanolamine, M-RCOO and

Table 26

Analysis of Eluted and Stock Palmitoyl-lysophosphatidylethanolamine by Thin Layer Chromatography

	Number of Bands	Rf's
Eluted LPE	6	0.14, 0.17, 0.32, 0.75, 0.82, 0.90
Stock LPE	2	0.16, 0.34 (minor)

Standard palmitoyllysophosphatidylethanolamine (0.20 mg) was applied on a TLC plate and was immediately eluted from silica by $CHCl_3/CH_3OH$ (2/1). The eluted lipid was applied to a TLC plate, along with stock lipid, and developed in $CHCl_3/CH_3OH/H_2O$ (65/25/4). The lipids were subsequently visualized by iodine staining.

glycerophosphorylethanolamine as well as an intense M + H peak (114). Field desorption mass spectrometry was the method of choice but because it was not readily available, electron ionization mass spectrometry was used instead.

Lysophosphatidylethanolamine was made volatile by silylation and a typical spectrum is shown in Fig. 22. The molecular ion was not found but several characteristic ions formed from the fatty acid and ethanolamine moieties were evident. Fragment 174 was indicative of ethanolamine (110) while fragments 211 and 239 appear to be derived from palmitic acid (Table 27). Fragments 627, 538, 449 and 385 may be useful for identification but not fragment 357 which was found in the spectra of various other silylated derivatives of phospholipids (110).

Several attempts were made to analyze LPE isolated from cytosol by mass spectrometry and in every instance, the spectrum obtained was very weak and was not suitable for analysis. And in light of the results which showed that LPE was partially degraded during elution from silica, this experiment was terminated.

8. The Fatty Acid Composition and the Amount of Lysophosphatidylethanolamine in Cytosol

The nature of the fatty acid of LPE markedly affects its ability to stimulate the cytidylyltransferase (25). Hence, the fatty acid residues from LPE were analyzed and were found to be similar in both groups of rats - 70% $C_{16:0}$ and 30% $C_{18:0}$ fatty acids. The total amount of LPE recovered from cholesterol/cholic acid-fed rat liver cytosol was greater than the amount isolated from control cytosol (6.9 ± 0.6 and 2.8 ± 0.8 µg/ml cytosol, respectively). The significance of the elevation of LPE in experimental cytosol remains to be established

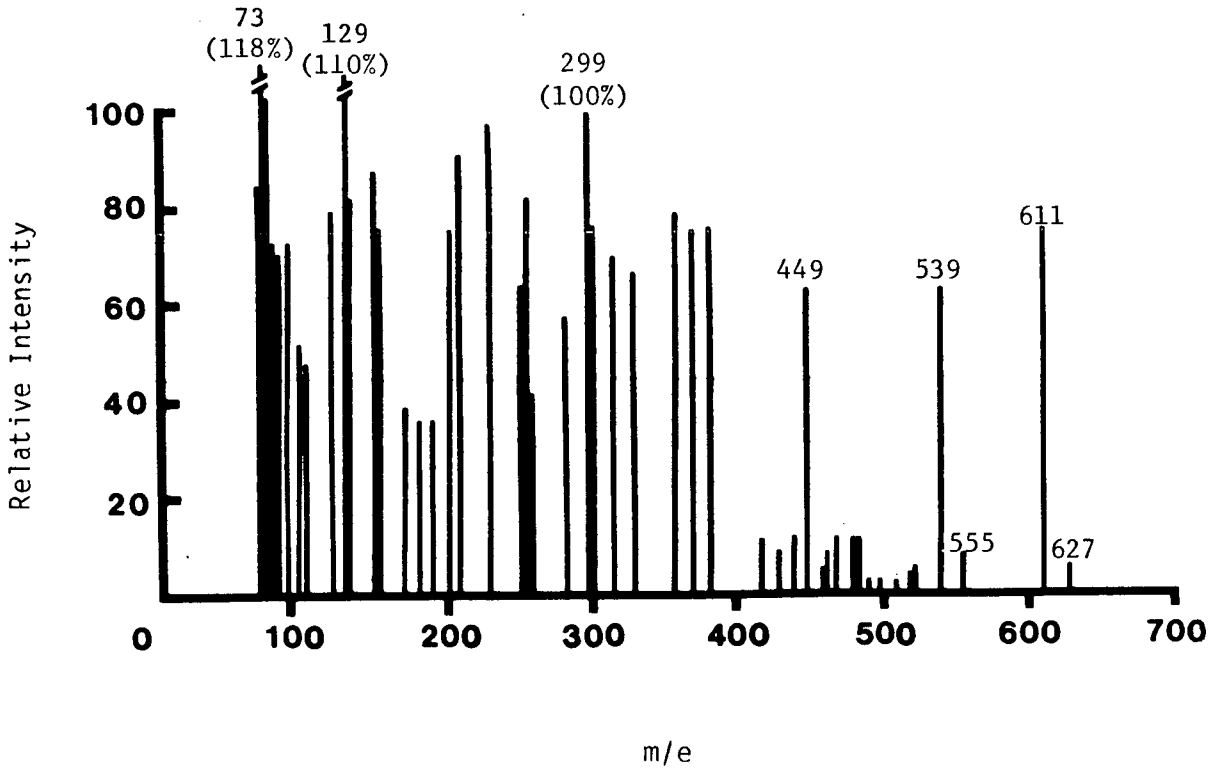
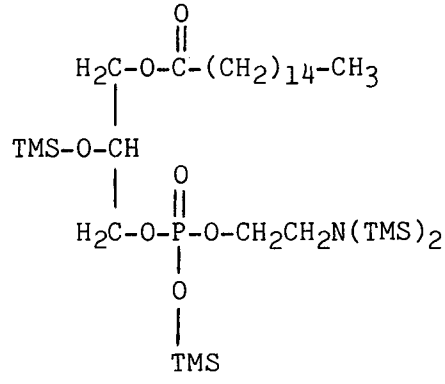
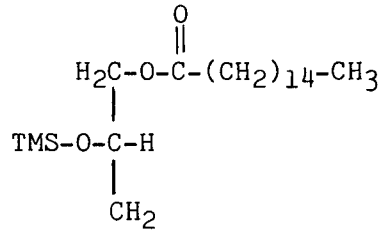


Figure 22. Mass Spectra of the Silylated Derivative of Palmitoyl Lysophosphatidylethanolamine. The sample was prepared by silylating 0.25 mg of standard palmitoyl-LPE and analysis was performed in an AEI-902 Mass Spectrometer. Other conditions are described in the Materials and Methods section.

Table 27

Structure and Predicted Fragments of the Silylated Derivative of Palmitoyl-lysophosphatidylethanolamine

M/E	Predicted Structure
741	
627	M-114($CHCH_2NTMSi$)
611	M-115(CH_2CH_2NTMSi)-15($-CH_3$)
555	M-186
538	M-188($CH_2CH_2N(TMSi)_2$)-15(CH_3)
449	M-203-90(HO-TMS)
385	
371	M-356-14(CH_2)

(cont'd)

M/E	Predicted Structure
341	357-16
239	$CH_3(CH_2)_{14}\overset{\overset{O}{\|\|}}{C}$
211	$CH_3(CH_2)_{14}$
174	$CH_2N(TMS)_2$
146 (147)	$TMS\text{-}O\text{-}\underset{\underset{CH_2}{\|}}{\overset{\overset{CH_2\text{-}O}{\|}}{CH}}$
103	$TMS\text{-}O\text{-}CH_2$

9. Lysophosphatidylethanolamine In Cytosol was not Generated During the Preparation of Subcellular Fractions

The LPE found in the cytosol could possibly be generated *in vitro* during the preparation of the cytosol and two experiments were done to test this possibility. It is well established that activity of phospholipases is dependent on the presence of Ca^{++} ions. Thus, control and cholesterol/cholate-fed rat liver cytosols were prepared in the presence of 10 mM EDTA and the cytidylyltransferase activities were measured and found to be 0.41 and 0.81 nmol/min/mg, respectively, not different from the activities found in cytosols prepared without EDTA (Table 20).

The second experiment involved the addition of ^{14}C-labelled PE, in the form of PE/rat liver phospholipid (1/1; w/w) sonicated vesicles, to the tissue and saline prior to homogenization. Of the 23,000 dpm per ml homogenate added, approximately 12,500 dpm were recovered in PE per ml of cytosol, and in both control and experimental lipid extracts, no radioactivity was found to be associated with LPE after fractionation of the phospholipids by 2-D chromatography. The unrecovered radioactivity was most probably associated with vesicles which were sedimented during the preparation of cytosol. It is possible that the added PE vesicles may be poor substrates for tissue phospholipase A when compared to endogenous PE. Nevertheless, the results strongly indicated that LPE found in cytosol was not generated *in vitro*.

IV. Cholesterol/Cholate Feeding: In Vivo Studies

The *in vitro* studies showed that the activity of the cytidylyltransferase, when measured under optimal conditions, was stimulated 2-fold in the livers of rats fed a high cholesterol/cholic acid diet and the stimulation of enzyme activity was not due to an increase in enzyme protein but to an activation of the enzyme by a lipid. The studies up to this point were limited to experiments with subcellular fractions and the actual status of PC synthesis in the intact liver remains unclear.

The *in vivo* studies were designed to correlate the 2-fold stimulation of the cytidylyltransferase *in vitro* with the rate of PC biosynthesis *in vivo*. The rate of biosynthesis in liver was measured by monitoring the rate of incorporation of [*Me*-^{3}H]choline into PC and the following sections describe the results.

1. *The Amount of Radioactivity in Choline, Phosphocholine, CDP-Choline, Betaine and Phosphatidylcholine at Several Time Periods After Intraportal Injection of Labelled Choline*

Radioactive choline was introduced into the rats by the portal vein as described in the Materials and Methods section. In the first experiment, 0.5 mM choline was injected (Fig. 23) and as expected, radioactivity incorporated into the various pools. The amount of radioactivity in choline reached a peak before 60 sec and this was followed by maximum incorporation of radioactivity into phosphocholine and betaine shortly after. Radioactivity in phosphocholine increased very rapidly and reached a maximum value within 1 min after injection. The radioactivity in phosphocholine remained almost unchanged between 1 and 4 min after injection and the rate of PC biosynthesis was measured within this time period in a later experiment. [*Me*-^{3}H]choline was incorporated into PC in a linear manner and half of the total injected choline was oxidized to betaine while a negligible amount (0.1%) was incorporated into the CDP-choline pool.

When [*Me*-^{3}H]choline was used in these experiments, there were two possible ways in which radioactivity could be incorporated into PC. Aside from the direct incorporation of labelled choline by the *de novo* pathway, radioactivity could also be introduced via the PE methylation pathway. Methyl labelled *S*-adenosylmethionine could be made *in vivo* from labelled

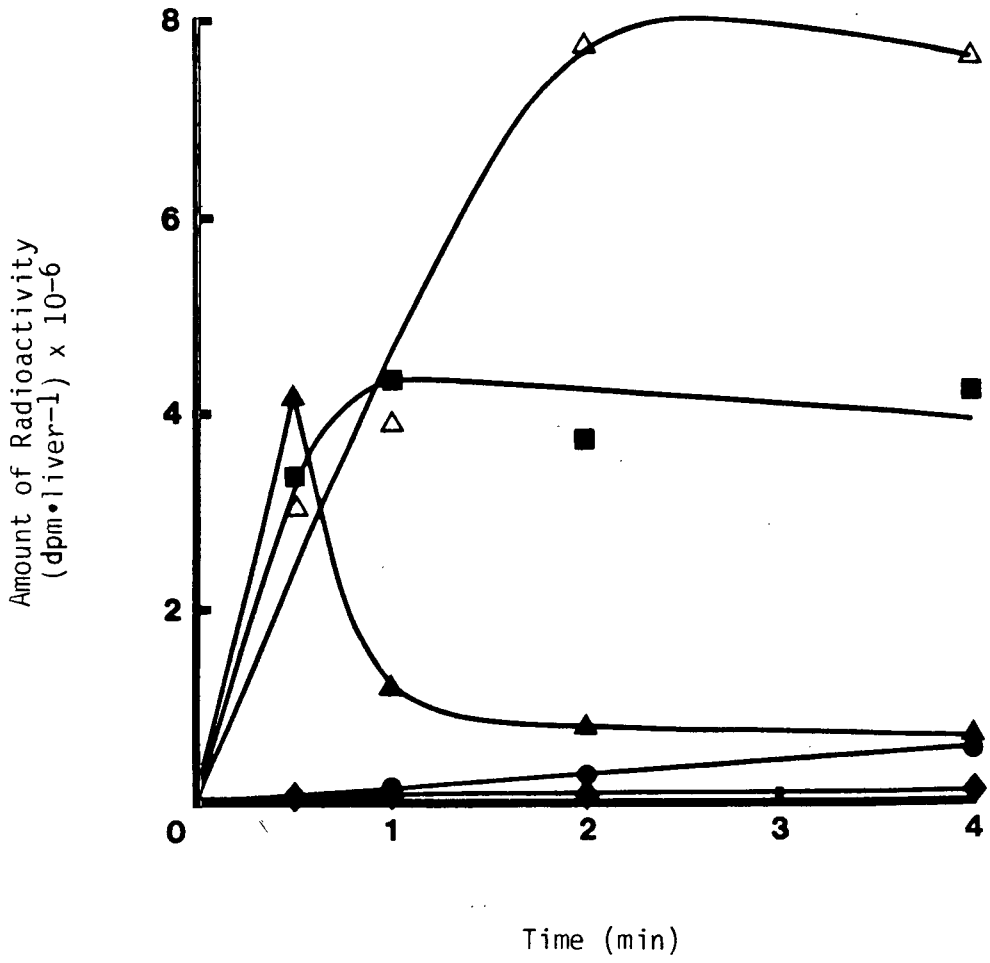


Figure 23. Incorporation of [Me-^{3}H]Choline into Hepatic Pools of Choline (▲), Phosphocholine (■), CDP-Choline (◆), Betaine (△) and Phosphatidylcholine (●). A 0.20 ml solution of labelled choline (0.5 mM, 360 µCi/µmol) was injected intraportally into control rats and the amount of radioactivity incorporated into various pools was determined as described in the Materials and Methods section.

methionine which could be synthesized by methylation of homocysteine with [Me-^{3}H]betaine as methyl donor. Therefore, to eliminate any possible incorporation of radioactivity into PC by the PE methylation pathway, ^{14}C-backbone labelled choline was used in the next experiment. The incorporation of the injected label into livers from control and hypercholesterolemic rats at the time of sampling varied between 30 and 50%. At 30 sec after injection, radioactivity in choline and betaine in the experimental group was only half that found in controls, while the incorporation of radioactivity into phosphocholine and CDP-choline was markedly increased in the experimental livers. In addition, incorporation of radioactivity into PC was three times higher in the experimental group (Table 28). These results suggested that hepatic PC biosynthesis was stimulated in the experimental animals which appear preferentially to channel injected choline into phospholipid synthesis.

The concentration of choline in the injection solution could affect the uptake of choline into the liver. Under normal circumstances, the concentration of choline in rat serum was between 3.6 and 21.4 μM (115). Therefore, in order to simulate *in vivo* conditions as closely as possible, choline concentration in the injection solution was adjusted to between 50 and 100 μM in all subsequent experiments. Commercially available ^{14}C-labelled choline had very low specific radioactivity and was not used in any further experiments. Instead, [Me-^{3}H]choline which had a much higher specific activity was used.

2. An Estimate of the Rate of Phosphatidylcholine Biosynthesis in Livers from Control and Cholesterol/Cholate-fed Rats

The rate of PC biosynthesis was determined by calculating the rate of conversion of phosphocholine to PC. A 100 μl solution of 60 μM

Table 28

The Incorporation of [1,2-^{14}C]Choline into Various Compounds in the Livers from Control and Cholesterol/Cholate-Fed Rats.

	Incorporated Radioactivity (dpm/Liver) x 10^{-4}				
	Choline	Phospho-choline	CDP-choline	Betaine	Phosphatidyl-choline
Control	70	90	0.7	168	7.2
Experimental	39	170	8.6	82	21

Each of a group of 3 control and 3 experimental rats was injected with a 0.25 ml solution of 4.5 mM [1,2-^{14}C]choline (3 µCi/µmol) containing 3.3 µCi of radioactivity. Both groups incorporated between 30 and 50% of the total injected dose and the amount of radioactivity incorporated into various pools was determined 30 sec post injection.

[Me-^{3}H]choline was injected intraportally into control and experimental rats and the amount of radioactivity incorporated into phosphocholine, PC and other related compounds at 1, 2 and 3 min post-injection were measured (Table 29 and 30). Livers from both groups incorporated about half of the injected dose and the same proportion of the incorporated radioactivity was found in betaine at all time points. In contrast, when 4.5 mM choline was used in the injection solution as in a previous experiment (Table 28), the experimental group had only half the amount of radioactivity in betaine when compared to the control group. The data seem to suggest that the proportion of exogenous choline oxidized to betaine in the liver depends on the rate of hepatic PC synthesis as well as on the abundance of choline.

Control and experimental rats incorporated radioactivity into choline and phosphocholine in a similar manner at 1 and 2 min (Table 29). Incorporation of radioactivity into PC was elevated by about 2-fold in the experimental rats at 1 and 2 min. At 3 min, however, the control data had very large experimental error and it was difficult to determine if a real difference existed between control and experimental values. Therefore, the rate of PC biosynthesis was determined between 1 and 2 min.

An estimate of the rate of PC synthesis in rat liver was calculated from the specific radioactivity of phosphocholine and the net amount of radioactivity incorporated into PC between 1 and 2 min post-injection. The rates calculated in this way were directly comparable since the percent of the injected dose incorporated into the livers of control and experimental rats were similar. The calculation of the rate was based on several assumptions. First, the rate of PC synthesis was assumed to be equal to the rate of conversion of phosphocholine to PC. Second, the specific radioactivity of CDP-choline and PC formed by the de novo pathway is equal

Table 29

Incorporation of Radioactivity into Various Choline-Containing Metabolites After Intraportal Injection of 60 μM [Me-^{3}H]Choline

Time (min)	Amount of Radioactivity (DPM x 10^{-6} $Liver^{-1}$)		
	Choline	Phosphocholine	Phosphatidyl-choline
Control			
1	9.4 ± 2.8 (7)	36.0 ± 14.6 (7)	0.98 ± 0.29 (7)
2	4.4 ± 2.3 (8)	31.4 ± 12.5 (8)	3.04 ± 1.93 (8)
3	3.2 ± 2.3 (7)	27.0 ± 11.0 (7)	7.2 ± 7.2 (7)
Experimental			
1	11.5 ± 3.3 (7)	35.4 ± 12.1 (7)	1.82 ± 0.82 (7)
2	3.1 ± 1.6 (6)	31.6 ± 12.6 (6)	8.53 ± 2.47 (6)
3	5.4 ± 4.5 (8)	33.0 ± 11.3 (8)	8.4 ± 3.4 (8)

Brackets indicate the number of animals used. Values are given as mean ± S.D.

Table 30

Incorporation of Radioactivity into Betaine and the Percent of Injected Dose Incorporated After Intraportal Injection of 60 μM [Me-^{3}H]Choline

Time (min)	Percent of Dose Incorporated	Radioactivity in Betaine (dpm x 10^{-6} Liver)
Control		
1	66 ± 15 (7)	25.5 ± 7.8 (7)
2	51 ± 12 (8)	17.3 ± 5.0 (8)
3	53 ± 21 (8)	18.0 ± 10 (8)
Experimental		
1	65 ± 17 (7)	23.4 ± 6.3 (7)
2.	54 ± 20 (6)	16.4 ± 6.6 (6)
3.	59 ± 16 (8)	20.0 ± 7.7 (8)

Percent of dose incorporated was calculated by dividing the sum of the total amount of radioactivity incorporated into the upper and lower phases of the Bligh and Dyer extraction by the amount of radioactivity injected. The numbers in brackets indicate the number of animals used. Values are given as mean ± S.D.

to that of phosphocholine. And last, within this same time period, the incorporation of radioactivity into PC by the phospatidylethanolamine methylation pathway was assumed to be negligible.

Estimates of the rates of PC biosynthesis and the parameters used in the calculations are given in Table 31. The concentration of phosphocholine as well as the amount of radioactivity in the pool as expressed per liver was similar in both groups. Thus, the calculated specific radioactivity of phosphocholine was also similar. From the rate of incorporation of radioactivity into PC and the specific radioactivity of phosphocholine, the rates of synthesis were calculated. The estimated rate of PC biosynthesis in rats fed the high cholesterol/cholate diet was three times higher than in the controls and this correlated well with the 2- to 3-fold stimulation of the cytidylyltransferase activity observed in vitro.

Table 31

The Effect of Cholesterol/Cholate Feeding on the Rate of Phosphatidylcholine Biosynthesis

	Control	Treated
Phosphocholine concentration (nmoles liver^{-1})	3,574 ± 1,052 (22)	3,522 ± 988 (21)
Average Radioactivity in Phosphocholine (dpm x 10^{-6} liver^{-1})	33.7 (15)	33.5 (13)
Specific Radioactivity (dpm x 10^{-3} x nmole^{-1})	9.4	9.5
Rate of Incorporation of Radioactivity into PC (dpm liver^{-1} min^{-1} x 10^{-6})	2.1 (15)	6.7 (13)
Rate of PC Biosynthesis (nmoles liver^{-1} min^{-1})	223	705

Each rat was injected with 50 μCi of [Me^{3}H]choline and the incorporation of radioactivity into phosphocholine and phosphatidylcholine was measured. Control rats had slightly larger livers than experimental rats (3.2 ± 0.5 and 2.7 ± 0.4 g per liver respectively). The numbers in brackets indicate the number of animals used.

DISCUSSION

I. Phosphatidylcholine Biosynthesis in Rats Fed a Normal and Cholesterol/Cholate-Rich Diet

A linear relationship between plasma cholesterol and phospholipids suggested that the metabolism of cholesterol and phospholipids in the liver, where the majority of blood lipoproteins are synthesized, may be closely related. Induction of hypercholesterolemia by dietary means resulted in elevated levels of phospholipids in the plasma while infusion of phosphatides to raise the level of plasma phospholipids resulted in increased levels of plasma cholesterol (see Introduction). On the basis of these observations, PC biosynthesis was investigated in rats fed a cholesterol/cholate-rich diet.

Phosphatidylcholine biosynthesis was perturbed in hypercholesterolemic rats. The observed effects were specifically induced by the intake of cholesterol and cholate and not by any other nutritional factors since the control and experimental diets have very similar caloric content and composition.

Results from the present investigation indicate that young rats have a higher rate of PC synthesis than adults. The estimated rate of PC biosynthesis in control rats (Table 31) was similar to rates determined

by various other workers (117). However, the rate per g liver obtained in the present study with young rats, was almost 3-times higher than published values for adult rats (116). This finding, however, was in agreement with a previous observation that liver slices from 25-day old rats incorporated 50 to 100% more radioactive choline into phosphatidylcholine than liver slices from adult rats (117). Together, the evidence indicated that the rate of PC biosynthesis per unit weight of liver was indeed markedly influenced by development. Growing rats require more phospholipids, not only for assembly of plasma lipoproteins, but also for the synthesis of new membranes in the liver (118).

The level of plasma phospholipids was increased in the cholesterol/cholate-fed rats. This increase was most probably the result of the 2- to 3-fold stimulation in the rate of hepatic PC biosynthesis. An earlier study showed that PC's in the liver and plasma are in rapid equilibrium (9) and suggested that increased synthesis may indeed by responsible for the hyperphospholipidemia.

II. Measurement of the Rate of Phosphatidylcholine Biosynthesis

In experiments where the rate of PC biosynthesis was measured, 2 different concentrations of choline were used in the injection solution and in both cases, rats fed the high cholesterol/cholate diet incorporated 2- to 3-times more radioactivity than rats fed the control diet. The manner in which choline was utilized in the livers, however, depended upon the concentration of choline in the injection mixture. When 60 μM choline was used, the amount of radioactivity incorporated into betaine and phosphocholine was similar in both groups (Tables 29 and 30). In contrast, when the concentration of choline in the injection solution was 4.5 mM, the amount of radioactivity in phosphocholine was several fold higher and the

radioactivity in betaine was 50% lower in the hypercholesterolemic rats when compared to control (Table 28). The data illustrated an aspect of regulation which may be important *in vivo*. When the rate of PC biosynthesis was stimulated, as during hypercholesterolemia, excess choline which was oxidized to betaine in normal livers, was converted to phosphocholine and committed for PC biosynthesis. The fate of choline in liver appeared to be regulated by the requirements of PC biosynthesis and the supply of choline. The preferential channeling of labelled choline into phosphocholine and PC has been observed in diethylstilbestrol-treated roosters which have an elevated rate of biosynthesis (119) and also in hepatocytes when the culture medium contained less than 20 μM choline (120). The mechanism which determines the fate of choline in liver remains an important subject for future research.

Results from experiments with 4.5 mM choline in the injection solution suggested that the activities of choline kinase and phosphocholine cytidylyltransferase were dramatically increased in the experimental animals. The amount of radioactivity in phosphocholine and CDP-choline was increased by 2- and 20-fold respectively in the hypercholesterolemic rats (Table 28). The activity of choline kinase *in vitro*, however, was not increased while the cytidylyltransferase activity was only increased 2- to 3-fold in the experimental cytosol (Table 20). The apparent stimulation of phosphocholine synthesis at high intracellular concentrations of choline remains unclear at present. An increase in the level of choline in the cell, would not result in stimulation of choline kinase activity since the normal intracellular concentration of choline (Table 1) is about seven times the Km of choline kinase for choline (Table 2).

The method used for the estimation of the rate of PC biosynthesis was subject to several limitations and the assumptions used in the calculation have been discussed previously (see Results). There was considerable variation in the amount of radioactivity incorporated into betaine and the various choline-containing intermediates (Table 29) in both groups of rats even though identical conditions were used throughout the whole study. This variation could be explained by several factors. First, the concentration of choline in the portal veins probably differed from one rat to another and consequently, the dilution of the injected isotope would vary and second, variation in the metabolism of the animals may also be a contributing factor.

III. Enzymes of Phosphatidylcholine Biosynthesis in Normal and Hypercholesterolemic Rats.

Rats fed the high cholesterol/cholate diet had a 2- to 3-fold stimulation of the activity of hepatic phosphocholine cytidylyltransferase when compared to controls (Table 20). The activities of the other enzymes in the de novo pathway as well as the activities of the PE-N-methyltransferases, however, were unchanged and these results provided evidence that dietary cholesterol and cholate does not cause a general stimulation of enzymes in cytosol or microsomes but that the diet specifically affected the activity of the cytidylyltransferase.

Phosphocholine cytidylyltransferase is the rate limiting enzyme in the de novo synthesis of PC during hypercholesterolemia. The 2- to 3-fold stimulation of PC synthesis in the experimental group was specifically the result of a 2- to 3-fold increase in phosphocholine cytidylyltransferase activity. The suggestion by Infante (see Introduction) that choline kinase was also rate-limiting in the de novo pathway was not consistent with the

results from the present study where choline kinase activity and the concentration of phosphocholine were not increased in the livers from cholesterol/cholate-fed rats (Tables 20 and 31). And even if the phosphocholine pool was increased, it is difficult to understand how the overall rate of the pathway could be stimulated when the concentration of phosphocholine in normal livers is more than five times the Km of the cytidylyltransferase for phosphocholine (Tables 1 and 3). The data show that choline kinase is not rate-limiting during hypercholesterolemia. The enzyme, however, may be involved in the regulation of the fate of choline in liver.

Fresh rat liver cytosol from cholesterol/cholate-fed rats had 2.5 times more H-form than control cytosol (Fig. 11) and this was correlated with a 2.5-fold increase in the concentration of DG in the experimental cytosol. These results agreed with a previous study which showed that DG was the only lipid from rat liver that aggregated the cytidylyltransferase (26).

The cytidylyltransferase is found both in cytosol and microsomes, and the soluble activity was resolved into 2 forms on the basis of molecular size. Studies with antibodies raised against the purified L-form showed that the H-form, L-form as well as the microsome associated enzyme were immunologically identical (74) and strongly suggested that all the cytidylyltransferase in rat liver was one and the same. In vivo, is the enzyme in the cytoplasm and on the endoplasmic reticulum of the cell or is the cytidylyltransferase exclusively membrane bound? There is no evidence to support either possibility at the present but various circumstantial evidence seems to support the latter hypothesis. When rat liver was homogenized in saline, the majority of the cytidylyltransferase was found in the cytosol. In contrast, most of the enzyme activity was found in the

microsomal fraction when distilled water was used while an intermediate distribution between microsomes and cytosol was obtained when 0.25 M sucrose was used (19). This suggested that the cytidylyltransferase is loosely bound to the endoplasmic reticulum *in vivo* and homogenization of the tissue in saline or sucrose promoted solubilization of the enzyme.

The H-form is a heterogeneous population of enzyme aggregates which possess a wide range of molecular weights (Table 3) and they appear to be formed by random aggregation of L-form with no apparent stoichiometry. The composition of phospholipids in cytosols from normal and hypercholesterolemic rats (Table 23) was very similar to the composition of phospholipids in microsomes where the mole per cent of PC, PE, PS and PI are 73, 19, 3 and 4 respectively (121). These observations suggested that the solubilized cytidylyltransferase which has a natural affinity for membranes, probably randomly aggregate on to cytomembrane components which are present in 100,000 x *g* supernatant (122).

In the present study, the cytosol from cholesterol/cholate-fed rats contain 2-fold more lipid phosphorus and DG than control cytosol and the lipids are most probably associated with membrane fragments. The increase in the amount of H-form in the experimental cytosol, therefore, could very well be the result of increased amounts of membrane fragments.

The H- and L-form are probably artifacts produced during the preparation of subcellular fractions and the cytidylyltransferase most likely exists as one species which is associated with the endoplasmic reticulum *in vivo*. However, the possibility that the enzyme exists in a free and membrane bound form *in vivo* has not been excluded.

The stimulation of phosphocholine cytidylyltransferase in experimental cytosol was investigated. The activity of the cytidylyltransferase was measured by determination of the amount of CDP-choline formed in the

assay. An apparent stimulation of the cytidylyltransferase activity would occur if CDP-choline was more rapidly degraded in control cytosol. The rates of degradation of CDP-choline in control and experimental cytosols were similar (Table 21) and the assay does truly reflect a stimulation of cytidylyltransferase activity in experimental cytosol.

The stimulation of cytidylyltransferase activity could be explained in a number of ways. First, the amount of enzyme protein could be increased in the experimental cytosol. Second, the amount of enzyme was unchanged but a more active species of enzyme was present in the experimental cytosol and third, a modulator of the cytidylyltransferase could be present in or absent from either of the cytosols.

The amount of enzyme in control and experimental cytosols was similar as determined by immunotitration experiments (Fig. 17) and the regulation of PC biosynthesis does not appear to involve an adaptive change in the concentration of the cytidylyltransferase in livers from hypercholesterolemic rats. This result is in line with findings from experiments with choline-deficient livers where the activity of the cytidylyltransferase was one half of that in controls and immunotitration studies showed that the quantity of enzyme was unchanged.

The possibility that an enzyme modulator was responsible for the stimulation of the cytidylyltransferase was explored. A trypsin-insensitive activator which is soluble in $CHCl_3/CH_3OH$ (2/1; v/v) was found in the cytosols. The activator appeared to be a lipid and there was twice as much activator in the experimental cytosol as in controls. The presence of a lipid activator is consistent with results from studies with lung and liver which showed that the cytidylyltransferase was strongly stimulated by phospholipids.

Several attempts were made to identify the activator by TLC (see Results) and this method was found to be unsuitable. When lipid extracted from cytosol was fractionated on silicic acid plates more than 70% of the activator was lost (Table 25). In direct contrast, however, palmitoyl lysophosphatidylethanolamine, which was implicated as a potent activator of the enzyme (25), was activated by spotting and elution from silica (Fig. 21). The results indicated two things: first, the data suggested that the activator of the cytidylyltransferase was not LPE in view of the effect of silica and second, another technique needed to be employed in future studies before the identity of the activator can be established.

IV. The Regulation of the De Novo Pathway for Phosphatidylcholine Biosynthesis

The regulation of *de novo* synthesis of PC is complex and every enzyme in the pathway is involved. The fate of choline as it enters the liver cell appears to be determined, at least in part, by choline kinase, the first enzyme of the pathway. The proportion of the total incoming choline that is converted to phosphocholine and thus committed for PC biosynthesis, is dependent upon the rate of PC biosynthesis and the supply of choline.

The second enzyme of the pathway, phosphocholine cytidylyltransferase, determines the overall rate of the pathway as demonstrated in the present study (Fig. 24). The first committed step in a pathway which involves a dissociated enzyme system is normally the rate limiting reaction whereas in a multienzyme complex, it has been proposed that any reaction in the pathway can be rate-limiting (123). A multienzyme complex composed of all three enzymes of the *de novo* pathway has not been demonstrated, but the cytidylyltransferase and phosphocholinetransferase have been shown to be situated in the cytoplasmic side of the endoplasmic reticulum (124).

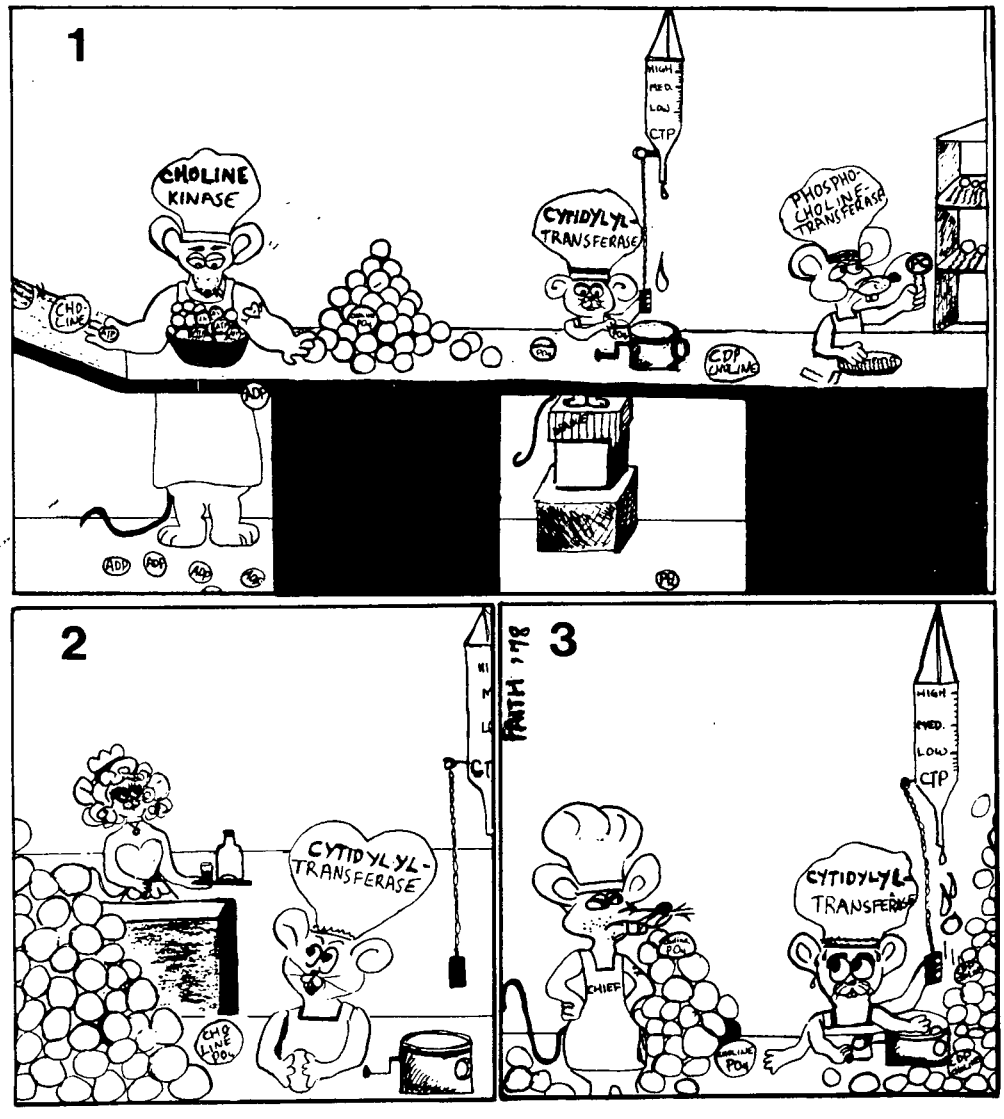


Figure 24. Regulation of the Rate of Phosphatidylcholine Biosynthesis. The de novo synthesis of PC is illustrated as an assembly line process where the cytidylyltransferase is the slowest worker in the chain (Frame 1). An alteration in the activity of the cytidylyltransferase (Frames 2 and 3) would result in a change in the overall rate of production of the assembly line.

Although the spatial relationship of the two enzymes on the membrane has not been investigated, it is tempting to speculate that the cytidylyltransferase and phosphocholinetransferase are associated in some manner which facilitates the utilization of CDP-choline by phosphocholinetransferase. This management would prevent wasteful accumulation of intermediates, as in a multienzyme complex (123), and the concentration of CDP-choline in vivo (Table 1) seem to support the idea that some physical relationship exists between the cytidylyltransferase and phosphocholinetransferase.

The effects of various metabolites on the activity of the cytidylyltransferase suggest that the rate of PC biosynthesis may be sensitive to the energy state of the cell. The cytidylyltransferase activity was inhibited by NAD and NADP (Table 17) which are metabolites indicative of low energy while enzyme activity was stimulated by NADH and NADPH. It seems logical that the cell would not synthesize more PC when energy is scarce and would permit synthesis where energy is adequate. The effects of the nucleotides on the cytidylyltransferase (Table 17), however, were more difficult to understand. Inhibition of enzyme activity by nucleoside triphosphates was difficult to rationalize and it remains to be established whether the effects on PC biosynthesis of the various metabolites used in the present study have any real significance in vivo.

The mechanism by which the rate of PC biosynthesis is stimulated during cholesterol/cholate feeding of rats remains unclear at the present but results from various studies suggest that a hormonal mechanism is probably involved. There are two main lines of evidence. First, diets containing high fat or high carbohydrate have been shown to markedly alter the level of glucagon in the blood (125) and second, the incorporation of labelled

choline into PC was affected by various hormones (see Introduction). A recent study with fetal rat lung in organ culture (126) showed that the incorporation of labelled choline into PC was stimulated 2-fold by aminophylline, a cyclic AMP phosphodiesterase inhibitor, and also by cyclic AMP. In another study, the total amount of PC and disaturated PC as well as the incorporation of labelled choline into these fractions were increased in alveolar type II cells treated with cyclic AMP (127). Various analogs of cyclic AMP increased phosphatidylcholine levels and the degree of stimulation by the different compounds was correlated to their ability to activate protein kinase (127). The data suggested that a protein phosphorylation mechanism may be involved. The activities of choline kinase and phosphocholinetransferase in fetal lung explants were not significantly affected by aminophylline (126). In contrast, phosphocholine cytidylyltransferase was increased in fetal rabbit lung when pregnant rabbits were treated with corticosteroids (128) and some of the effects of this hormone have been suggested to be mediated by cyclic AMP (129). Together, the evidence suggest that a hormonal mechanism which involves cyclic AMP as second messenger may be involved in controlling the rate of PC biosynthesis and phosphorylation is one way by which the activity of phosphocholine cytidylyltransferase may be regulated.

The last enzyme of the de novo pathway is phosphocholinetransferase and it determines the degree of unsaturation of PC by selectively utilizing certain DG. The phosphocholinetransferase is a potential point for regulation even though the enzyme appears to poorly utilize disaturated species of DG in lung where dipalmitoyl PC is required for surfactant synthesis (see Introduction). Nevertheless, it is conceivable that the specificity of the enzyme could be regulated so that DG's having certain

saturated fatty acids on C-1 or unsaturated fatty acids on C-2 may be preferentially utilized. Whether the species of fatty acids on PC are altered during hypercholesterolemia remains to be determined.

V. Suggestions for Further Studies

There are several areas in the present study which warrant further investigation. First and foremost, the modulator responsible for the activation of the cytidylyltransferase in cytosol from cholesterol/cholate fed rats should be identified. Subsequent investigations should be directed ultimately towards elucidation of the chain of events which lead to the activation of the cytidylyltransferase and stimulation of PC synthesis. These studies could also yield some insight into the relationship between the regulation of cholesterol and PC biosynthesis and may facilitate in the understanding of lipoprotein synthesis in the liver.

The mechanism which determines the fate of choline in liver also warrants further study. The various isozymes of choline kinase may very likely be involved in this process.

The liver synthesizes PC's which are exported as bile or blood lipoproteins and how PC synthesis is regulated to fulfill the various requirements for export and for the needs of the liver remains to be investigated.

Lastly, the biosynthesis of other phospholipids during hypercholesterolemia should be studied. Results from this investigation will not only help explain how the phospholipid composition in membranes is maintained but they will also be invaluable in the elucidation of the synthesis of bile and various plasma lipoproteins in the liver.

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