ISOLATION OF RAT LIVER CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE AND REGULATION OF HEPATIC PHOSPHATIDYLCHOLINE BIOSYNTHESIS

by

ZEMIN YAO

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Department of Biochemistry

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date April, 26, 1985

ABSTRACT

Two kinds of affinity chromatography, CDP-choline- and CTP-Sepharose 4B, were investigated for purification of the cytosolic CTP:phosphocholine cytidylyltransferase from rat liver. The enzyme did not show strong affinity for the CDP-choline Sepharose resin, but bound to the CTP-Sepharose column in the presence of 14 mM magnesium acetate. The combination of CTP affinity chromatography with ion-exchange techniques provided about 70-fold purification of the cytosolic enzyme with a specific activity of about 90 units per milligram protein.

The influence of diphenylsulfone compounds on the synthesis of phosphatidylcholine by the CDP-choline pathway was examined in isolated rat hepatocytes and HeLa cells. The administration of the sulfones (100 ug/ml), except dapsone, to HeLa cells inhibited the total [methyl-^{3}H]choline incorporation into the cells, but did not change the rate of conversion of choline to phosphatidylcholine. The addition of the sulfones (100 ug/ml) to rat hepatocytes did not inhibit the biosynthesis of phosphatidylcholine and choline metabolism.

The effect of vasopressin on the distribution of cytidylyltransferase between cytosol and microsomes in rat hepatocytes was also investigated. The digitonin-mediated release of cytosolic cytidylyltransferase was reduced from the cells treated with vasopressin (5-20 nM), while the enhanced rate of incorporation of [methyl-^{3}H]choline into phosphatidylcholine was not observed.

TABLE OF CONTENTS

Chapter I. Introduction..... 1

1. Structure of phosphatidylcholine..... 1

2. Biological functions of phosphatidylcholine..... 1

3. Pathways of phosphatidylcholine biosynthesis in mammals..... 4

4. Enzymes of phosphatidylcholine biosynthesis via Kennedy pathway..... 4

5. Regulation of phosphatidylcholine biosynthesis via Kennedy pathway..... 7

6. The thesis investigations..... 8

Chapter II. Purification of CTP:phosphocholine cytidylyltransferase from rat liver cytosol by affinity chromatography..... 10

Materials and methods..... 13

Results..... 18

Discussion..... 28

Chapter III. Effects of diphenylsulfone compounds on the metabolism of [methyl-^{3}H]choline in rat hepatocytes and HeLa cells..... 32

Materials and methods..... 36

Results..... 39

Discussion..... 48

Chapter IV. Effects of vasopressin on CTP:phosphocholine cytidylyltransferase in rat hepatocytes..... 52

Materials and Methods..... 57

Results..... 59

Discussion..... 67

References..... 72

LIST OF TABLES

Table		Page
1.	Purification of CTP:Phosphocholine Cytidylyltransferase from Rat Liver Cytosol.....	27
2.	Effects of Dapsone and AUS on the [Methyl-^{3}H]Choline Uptake by HeLa Cells.....	40
3.	Effects of Diphenylsulfone derivatives (III and IV) on The [Methyl-^{3}H]Choline Uptake by HeLa Cells.....	41
4.	Effect of Vasopressin on The Incorporation of [Methyl-^{3}H] Choline into PC.....	66

LIST OF FIGURES

Figure		Page
1.	Space-filling Model of Phosphatidylcholine.....	2
2.	Kennedy Pathway for Phosphatidylcholine Biosynthesis from Choline.....	5
3.	Phosphatidylcholine Biosynthesis by Successive N-Methylation of Phosphatidylethanolamine.....	6
4.	Scheme of The Synthesis of CDP-Choline Sepharose 4B.....	19
5.	DEAE-Cellulose chromatography of Rat Liver Cytosolic Cytidylyltransferase.....	22
6.	CDP-Choline Sepharose 4B Chromatography of Rat Liver Cytosolic Cytidylyltransferase.....	23
7.	Phospho-Cellulose Chromatography of Rat Liver Cytosolic Cytidylyltransferase.....	25
8.	CTP Sepharose 4B Chromatography of Rat Liver Cytosolic Cytidylyltransferase.....	26
9.	Structure of Dapsone (I), AUS (II) and Analogs (III and IV).....	35
10.	Influence of AUS and IV on The Metabolism of [Methyl-^{3}H]Choline in HeLa Cells.....	44
11.	Influence of Dapsone and III on the Metabolism of [Methyl-^{3}H]Choline in Rat Hepatocytes.....	46
12.	Influence of AUS and IV on The Metabolism of [Methyl-^{3}H]Choline in Rat Hepatocytes.....	47
13.	Digitonin-mediated Release of CT from Rat Hepatocytes...	60
14.	Effect of Different Concentrations of Vasopressin on The Digitonin-mediated Release of CT from Rat Hepatocytes.....	61
15.	Effect of Vasopressin on The Incorporation of [Methyl-^{3}H]Choline into Phospholipids.....	63
16.	Influence of Vasopressin on The Incorporation of [Methyl-^{3}H]Choline into PC.....	64

LIST OF ABBREVIATIONS

A	absorbance
ACS	aqueous counting scintillant
ATP	adenosine 5'-triphosphate
AUS	1-[4-(4-sulfanilyl)phenyl]urea
BHK	baby hamster kidney
cAMP	adenosine 3',5'-monophosphate
CHO	chinese hamster ovary
CoA	coenzyme A
CPT	cholinephosphotransferase
CT	phosphocholine cytidylyltransferase
CTP	cytidine 5'-triphosphate
DEAE	diethylaminoethyl
DES	dietheylstilboestrol
DG	diacylglycerol
DMSO	dimethylsulfoxide
dpm	disintegrations per minute
DTT	dithiothreitol
E.R.	endoplasmic reticulum
FCS	fetal calf serum
Fig.	figure
g	gravity
g(m)	gram
hr	hour
HDL	high density lipoprotein
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid
HMG-CoA	beta-hydroxy-beta-methyl glutaryl-CoA
Ig	immunoglobulin
Km	Michaelis-Menten constant
LCAT	lecithin cholesterol acyltransferase
LDL	low density lipoprotein
lyso-PC	lysophosphatidylcholine
lyso-PE	lysophosphatidylethanolamine
m	meter
M	molar
MEM	modified Eagle's medium
min	minute
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PGs	prostaglandins
PI	phosphatidylinositol
PMSF	phenylmethylsulphonyl fluoride
PS	phosphatidylserine
PtdIns	1-(3-sn-phosphatidyl)-L-myo-inositol
PtdIns4P	1-(3-sn-phosphatidyl)-L-myo-inositol 4-phosphate
PtdIns(4,5)P_2	1-(3-sn-phosphatidyl)-L-myo-inositol 4,5-biphosphate

R_f	ratio of distance moved by a solute relative to the solvent front
rpm	revolutions per minute
S.D.	standard deviation
TLC	thin-layer chromatography
TTP	thymidine 5'-triphosphate
Tris	tris (hydroxymethyl) aminomethane
UTP	uridine 5'-triphosphate
UV	ultraviolet
VLDL	very low density lipoprotein

Notes: Standard prefixes are: m (milli) - 10^{-3}; u (micro) - 10^{-6}; n (nano) - 10^{-9}

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CHAPTER I. INTRODUCTION

1. STRUCTURE OF PHOSPHATIDYLYLCHOLINE

Phosphatidylcholine (PC) is the major phospholipid present in eukaryotes but rarely occurs in prokaryotes. The structure of PC is distinguished from the other phospholipids by its choline headgroup, although a variety of fatty acids can be esterified to the glycerol backbone. Usually, saturated fatty acids are esterified at the C1 position of glycerol while unsaturated fatty acids are at C2 position (Figure 1).

2. BIOLOGICAL FUNCTIONS OF PHOSPHATIDYLYLCHOLINE

The primary function of PC is structural, which arises from its amphipathic nature---the choline headgroup is hydrophilic whereas the fatty acid acyl chains are hydrophobic. The ability of PC and other phospholipids to assume spontaneously a bilayer organization in the presence of excess water and at concentrations above the "critical micellar concentration" (CMC) might be the dominant reason for Nature's choice of phospholipids as a universal constituent of cell membranes. The most abundant phospholipid in eukaryotes is PC, which accounts for nearly 50% of the total membrane phospholipid (1). PC has also been described as a requirement for the activity of several membrane-bound enzymes (2). Apparently, PC offers a suitable environment for maintaining the functional conformation of those enzymes. In

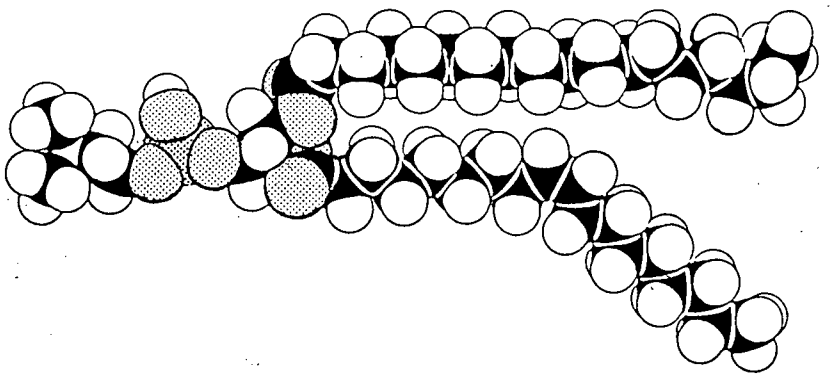

Fig.1. Space-filling model of phosphatidyl-choline.

addition to the membrane component, PC also functions as a constituent of bile, plasma lipoproteins and lung surfactant. In bile, PC accounts for 90% of the total phospholipid component in the rat (3) and 68% in humans (4). The bile PC together with conjugated bile acids functions in solubilization of cholesterol in bile. The function of PC in blood plasma lipoprotein is not clear. It is thought PC coats the surface of lipoprotein to render the core of triacylglycerol and cholesterol ester soluble in plasma, and also, PC itself is transported from liver to target tissues via lipoproteins. PC accounts for approximately 70% of lung surfactant by weight and lines the alveoli of lung, which lowers the surface tension and prevents the collapse of the alveolar tissue during expiration (5). Failure to maintain an adequate supply of this material in lung alveoli after birth is thought to be responsible for Respiratory Distress Syndrome, the major cause of mortality and morbidity in premature infants (6). As well as being a structural component, PC also functions as an important metabolic precursor involved in many biochemical pathways. PC serves as a donor of the fatty acyl moiety for the synthesis of cholesterol ester in a reaction catalyzed by lecithin-cholesterol acyltransferase (LCAT). Arachidonic acid from PC is utilized for the synthesis of prostaglandins, thromboxanes and leukotrienes. Recently, a particular molecular species of PC (1-alkyl-2-acetyl-PC) has been described as a platelet activating factor which causes platelet aggregation (7).

3. PATHWAYS OF PHOSPHATIDYLYLCHOLINE BIOSYNTHESIS IN MAMMALS

There are five separate pathways which lead to the formation of PC (1). They are: 1) the major de novo pathway (also known as CDP-choline pathway) described by Kennedy in the 1950's (8), which involves the conversion of choline to phosphocholine, CDP-choline and PC (Figure 2); 2) the stepwise methylation of phosphatidylethanolamine (PE) which is catalyzed by PE methyltransferase (9) (Figure 3); 3) the base exchange reaction in which free choline can displace ethanolamine or serine from appropriate diacylglycerophospholipid (10,11); 4) the acylation of lysophosphatidylcholine (lyso-PC) by acyl-CoA (12,13); and 5) transacylation between two molecules of lyso-PC (14). In rat liver, about 70% of the total PC is synthesized by the CDP-choline pathway and the remaining 30% by the methylation pathway (15), whereas the other three mechanisms are not important for de novo biosynthesis.

4. ENZYMES OF PHOSPHATIDYLYLCHOLINE BIOSYNTHESIS VIA KENNEDY PATHWAY

Studies on PC metabolism are complicated since the majority of enzymes involved in phospholipid biosynthesis are membrane bound or require lipid for their activity. The only exception is choline kinase (EC 2.7.1.32) which catalyzes the phosphorylation of choline by ATP in the Kennedy pathway. Choline kinase is neither associated with membranes nor requires phospholipids for activity, and has recently been purified to homogeneity from rat kidney in a dimeric form with the molecular weight of 80,000(16).

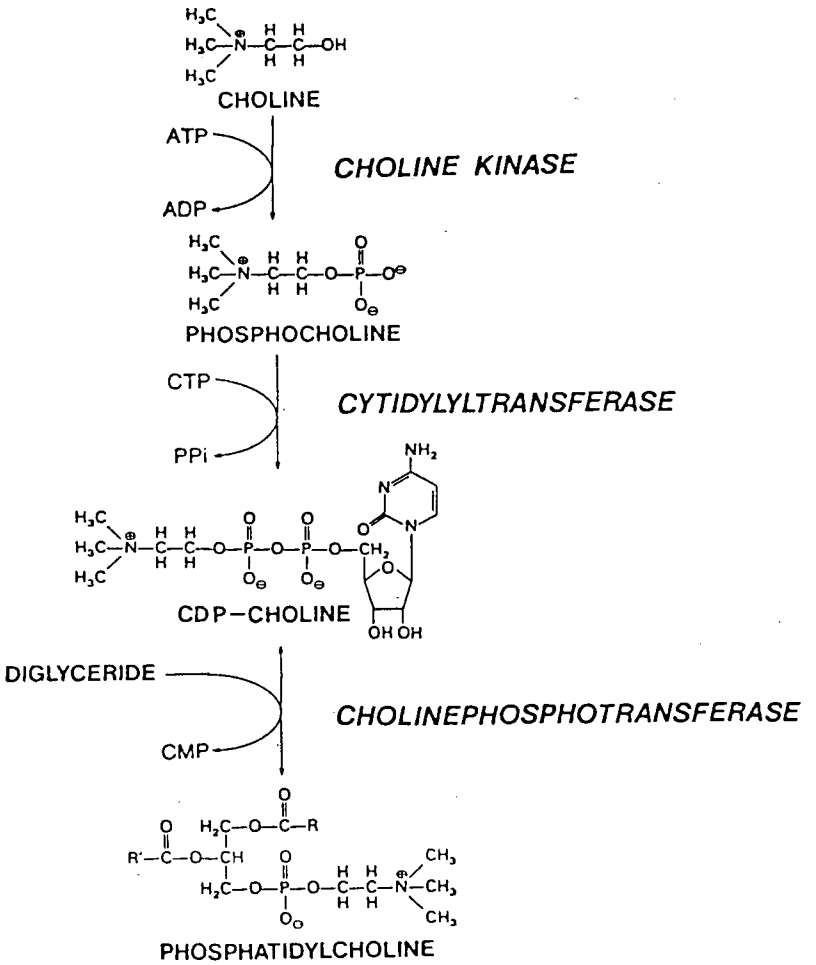

Fig.2. Kennedy pathway for phosphatidylcholine biosynthesis from choline.

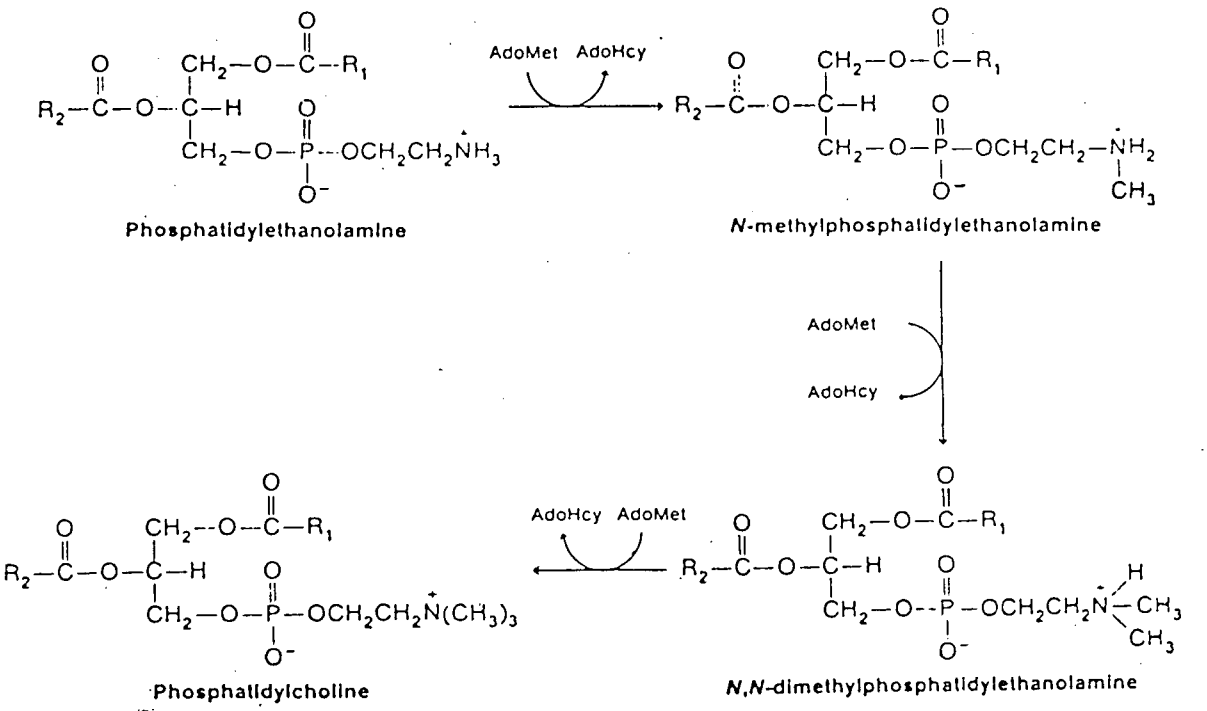

Fig.3. Phosphatidylcholine biosynthesis by successive N-methylation of phosphatidyl-ethanolamine.

Once choline has been phosphorylated to form phosphocholine the only fate of this product is for PC formation. The enzyme CTP:phosphocholine cytidylyltransferase (CT) (EC 2.7.7.15) catalyzes the formation of CDP-choline and PPi from phosphocholine and CTP, the dominant energy form utilized in lipid metabolism. Attempts at purification of CT so far have been only partially successful. The properties of CT in rat liver will be discussed in Chapter II. The final step in the Kennedy pathway is the formation of PC and CMP from diacylglycerol (DG) and CDP-choline, catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) (EC 2.7.8.2). The activity of CPT is mostly recovered from E.R. (microsomes) and mitochondria in mammalians tissues(10,17), while a report from chick macrophages showed that CPT was located on the plasma membrane (18). Partial purification of this membrane bound enzyme has been reported (19). The enzyme requires magnesium as a cofactor and microsomal phospholipids for its maximum activity (19).

5. REGULATION OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS VIA KENNEDY PATHWAY

Towards the ends of 1970's, a number of approaches have provided evidence that the rate of PC biosynthesis via the Kennedy pathway is usually determined by the activity of CT (20). It has been postulated that the activity of CT is regulated by translocation of CT from the cytosol, where it is inactive, to the E.R., where it is activated (20). The conclusion largely arises from the observations that: 1) CT is ambiquitous in that

it is recovered in both the cytosolic and microsomal fractions; 2) the cytosolic CT is inactive and needs phospholipid for activity while the microsomal CT is active and insensitive to phospholipid activation; 3)there are parallel changes between PC biosynthesis and the activity of microsomal CT in different physiological conditions. Supportive evidence has been obtained from studies in a variety of mammalians organs, liver (21,22), heart (23), lung (24) and intestine (25), as well as in some cell lines (26). The translocation process of CT may be regulated by reversible enzyme phosphorylation (27) and by fatty acids (28), yet proof of this hypothesis is still waiting for the complete purification of the enzyme.

On the other hand, some experimental and theoretical evidence suggests that the first reaction in the de novo synthesis pathway catalyzed by choline kinase might be a rate-limiting enzyme for PC biosynthesis (29,30).

6. THE THESIS INVESTIGATIONS

Liver is a major organ for phospholipid biosynthesis and the phospholipids synthesized in liver participate not only in membrane formation within the organ but also are transferred into bile or plasma lipoproteins (31). Hence, many efforts of this laboratory in the last decade were focused on revealing the regulatory mechanisms behind the PC biosynthesis, the relationship between PC biosynthesis and lipoprotein metabolism, and on purification of the enzymes involved in PC biosynthesis from rat liver. In this thesis, I have investigated the regulatory mecha-

nism of PC biosynthesis in cultured rat hepatocytes by using diphenylsulfone compounds (Chapter III) or vasopressin (Chapter IV), and also attempted to purify CT from rat liver by different techniques including affinity chromatography (Chapter II).

CHAPTER II. PURIFICATION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE FROM RAT LIVER CYTOSOL BY AFFINITY CHROMATOGRAPHY

CTP:phosphocholine cytidylyltransferase (CT) (E.C.2.7.7.15) catalyzes the reversible formation of CDP-choline and pyrophosphate from phosphocholine and CTP, and may control the rate of phosphatidylcholine (PC) biosynthesis (15,32,33,34). Deoxy-CTP can also serve as a substrate instead of CTP in this reaction, but the other nucleoside triphosphate (ATP, GTP, UTP, or TTP) do not (35).

CT has been described as an ambiquitous enzyme which is associated with both the soluble and microsomal fractions of the tissue homogenates (34). The cytosolic CT isolated from different organs is inactive and needs phospholipid for its activity, although it is the dominant form of the enzyme in cells. The microsomal CT, however, is the active form and is not further activated by exogenous phospholipid although it is always the minor enzyme form within cells (20). The relative distribution of the enzyme between cytosol and microsomes varies in different developmental stages of individual animals. In prenatal liver of rat, over 70% of the total CT protein is detected in the cytosolic fraction, while on the day of birth, most of the enzyme associates with the microsomes (21). A similar phenomenon has been observed in lung (36). In fetal rats delivered one or two days prematurely, CT is redistributed from cytosol to microsome so that the microsomal activity is increased by 60%, while there

is no change in the total activity of CT (36).

In addition, the relative distribution of CT between cytosol and microsomes is dependent on conditions used in homogenization (37). If adult rat liver is homogenized in isotonic saline (0.145 M NaCl), more then 90% of the enzyme activity is recovered from the 170,000 x g, 1 hr supernatant (cytosol), whereas homogenization performed in distilled water results in 85% of the activity being asssociated with the pellet fraction (microsomes) (37).

The strategies in CT purification published so far have all taken advantage of the soluble property of the cytosolic enzyme (38,39,40,41). The occurrence of CT in the cytosol varies from organ to organ, of which liver exhibits the greatest cytosolic CT activity in terms of either units per gram of tissue or total units per organ (42). Thus, liver appears to be an ideal starting material for preparative purposes. However, the purification of the cytosolic CT is complicated since the soluble enzyme has a strong tendency to aggregate in the presence of phospholipid which is unavoidably present in cytosol due to the breakage of membranes during homogenization. The aggregated CT forms polymers with diverse molecular weights ranging between 5×10^5 and 1.3×10^7, and behaves like the microsomal enzyme which is insensitive to phospholipid activation, while the unaggregated enzyme form is estimated to have a molecular weight of 2×10^5 (28). The aggregation of CT with phospholipid seems irreversible since attempts to displace the enzyme activity from the particulate material isolated from water homogenates of rat liver were unsuc-

cessful (21,37). On the other hand, phospholipid appears to stabilize the partially purified CT. The addition of phospholipid to the enzyme preparation at later purification stages can maintain the enzyme activity which is lost dramatically in the absence of phospholipid.

In vitro studies on partially purified CT from rat liver cytosol showed that the reversible reaction catalyzed by CT had an equilibrium constant of 0.80 in favour of the formation of CTP and phosphocholine (43). The Michaelis constants for CTP and CDP-choline of the partially purified CT are the same (Km = 0.21 mM) when assayed in the presence of phospholipid (43). Since CDP-choline is a substrate for only CT and CPT, while CTP is a substrate for several enzymes involved in lipid biosynthesis, CDP-choline should be an ideal candidate for affinity column purification of cytosolic CT. CPT is a membrane-associated enzyme which is absent in cytosol.

In the present studies, two kinds of affinity chromatography, CDP-choline and CTP-Sepharose 4B, have been investigated for purification of the cytosolic CT from rat liver. Preliminary results showed cytosolic CT did not have strong affinity to the CDP-choline Sepharose resin but did bind to the CTP affinity column in the presence of magnesium acetate. The combination of CTP affinity chromatography with ion-exchange techniques provided about 70-fold purification with a specific activity of about 90 units per milligram protein. The problem of losing activity at the later purification stages was still unsolved.

MATERIALS AND METHODS

Chemicals- DE-52 and P-11 ionic exchange resins were the products of Whatman. ATP, CTP, CDP-choline, adipic acid dihydrazide and choline kinase were purchased from Sigma. [methyl-^{3}H]Choline and Aqueous Counting Scintillant (ACS) were obtained from the Radiochemical Center, Amersham. [methyl-^{3}H]Phosphocholine (10 uci/umol) was synthesized enzymatically from [methyl-^{3}H]choline and ATP with choline kinase based on the method of Vance et al. (43). 2',7'-dichloro-fluorescein was a product of Eastman Kodak Co.

Preparation of Rat Liver Cytosol- Rat liver cytosol was prepared based on the method described by Choy et al. (40). Wistar rats (about 200 g) from the University of British Columbia Animal Unit were decapitated, and the livers were removed immediately. A 25% homogenate of liver was prepared in ice-cold isotonic saline (0.145 M NaCl) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) by 5 strokes in a Potter-Elvehjem homogenizer, and centrifuged at 170,000 x g for 60 min at 4°C. The supernatant was filtered through two layers of cheese cloth to remove the majority of the lipid-rich layer which floated at the air-solution interface after ultracentrifugation. CT in this sample is very stable, as freezing at -70°C and thawing at 37°C causes no detectable loss of enzyme activity. The sample was designated as cytosolic fraction.

DEAE-Cellulose Chromatography of Cytosolic CT- All the procedures described below were performed at 4°C. The liver cytosol stored at -70°C was thawed at 37°C and quickly cooled in ice, as prolonged incubation in 37°C will cause aggregation of the cytosolic CT. The sample was brought to 30% ammonium sulfate saturation in the presence of 0.05% Triton X-100, and the precipitate was discarded after centrifugation at 10,000 x g for 10 min. The supernatant was brought to 40% ammonium sulfate saturation and the precipitate collected after centrifugation under the same conditions. The pellet was suspended in 20 mM Tris-succinate (pH 6.5) buffer containing 0.025% Triton X-100, 0.5 mM PMSF and 0.5 mM dithiothreitol (DTT) to the same volume as that of the original cytosol, thus ensuring a low conductivity. This was called the 40% fraction.

The 40% sample (approx. 130 ml) was applied to a DE-52 column (3 x 35 cm) which had been equilibrated with the sample buffer. Subsequently, the column was eluted with 50 ml of buffer and then with a 400 ml NaCl gradient ranging between 0-0.5 M in the same buffer. Fractions (9 ml) were collected and enzyme activity was assayed. The enzyme activity eluted in the conductivity region around 3 mMHO. This protocol is routinely used in this laboratory and gives reproducible results of appoximately 15-fold purification with 20-40% yield relative to the cytosolic fraction. The enzyme could be stored at -70°C for at least 2-3 months with negligible loss of enzyme activity.

Phospho-Cellulose Chromatography of CT- The procedure of CT purification by phospho-cellulose was developed in this laboratory

(Sanghera, J., unpublished result). The enzyme pooled from DE-52 chromatography was applied to a P-11 column (2.5 x 8 cm) which had been equilibrated with the sample buffer as used in DE-52 column. The bulk of protein was eluted with 80 ml of 0.2 M NaCl and the enzyme was removed with a 100 ml gradient of 0-0.6 M NaCl into 4.5 ml fractions. The enzyme peak was located in the conductivity range between 7 and 8.5 mMHO. CT in this sample is extremely unstable and should be subjected to the successive purification step as quickly as possible.

Enzyme Assay- The measurement of CT activity was essentially based on the methods described by Vance et al. (43). The reaction mixture contained in a final volume of 100 ul: 0.2 mg of total rat liver phospholipid; 10 nmol of oleate; 7.5 umol of Tris-succinate, pH 6.5; 0.75 umol of magnesium acetate; 0.2 umol of CTP; 0.15 umol of [methyl-^{3}H]phosphocholine (10 uci/umol) and an appropriate amount of enzyme protein (2-40 ug). The reaction was performed at 37°C for 15-30 min, and stopped by immersion of the tubes in boiling water for 2 min. The protein precipitate was pelleted by centrifugation at 2,500 rpm (Western H-103N centrifuge) for 5 min, and 40 ul of the supernatant was applied to Silica G-60 plates (Merck) with 3 x 10 cm per lane. CDP-choline (0.1 mg) and phosphocholine (0.6 mg) carriers were applied for each lane. The TLC plates were developed in CH_3OH /0.6% NaCl/NH_3 (10:10:1; v:v:v) for 50 min. CDP-choline was visible under short-wave UV light after spraying with 2',7'-dichlorofluorescein (0.01% in CH_3OH), and scraped from the plates into a plastic scintillation vial with 0.5 ml of water and 4.5 ml of ACS fluid.

The samples were counted for radioactivity with 20-30% counting efficiency. The region of phosphocholine on the plates was visualized in an iodine vapour in order to confirm the separation of CDP-choline from phosphocholine. One unit of enzyme activity is defined as one umole of CDP-choline formed per min.

Protein Assay- The Bio-Rad assay based on the method of Bradford (44) was used for the estimation of protein concentration. Bio-Rad stock reagent was diluted in distilled water (4:13; v:v), and 2.5 ml of the diluted reagent was added to 0.5 ml of protein sample with IgG (Bio-Rad Standard) as the protein standard. The assay was linear in the range between 5-80 ug of protein. For some assays, the microassay procedure was adopted to determine protein concentrations less then 10 ug/ml. In this case, 0.2 ml of the concentrated Bio-Rad stock reagent was added to 0.8 ml of protein sample. The absorbance was read at 595 nm after 15 min but before 60 min.

Preparation of Total Rat Liver Phospholipid- Total rat liver phospholipid was extracted from the 170,000 x g x 1 hr pellet of rat liver homogenate by the Bligh and Dyer method (45). The pellet was re-homogenized in 80 ml of water, and mixed with 100 ml of chloroform and 200 ml of methanol by stirring for 1 hr at room temperature. The mixture was centrifuged at 8,000 rpm (in a Sorvall Type GSA rotor) for 20 min, and the supernatant was filtered through glasswool. The filtrate was brought to the final ratio of $CHCl_3/CH_3OH/H_2O$ to 1:1:0.8 (v:v:v) by the addition of 80 ml of water and 100 ml of chloroform. The filtrate was centri-

fuged under the same condition, and the chloroform phase was transferred to a preweighed round-bottom flask to remove the chloroform by rotary evaporation. The flask was cooled at -20 ^{o}C for 20 min and rinsed with ice-cold acetone to extract the neutral lipids. The remaining acetone was evaporated under nitrogen. The phospholipid was dissolved in chloroform at a final concentration of 20 mg/ml and stored at -20^{o}C.

RESULTS

Preparation of CDP-Choline Sepharose 4B A modification of a method originally used for the coupling of CDP-diglyceride to Sepharose 4B (46,47) was used to link covalently an oxidized derivative of CDP-choline to the gel. The scheme of the reactions are summarized in Fig.4.

1) Preparation of Cyanogen Bromide Activated Sepharose 4B. Cyanogen bromide activated Sepharose 4B was prepared by the method described by Cuatrecasas (48). Sepharose 4B (50 ml) was prewashed with 1 M NaCl and H_2O and finally suspended in 50 ml of H_2O. Ground CNBr powder (10 g) was poured into the resin suspension and mixed quickly. The reaction was maintained at pH 11 with 6 N NaOH, and the temperature was controlled below 20°C by adding ice. The reaction time was about 15 min and stopped by the addition of ice to the mixture. The activated resin was washed quickly with ice-cold 0.1 M Na_2CO_3 (pH 9.5) and finally resuspended in the same solution.

2) Preparation of Sepharose 4B Adipic Acid Dihydrazide. Adipic acid dihydrazide (4.5 g) was mixed into the CNBr activated resin, and the reaction was performed for 17 hr at 4°C by gently stirring the suspension. The resin was washed extensively with 1M NaCl and then 0.1 M sodium acetate (pH 5.0), and tested for the presence of covalently bound hydrazide as described by Cuatrecasas (48). The resin with ligand turned a dark red in saturated sodium borate containing several drops of 3% 2,4,6-

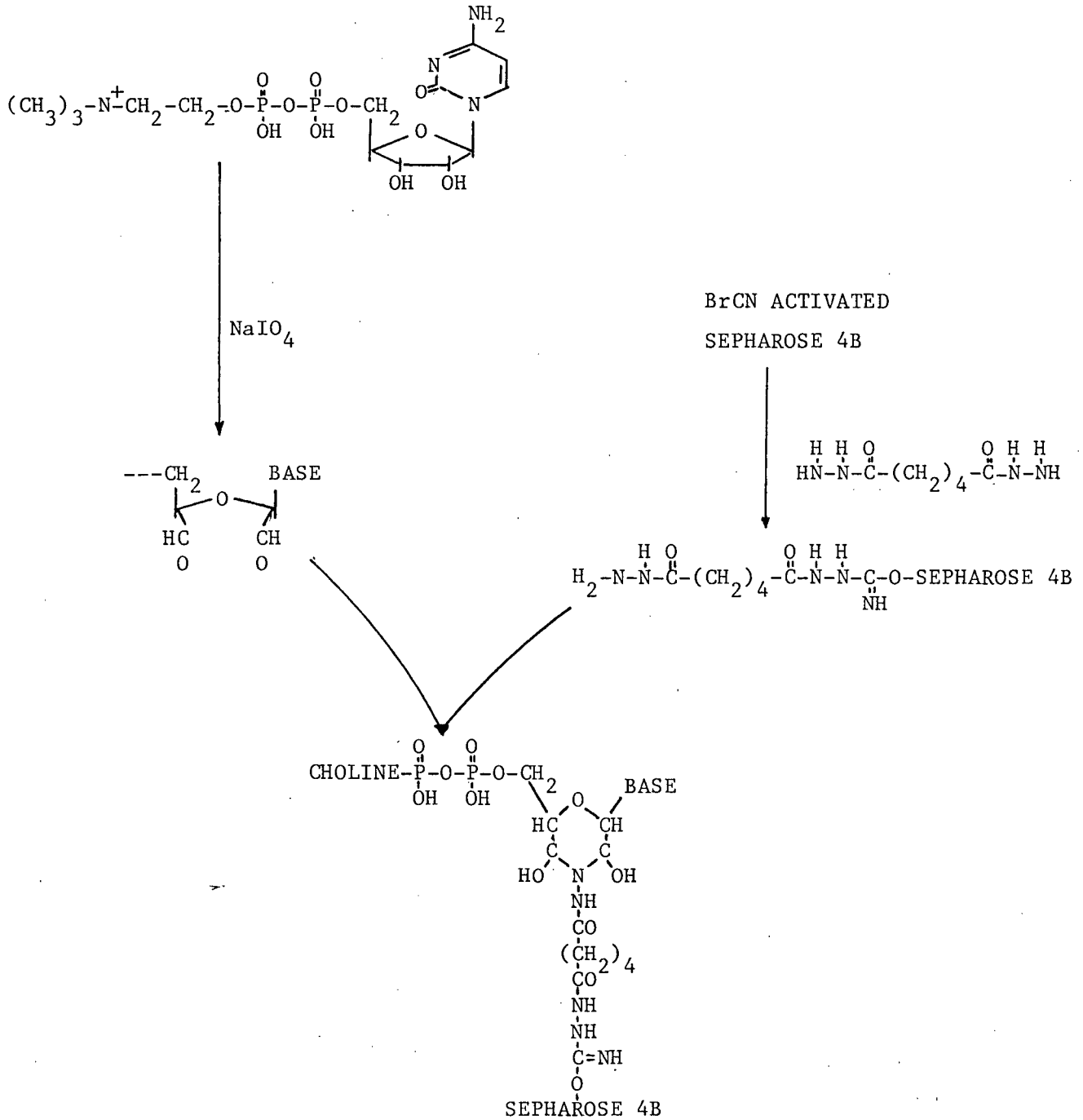

Fig 4. Scheme of The Synthesis of CDP-Choline Sepharose 4B.

trinitrobenzene sulfate, while unsubstituted resin was pale yellow.

3) Oxidation of CDP-Choline. CDP-choline (0.25 mmol; i.e.,5 umol per ml of resin) was dissolved in 25 ml of H_2O, and the ribosyl hydroxyls were oxidized to aldehydes by the addition of $NaIO_4$ (0.25 mmol). The mixture was adjusted to pH 5.55 with acetic acid. The reaction was carried out in the dark for 2 hr at room temperature and then overnight at 4°C. Formation of the dialdehyde was determined by TLC on Silica gel with the solvent CH_3OH/ 0.6% NaCl/NH_3 (10/10/1; v/v/v). The oxidized product had an increased R_f (0.73) relative to CDP-choline (0.59). [methyl-^{3}H]CDP-choline was used to estimate the oxidation efficiency.

4) Coupling of Oxidized CDP-Choline to Sepharose 4B Adipic Acid Dihydrazide. The oxidized CDP-choline solution and the Sepharose 4B adipic acid dihydrazide were combined in 0.1 M sodium acetate (pH 5.0) to give a final volume of 90 ml and mixed overnight at 4°C. The resin was washed with 0.1 M sodium acetate (pH 5.0) containing 0.5 M KCl followed by H_2O and finally with the buffer to be used for chromatography. Analysis of the CDP-choline content (determination of the radioactivity of [methyl-^{3}H] CDP-choline on the resin) indicated about 1.7 umol of CDP-choline per ml of gel.

Preparation of CTP-Sepharose 4B. CTP-Sepharose 4B was prepared similarly to that of CDP-choline Sepharose 4B with similar coupling efficiency (2.0 umol of CTP per ml of gel).

Separation of Cytidylyltransferase by CDP-Choline Sepharose 4B Chromatography. The following procedures were carried out at 4°C. Cytosolic CT collected by stepwise ammonium sulfate fractionation (30-40%) was purified by DEAE-cellulose chromatography (Fig.5) and the specific activity of the enzyme after the chromatography was usually 10-20 units/mg. A fraction of the enzyme (12-15ml) after DEAE-cellulose chromatography was brought to a final concentration of 14 mM magnesium acetate and then applied to a CDP-choline Sepharose 4B column (1.4 x 15 cm) which had been equilibrated with 20 mM Tris-succinate (pH 6.5) buffer containing 0.025% Triton X-100, 0.5 mM PMSF, 0.5 mM DTT and 14 mM magnesium acetate. The flow of the column was stopped for 30 min to achieve equilibrium between enzyme and ligand of the resin before starting to wash the column. The column was eluted with the buffer until the first protein peak diminished (monitored by recording the absorbance at 280 nm), and then with 0.5 M NaCl in the same buffer. The chromatogram in Figure 6 shows that less then 15% of the total enzyme activity loaded was retained by the column. The activity peak eluted with 0.5 M NaCl had a specific activity of 33 units/mg and showed 3-fold purification (The specific activity of original enzyme sample before CDP-choline Sepharose 4B chromatography was 10 units/mg in this particular experiment). However, since the bulk of enzyme did not bind to the CDP-choline resin, the total activity recovery was only 3.5%. Prolonged incubation of the sample within the column for up to 1 hr did not improve enzyme binding to the column. In an attempt to improve the affinity of enzyme to the CDP-choline ligand, the sample was

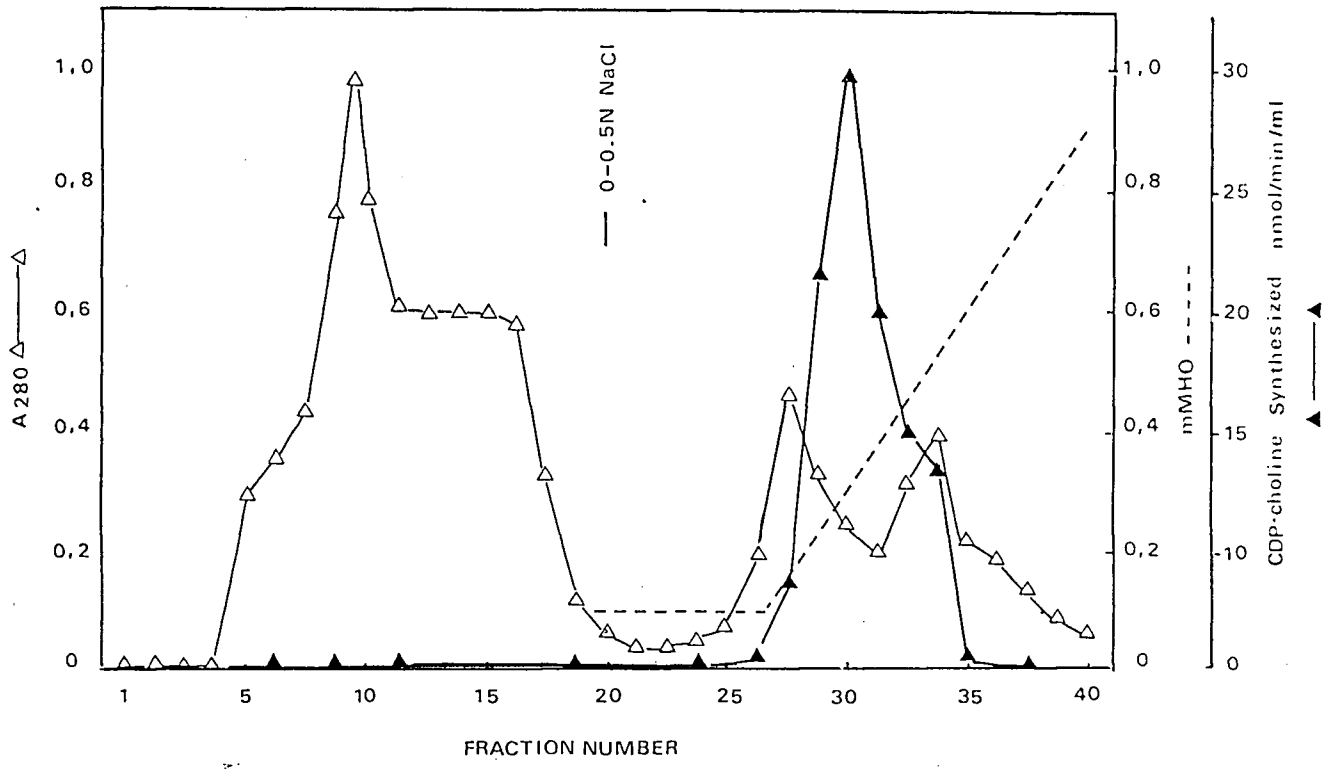

Fig.5. DEAE-cellulose Chromatography of Rat Liver Cytosolic Cytidylyltransferase. The 40% ammonium sulfate precipitate of rat liver cytosol was suspended in 20 mM Tris-succinate (pH 6.5) containing 0.025% Triton X-100, 0.5 mM PMSF and 0.5 mM DTT. Approx. 130 ml of the sample was applied to a DE-52 column (3 x 35 cm) which had been equilibrated with the sample buffer. The sample-loaded column was washed with 50 ml of the sample and then eluted with a 0-0.5 N NaCl gradient in the same buffer into 9 ml fractions.

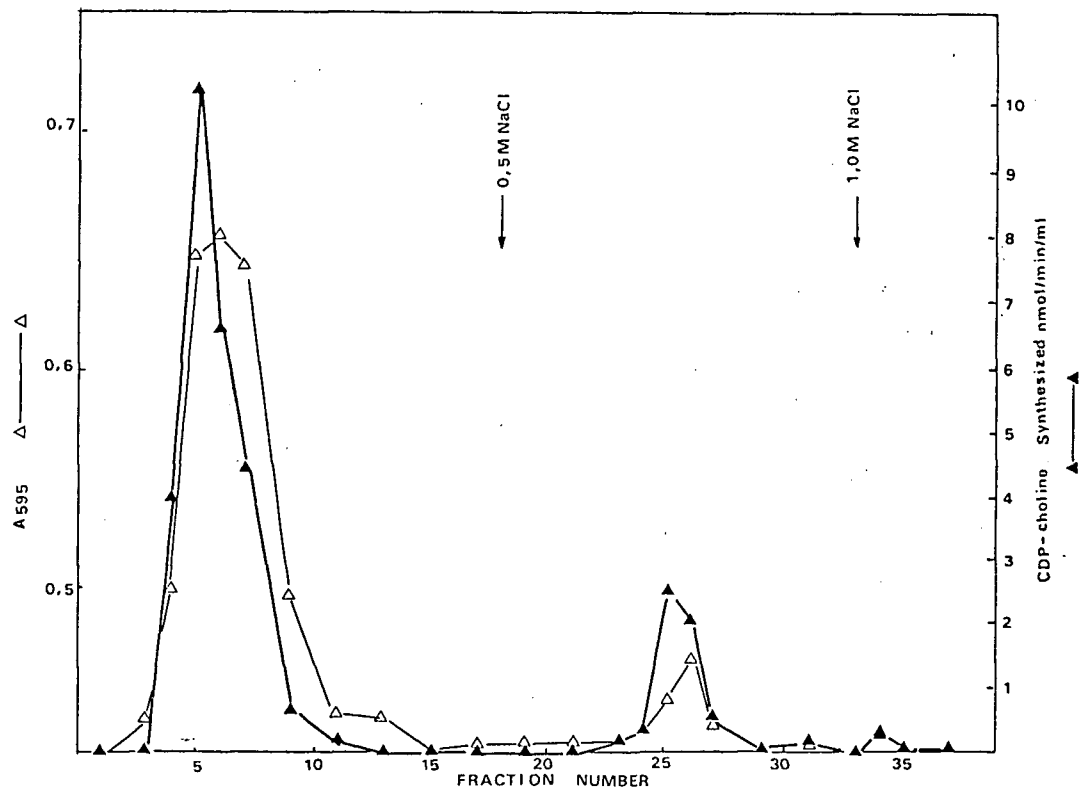

Fig.6. CDP-Choline Sepharose 4B Chromatography of Rat Liver Cytosolic Cytidylyltransferase. The enzyme fractions (12-15 ml) pooled from DE-52 column were brought to final concentrtion of 14 mM magnesium acetate and then applied to a CDP-choline column (1.4 x 13 cm) which had been equilibrated with 20 mM Tris-succinate(pH 6.5) containing 0.025% Triton X-100, 0.5 mM PMSF, 0.5 mM DTT and 14 mM magnesium acetate. The column was washed with the same buffer until A_{280} diminished and then eluted with 0.5 N NaCl. Fractions in the volume of 3.5 ml were collected. The column was regenerated by washing with 1 N NaCl.

brought to a final concentration of 0.2 mg/ml total rat liver phospholipid before being applied to the column. However, the protein and activity profile of the chromatogram was similar to those observed in the absence of phospholipid (data not shown).

Separation of Cytidylyltransferase by CTP-Sepharose 4B Chromatography. In contrast to CDP-choline Sepharose 4B column, the CTP-Sepharose 4B resin showed a fairly strong affinty for the enzyme. In order to improve on the purification, the enzyme preparation was fractionated by a phospho-cellulose column (pH 6.5) which followed ammonium sulfate precipitation and DEAE-cellulose chromatography. Figure 7 represents a typical chromatogram of CT purification by phospho-cellulose column. The enzyme pooled from phospho-cellulose column was dialyzed against 20 mM Tris-succinate buffer (pH 6.5) containing 0.025% Triton X-100, 0.5 mM PMSF and 0.5 mM DTT to lower the sample conductivity to 3 mMHO. This procedure caused a severe loss of enzyme activity, thus only one third of the activity was recovered after dialysis (see Table 1). The dialyzed sample (30 ml) was brought to a final concentration of 14mM magnesium acetate, and applied to a CTP-Sepharose 4B column (1.5 x 15 cm) which had been equilibrated with the sample buffer. After the bulk of the protein was eluted with 0.04 M NaCl, the enzyme was removed by a 200 ml gradient of 0.04-0.4 M NaCl in the same buffer (Figure 8). The chromatogram shows that there was no detectable enzyme activity in the first protein eluant, whereas the activity was eluted at a low conductivity range (2-3 mMHO) followed by a long tail. The results of a

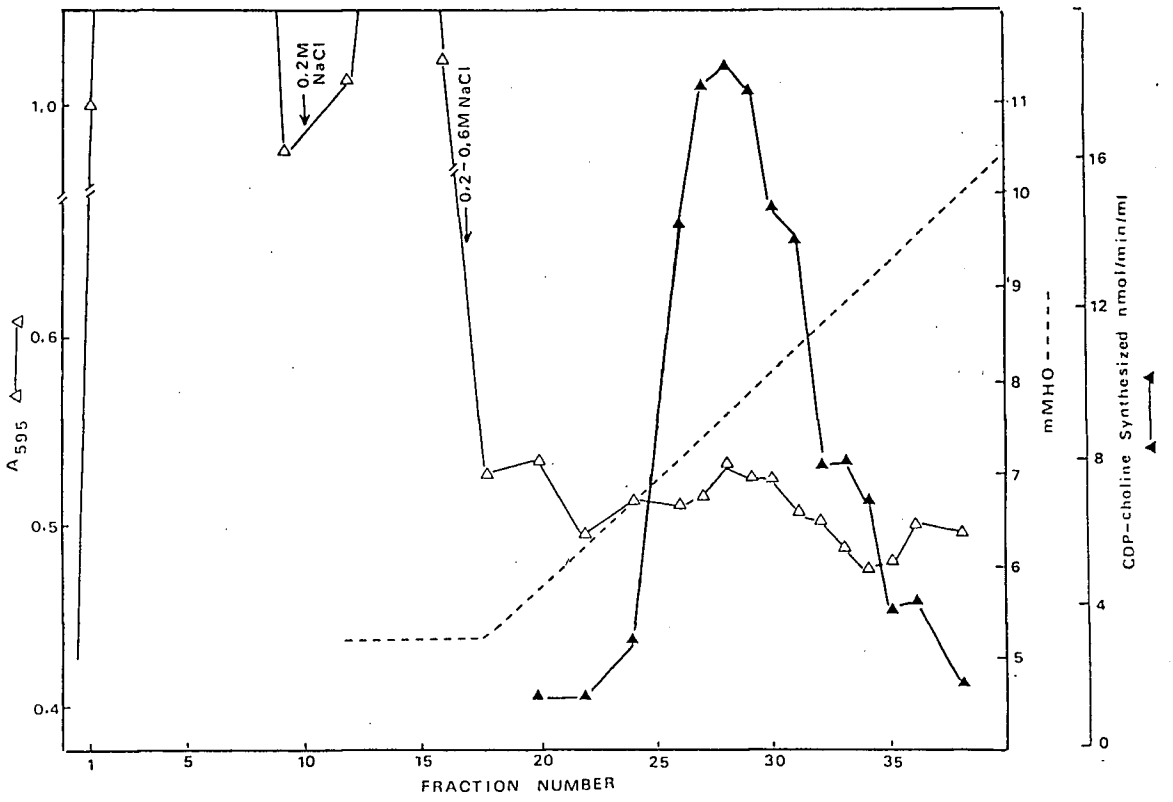

Fig.7. Phospho-Cellulose Chromatography of Rat Liver Cytosolic Cytidylyltransferase. The enzyme sample (40 ml) pooled from DE-52 column was applied to a P-11 column (2.5 x 8 cm) which had been equilibrated with 20 mM Tris-succinate (pH 6.5) containing 0.025% Triton X-100, 0.5 mM PMSF and 0.5 mM DTT. The column was washed with 80 ml of 0.2 M NaCl and then eluted with 100 ml of 0-0.6 M NaCl gradient in the same buffer into 4.5 ml fractions.

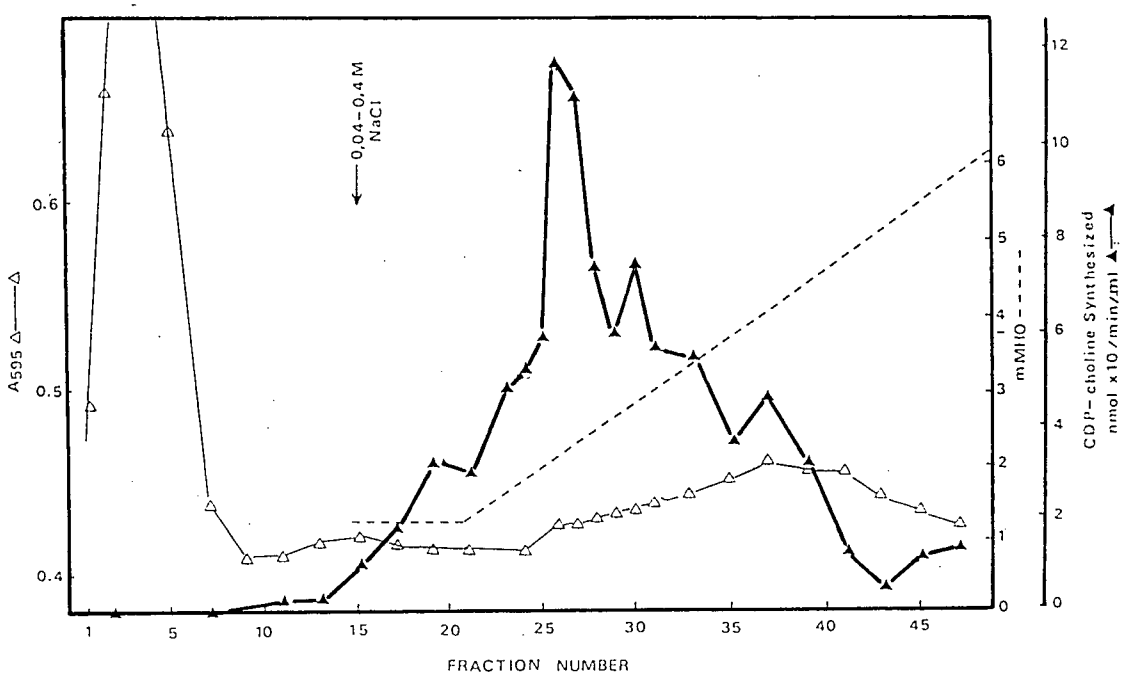

Fig.8. CTP-Sepharose 4B Chromatography of Rat Liver Cytosolic Cytidylyltransferase. The enzyme fractions (30 ml) from phospho-cellulose column were dialyzed against 20 mM Tris-succinate (pH 6.5) containing 0.025 % TritonX-100, 0.5 mM PMSF and 0.5 mM DTT for 1 hr and brought to the final concentration of 14 mM magnesium acetate before application to a CTP-Sepharose 4B column (1.5 x 15 cm) which had been equilibrated with the dialysis buffer containing 14 mM magnesium acetate. The sample-loaded column was washed with 0.04 M NaCl in the sample buffer and then eluted with 200 ml of 0.04-0.4 M NaCl gradient into 4 ml fractions.

purification scheme are summarized in Table 1. The CTP affinity chromatography exhibited a three-fold purification and 10% yield relative to the enzyme sample applied to the column. However, due to the loss of activity in the dialysis step, the overall purification was not improved.

Table 1. Purification of CTP:phosphocholine cytidylyltransferase from rat liver cytosol

Fraction	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Cytosol	128	3942	5558	1.4	100	1
$(NH_4)_2SO_4$ 40%	130	621	3916	6.3	70.5	4.5
DE-52	45	54	1165	21.6	21.0	15.4
P-11	31	5	471	94.2	8.5	67.3
P-11(after dialysis)	31	5	151	30.2	2.7	21.6
CTP-Sepharose 4B	16	0.14	13	92.9	0.2	66.4

DISCUSSION

The major purpose of this work was to find a method for purification of CT in the soluble, unaggregated, low molecular weight form. Since there is always some phospholipid in the cytosol fraction of 100,000 x g x 1 hr rat liver homogenate, part of the CT has associated with lipid spontaneously and there are two forms of CT present in the cytosol (40). These two forms of cytosolic CT can be distinguished by their different sensitivity to phospholipid activation, and can also be separated by stepwise ammonium sulfate precipitation (40). Most of the aggregated CT was pelleted with 30% ammonium sulfate, whereas the unaggregated CT is precipitated by 40% ammonium sulfate. In order to obtain the unaggregated form, previous studies done in this laboratory found that the addition of 0.5% Triton X-100 in the cytosol during ammonium sulfate precipitation significantly improved the recovery (70-85%) of the unaggregated form from cytosol, and caused no loss of activity. The presence of 0.025% Triton X-100 in the buffers used in the rest of the purification procedure seemed to be helpful in preventing enzyme from aggregating and stablizing the enzyme at early purification stages.

The two ion-exchange chromatography techniques (DEAE-cellulose and phospho-cellulose) used in this laboratory have been successful in the partial purification of unaggregated CT from rat liver cytosol. Figure 5 and Figure 7 represent the typical chromatograms of these two procedures respectively. The advan-

tage of using DEAE-cellulose chromatography as the first purification step is the high sample capacity of the column, and also, the enzyme preparation is quite stable in the presence of 0.025% Triton X-100 and no significant aggregation occurs. The phosphocellulose chromatography step provided a high fold purification and offered a higher recovery of activity (Table 1). But the enzyme eluted from the column contained high concentration of salt hence could not be subjected directly to the affinty chromatography without dialysis which, however, caused a severe loss of enzyme activity. The loss of activity after phospho-cellulose chromatography is possibly due to the almost complete removal of phospholipids from the enzyme through the two succesive ion-exchange columns. The addition of total rat liver phospholipid to the sample can maintain the enzyme activity, although the relationship between phospholipid and the enzyme is unclear. Since phospholipid causes the aggegation of CT and therefore affects the further purification of the unaggregated form of the enzyme, so we could not use phospholipid as a stabilizer. Choosing a detergent to offer an appropriate environment for maintaining CT functional structure and also prevent CT from aggregating remains an unsolved problem. So far, Triton X-100 appears the best candidate for this purpose.

An initial investigation on CT purification by affinity chromatography was reported from this laboratory 10 years ago (41) when glycerophosphocholine was covalently linked to epoxy-activated Sepharose 6B. The technique was not easily reproduced since the reaction of glycerophosphocholine coupling to the resin

was difficult to manipulate. The affinity columns prepared for this thesis used the natural substrates of CT as ligands. Hence we adopted a method to link covalently CTP or CDP-choline to Sepharose via a ribosyl group in order to expose both cytidine and phosphate (or phosphocholine) moieties to the enzyme. The putative structure of the affinity resin (Figure 4) might be the best representation based on the studies done by Hanske et al. on the structure of AMP and carboxylic acid hydrazides (49).

The difference in binding affinity for cytosolic CT to CTP- and CDP-choline-Sepharose resin is not explained. Obviously, the oxidized nucleotide derivatives have altered their original ribosyl structure in the coupling reaction. These structural changes might account for the failure of CT to bind to the CDP-choline resin, but not for the binding to the CTP resin. Choy et al. (40)speculated that the ability of the polymer form and unaggregated form of the enzyme to bind to an affinity column with glycerophosphocholine as ligand was different---while the unaggregated form was retained by the affinity material, the polymer form was not. The observation with the CDP-choline column experiment that addition of phospholipid to the cytosolic enzyme sample did not improve the enzyme binding to the affinity material might be due to the aggregation effect on the enzyme caused by phospholipid. The Michaelis constant of partially purified CT for CDP-choline (Km = 0.21 mM) was obtained in the presence of phospholipid (40) because of no detectable activity of the enzyme in the absence of phospholipid. Thus, the Km value reflected the affinity of the aggregated form of the enzyme to CDP-choline. Little is known about the affinity of unaggregated

enzyme for CDP-choline. However, the result from the CDP-choline Sepharose 4B chromatography shows that both the unaggregated and aggregated enzyme forms have no strong affinity to CDP-choline.

Preliminary studies showed that the CTP-Sepharose prepared as described previously (46,47) was of benefit to the cytosolic CT purification, while the commercially available CTP affinity resin (Sigma) did not retain the enzyme. Magnesium ions are required for activity (35), and are also necessary for promoting the enzyme affinity to the resin. The mechanism of the interaction between the enzyme and CTP is unclear. One of the probabilities is that there is a hydrophobic interaction involved, because the enzyme can be eluted from the affinty resin by the buffer with a lower ionic strength (2-3 mMHO) than that of the original sample (3 mMHO) (Figure 8).

As encountered by other groups (38,39), the severe loss of activity in later purification stages is still a problem. The partially purified enzyme with specific activity of about 100 units per mg protein was extremely unstable and more than 50% of the activity was lost within 3 hr at $4^{o}C$ during the dialysis step. Further studies are needed to discover a material(s) other than phospholipid which not only maintains the enzyme activity, but also prevents aggregation of the enzyme.

In conclusion, the CTP-sepharose 4B affinity chromatography may be an encouraging technique for cytosolic CT purification in combination with other ion-exchange chromatographies.

CHAPTER III. EFFECTS OF DIPHENYLSULFONE COMPOUNDS ON THE METABOLISM OF [METHYL-^{3}H]CHOLINE IN RAT HEPATOCYTES AND HELA CELLS

The biosynthesis of PC has been extensively studied in hepatocytes, because the PC synthesized in liver is not only the major membrane component within cells but also secreted as a constituent of bile or plasma lipoproteins. Rat liver has been reported to be a major organ for plasma lipoprotein synthesis as 80% of the lipoprotein pool in rat plasma originates from the liver (50) and the remainder is from intestine. The abundant forms of lipoprotein secreted by liver are either VLDL or HDL (51,52,53), in which phospholipid accounts for around 20% of the material by weight (54). The physiological function of the protein components in serum lipoproteins are somewhat understood. The abnormality of some lipoprotein apoproteins (55) or the deficiency of lipoprotein receptors on membranes of the target cells causes circulatory diseases. The function of phospholipids in the lipoprotein is probably mainly structural. However, the biological role of phospholipids in the process of lipoprotein secretion is of intrest since the secretion of products from cells must involves processes mediated by membrane components. Studies on the synthesis and assembly of plasma lipoproteins showed that lipid and apoproteins were assembled and glycosylated in Golgi apparatus before being secreted into plasma (57). Secretion of newly synthesized lipoproteins from liver into plasma was stopped very quickly after the administration of

puromycin, a specific inhibitor of protein synthesis (54), but the hepatic secretion of lipoproteins was not dependent on prior protein glycosylation (58). However, little is known about the biological role of phospholipids in the lipoprotein secretion process. One approach for studying the control of the lipoprotein secretion is to correlate changes in biosynthesis of PC, the major phospholipid component of lipoproteins, with lipoprotein secretory activity. This could be accomplished by using specific, nontoxic inhibitors of PC biosynthesis.

A series of diphenylsulfone compounds, dapsone and its derivatives, were reported to inhibit choline incorporation into PC. The studies done by Shigeura et al (18) in chick macrophages by using one of these compounds (AUS) showed this material markedly inhibited the synthesis of PC but exhibited no effects on the biosynthesis of DNA, RNA or protein. Bonney et al (59) also showed that the release of inflammatory mediators, prostaglandins (PGs), leukotrienes (LT) and lysosomal acid hydrolases (LAH) by mouse peritoneal macrophages, which is stimulated by endocytic stimuli such as zymosan, could be inhibited by the administration of diphenylsulfone compounds in a nontoxic manner. Correspondingly, the inhibition of PC biosynthesis was also observed in mouse macrophages. The reason for the inhibition of the product release by macrophages treated with the sulfones has been speculated to be due to the specific inhibition of PC biosynthesis via the CDP-choline pathway, since the release of products from cells certainly involves events mediated by plasma membrane components (59,60). Considering the similarity of the situations between the release of the products from macrophages and the secretion of

lipoproteins from hepatocytes, we hoped the diphenylsulfone compounds would help us to establish a model system for studying the relationship between an inhibition of PC biosynthesis and lipoprotein secretion in rat hepatocytes.

The isolation of hepatocytes and their maintenance in primary monolayer culture has enabled the studies of hepatic PC biosynthesis and lipoprotein secretion under conditions which reflect lipid metabolism *in vivo* yet permit subtle modifications in their nutritional, hormonal (61), and pharmacological states (62). Radioactively labelled (*methyl*-^{14}C or ^{3}H) choline is widely used as the precursor of PC in the CDP-choline biosynthesis pathway, and the distribution of radioactive in the cellular choline containing materials reflects the synthetic rate. But in rat hepatocytes, choline will also be oxidized to betaine. The conversion of choline to betaine is a two-step reaction catalyzed by choline dehydrogenase and betaine aldehyde dehydrogenase (63), and the product is rapidly released from rat hepatocytes (28,32,64) into medium.

Results reported here are from experiments in which we have attempted to use the diphenylsulfone compounds to perturb PC biosynthesis via the CDP-choline pathway in rat hepatocytes, as well as in HeLa cells. The preliminary studies have shown that the administration of these diphenylsulfone compounds, except dapsone, to HeLa cells inhibited the total [*methyl*-^{3}H]choline incorporation into the cells, but did not change the rate of conversion of choline to PC. Also, the effects of these compounds on PC biosynthesis in rat hepatocytes were investigated, but no

inhibition was observed.

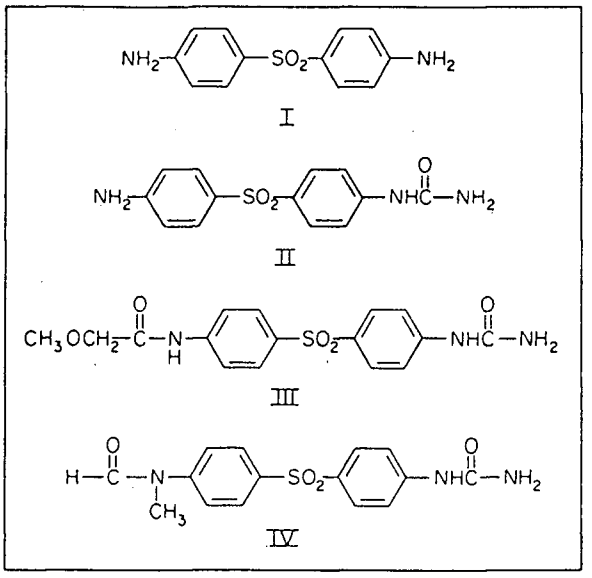

Fig. 9. Structures of dapsone (I), AUS (II) and analogs (III and IV).

MATERIALS AND METHODS

Chemicals- Female Wistar rats were from the University of British Columbia Animal Unit. Dulbecco's modified Eagle's medium (MEM) and fetal calf serum were bought from the Grand Island Biological Co., Grand Island, NY. [Methyl-^{3}H]Choline was obtained from Amersham. The diphenylsulfones, p,p'-diaminodiphenylsulfone (dapsone), [1-[4-(4-sulfanilyl)phenyl]urea (AUS), 4-Methoxyacetamido-4'-ureidodiphenyl sulfone (III) and 4-(N-Methylformamido)-4'-ureidodiphenyl sulfone (IV),were obtained from Merck Sharp & Dohme Research Lab., Division of Merck & Co., Inc. Rahway, NJ. The structures of the four compounds are shown in Figure 9.

Preparation of Rat Hepatocytes- Hepatocytes were isolated from female Wistar rats (180 g) by a collagenase perfusion technique as described by Davis et al (65), and cultured in plastic culture dishes (Lux Contur, 60 mm, 2-3 x 10^6 cells/dish) in arginine-free MEM with 28 uM choline chloride, 100 nm insulin, 0.4 mM ornithine, 100 ug/ml of streptomycin sulfate, 100 units/ml of penicillin G, 10 mM Hepes (pH 7.4) and 5% FCS at 37^o C under an atmosphere of 95% air/5% CO_2. The cells were maintained in monolayer culture for approximate 24 hr prior to all experiments.

Pulse-Chase Studies- Monolayer cultures of rat hepatocytes in 60 mm dishes (3 x 10^6 cells/dish) were washed twice with serum-free MEM and pulsed with 10 uCi [methyl-^{3}H]choline (0.12 Ci/mmol) for

1 hr. The cells were subsequently chased with MEM that contained 28 uM choline in the absence or presence of 100 ug/ml of diphenylsulfone compounds. At various times up to 4 hr, the cells were harvested and the radioactivity incorporated into cellular PC and the water soluble metabolites (choline, phosphocholine and CDP-choline) was determined. The radioactivity in the water soluble choline metabolites was separated by TLC on Silica gel G-60 with CH_3OH/0.6% NaCl/NH_3 (50:50:5; v:v:v) as solvent.

Phosphatidylcholine Extraction- The cellular PC was extracted by Bligh and Dyer's method (45). The culture medium was removed, and 0.7 ml of cold CH_3OH/H_2O (10:4; v:v) was added to each dish. The cells were harvested and the dish was washed with another 0.7 ml of the same solution. The sample was sonicated in a water bath sonicator for 10 min and an aliquot (50 ul) of sample was removed for protein assay. The remainder was mixed with 0.5 ml $CHCl_3$, 0.5 ml $CHCl_3$, and 0.5 ml H_2O sequentially, and then centrifuged at 2,500 rpm (Western H-103N centrifuge) for 5 min. The methanol-water (upper) phase was removed, and the chloroform (lower) phase was washed with 0.75 ml of theoretical upper phase ($CH_3OH/CHCl_3/H_2O$; 48/3/47; v/v/v) by centrifugation. The upper phase, which contains water soluble choline metabolites, was combined, and an aliquot was applied to TLC plates. An aliquot of the lower phase containing cellular PC was dried under nitrogen. The samples were counted for radioactivity as described previously (Chapter II, Materials and Methods section).

Growth of HeLa cells- HeLa cells were obtained from Flow Labora-

tories; they originally came from American Type Cell Culture Collection HeLa CCL-2. The routine growth conditions were as described by Pelech et al (66). HeLa cells were grown on plastic culture dishes (Lux Contur, 60 mm) in MEM (contains 28 uM choline chloride) with 0.4 mM ornithine, 100 ug/ml streptomycin sulfate, 80 ug/ml arginine, 100 units/ml penicillin G, 10 mM Hepes (pH 7.4) and 5% FCS at 37°C under an atmosphere of 95% air/5% CO_2. Confluent monolayer cell cultures were used for [methyl-^{3}H] choline incorporation or pulse-chase experiments as described above, except 80 ug/ml arginine was always present in all culture media.

RESULTS

Influence of Diphenylsulfone Compounds on [Methyl-^{3}H]Choline Uptake by Hela Cells : Pulse Labeling Studies- Bonney et al. (60) have shown that diphenylsulfones dapsone and AUS inhibited incorporation of [1,2-^{14}C]choline into PC, but not the formation of PE from [1,2,-^{14}C]ethanolamine in both mouse macrophages and HeLa cells. Similar results were obtained in the studies on chick macrophages by using AUS (18). I have studied the effect of these drugs, dapsone and AUS, as well as the related compounds III and IV, on the PC synthesis in HeLa cells. The experimental data observed are summarized in Table 2 and Table 3. After a 1 hr pulse, 90% of the radioactivity in the chloroform phase was in PC and likewise, 90% of radioactivity in the methanol-water phase was in phosphocholine. The radioactive labeling of CDP-choline was very small. This implies that the conversion of CDP-choline to PC is fast in this biosynthetic pathway. The data showed that dapsone (100 ug/ml) had no inhibitory effect on the incorporation of labeled choline, as measured by the total radioactivity of the cellular materials in both chloroform phase and methanol-water phase (Table 2). The other three compounds, AUS, III and IV, inhibited labeled choline incorporation to varying degree (20- 50%) following a pretreatment period of 0 or 4 hr (Table 3). Among these compounds, IV exhibited the highest inhibitory effect on [methyl-^{3}H]choline incorporation, since no pretreatment of the cells with the compound was necessary to

Table 2. Effects of Dapsone and AUS on the [Methyl-^{3}H]Choline Uptake by HeLa Cells*

Addition	Lower phase (dpm x 10^{-5}/mg)		Upper phase (dpm x 10^{-5}/mg)		Total (dpm x 10^{-5}/mg)	
	0 hr	4 hr	0 hr	4 hr	0hr	4hr
Control	0.97±0.09	1.05±0.12	35.6±4.8	29.1±1.5	36.7	30.2
Dapsone	1.08±0.09	0.97±0.05	39.9±0.3	31.1±1.8	41.0	32.1
	(-)	(-)	(-)	(-)	(-)	(-)
AUS	0.79±0.11	0.68±0.06	28.2±3.9	20.5±2.4	29.0	21.2
	(19)	(35)	(21)	(30)	(21)	(30)

* HeLa cells were cultured as described in Material and Methods. Cells were preincubated in serum-free MEM containing 100 ug/ml of dapsone or AUS dissolved in DMSO for 0 or 4 hr and then labeled with 11 uCi of [methyl-^{3}H]choline (0.13 Ci/mmol) per dish for an additional 1 hr in the presence of dapsone or AUS prior to harvesting. All cultures contained a final concentration of 0.1% DMSO. The [methyl-^{3}H]choline uptake was estimated by quantitation of the cellular radioactivity in both water-methanol (upper) phase and chloroform (lower) phase extracted by the Bligh and Dyer's method. Protein was estimated by Bio-Rad assay. The results are the averages±S.D.; N=3. Per cent inhibition by AUS with respect to control is given in parentheses.

Table 3. Effects of Diphenylsulfone Derivatives (III and IV) on the [Methyl-^{3}H]Choline Uptake by HeLa Cells*

	Lower phase (dpm x 10^{-5}/mg)		Upper phase (dpm x 10^{-5}/mg)		Total (dpm x 10^{-5}/mg)	
Addition	0 hr	4 hr	0 hr	4 hr	0 hr	4 hr
Control	4.02±0.51	1.93±0.38	39.7±1.8	32.2±3.5	43.7	34.1
III	3.61±0.29	1.08±0.20	27.4±1.0	19.0±2.3	31.0	20.0
	(10)	(44)	(31)	(41)	(29)	(41)
IV	2.21±0.12	1.17±0.06	20.4±0.4	16.6±0.6	22.6	17.8
	(45)	(39)	(49)	(48)	(48)	(48)

* The expermental conditions were the same as described in the legend of Table 2, except the compounds III and IV were used instead of dapsone and AUS. The results are the averages ±S.D.; N=3. Per cent inhibition by III and IV with respect to control is given in parentheses.

give the greatest inhibition (Table 3). The total [methyl-^{3}H] choline incorporation was reduced in the cells treated with AUS, III or IV, but the propotion of radioactivity in the chloroform and the methanol-water phases were similar for all three drugs, which suggests that the conversion of choline into PC was not affected. The studies done with mouse (60) or chick (18) macrophages suggested that these compounds inhibited the PC biosynthesis via CDP-choline pathway at steps following phosphocholine, i.e. at either CT or CPT. However,the data presented here imply that the reduced incorporation of radioactivity into PC was due to the reduction of total [methyl-^{3}H]choline incorporation into the cells treated with the compounds, rather than the inhibition of the conversion of choline into PC.

In order to determine the influence of diphenylsulfone compound on the [methyl-^{3}H]choline metabolism in HeLa cells, pulse-chase studies were done in the presence of AUS or IV. The reason for choosing these two compounds in this experiment was they exhibited higher inhibition of choline uptake. Since there is neither significant oxidation of choline to form betaine nor biosynthesis or secretion of lipoprotein in HeLa cells, the radioactivity in methanol-water and chloroform phases could represent the major choline metabolites, phosphocholine and PC, respectively, while the counts in medium compounds might be negligible. After 1 hr pulse of [methyl-^{3}H] choline in the presence of AUS and IV, the inhibition of choline incorporation into the cells was observed. The radioactivity labeling in both chloroform (Fig.10 A) and methanol-water phases (Fig.10 B) at the

zero time of the chase were lower in treated cells than in the controls. The rate of PC biosynthesis in the cells could still be estimated by comparing the rate of [methyl-^{3}H]choline incorporation into the chloroform phase between treated and control cells. There were little or no differences in the decrease of radioactivity from phosphocholine (Fig.10, B) and the increase of radioactivity in PC (Fig.10, A) by comparing the treated and control cells. The data confirmed that the diphenylsulfone compounds inhibited only the total incorporation of choline into HeLa cells but had no effect on the conversion of choline into PC. However, as AUS and IV inhibited the incorporation of choline, hence altering the specific activity of cellular choline, the slope of the incorporation of [methyl-^{3}H]choline into PC might not reflect the actual rate of PC biosynthesis.

Influence of Diphenylsulfone Compounds on [Methyl-^{3}H]Choline Metabolism in Rat Hepatocytes : Pulse-Chase Studies- Since the major organ for lipoprotein synthesis and secretion in rat is liver, cultured rat hepatocytes were treated with diphenylsulfone compounds (100 ug/ml) to show whether these drugs could inhibit specifically PC biosynthesis via the CDP-choline pathway, and determine the effect on PC biosynthesis and lipoprotein secretion. The cells were prelabeled with [methyl-^{3}H]choline for 1 hr in the absence of any kind of diphenylsulfone compound to avert the potential problem of apparent changes in the labeling of PC due to alterations in choline incorporation, as seen in HeLa cells (Fig.10). [Methyl-^{3}H]Choline is taken up by the cells and rapidly phosphorylated to phosphocholine or oxidized to

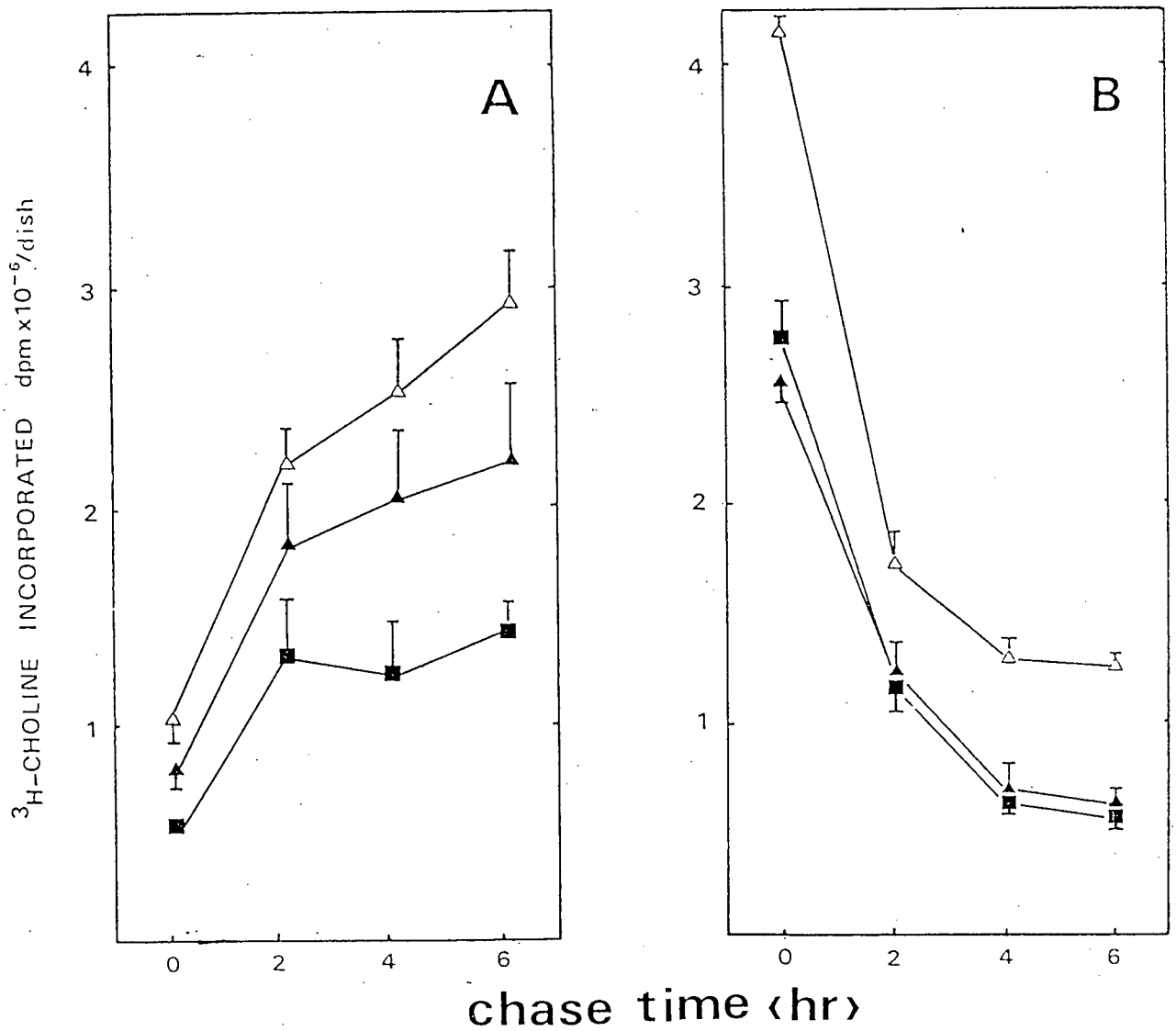

Fig.10. Influence of AUS and IV on the metabolism of [methyl-^{3}H]choline in HeLa cells. HeLa cells cultured in 60 mm dishes were washed with serum-free MEM and pulsed with 10 uCi [methyl-^{3}H]choline (0.12 Ci/mmol) per dish in the absence (Δ) or presence of 100 ug/ml AUS (▲) or IV (■) dissolved in DMSO for 1 hr. The cells were subsequently chased with 28 uM choline in the unlabeled medium for up to 6 hr prior to harvesting. All culture media contained a final concentration of 0.1% DMSO. Radioactivities in the chloroform phase (A) and methanol-water phase (B) extracted by Bligh and Dyer's method correspond to PC and phosphocholine, respectively. Each point represents the mean of three dishes.

betaine (Fig.11 and Fig.12). After 1 hr pulse, approximately 50% of the radioactivity was converted to betaine, whereas phosphocholine, PC, CDP-choline and choline accounted for the remaining 50%, in which about 70% was phosphocholine and 20% was PC. The cells secreted betaine into the medium, so after 1 hr of chase, more than half of the cellular betaine disappeared from the cells. The radioactivity in medium compounds increased rapidly within the first hours of the chase period, which represented the release of not only cellular betaine, but also phosphocholine and lipoprotein PC. All of these processes are not influenced by dapsone, AUS, III or IV, since neither the rate of decrease of radiaoctivity in cellular betaine nor the rate in the increase of radioactivity in the medium compounds were significantly altered. The decrease in labeled phosphocholine and increase in PC represented the enzymatic activity involved in the successive conversions of phosphocholine to CDP-choline and PC. Still, no altered rate of these conversions was observed in either diphenylsulfone compound treated cells compared to the control. The slower increase in cellular PC radioactivity, compared with the rate of decrease in cellular phosphocholine, might be explained as the secretion of lipoprotein PC to the medium. These results suggested that the activity of PC biosynthesis and lipoprotein secretion in rat hepatocytes were not affected by these diphenylsulfone compounds in these experimental conditions.

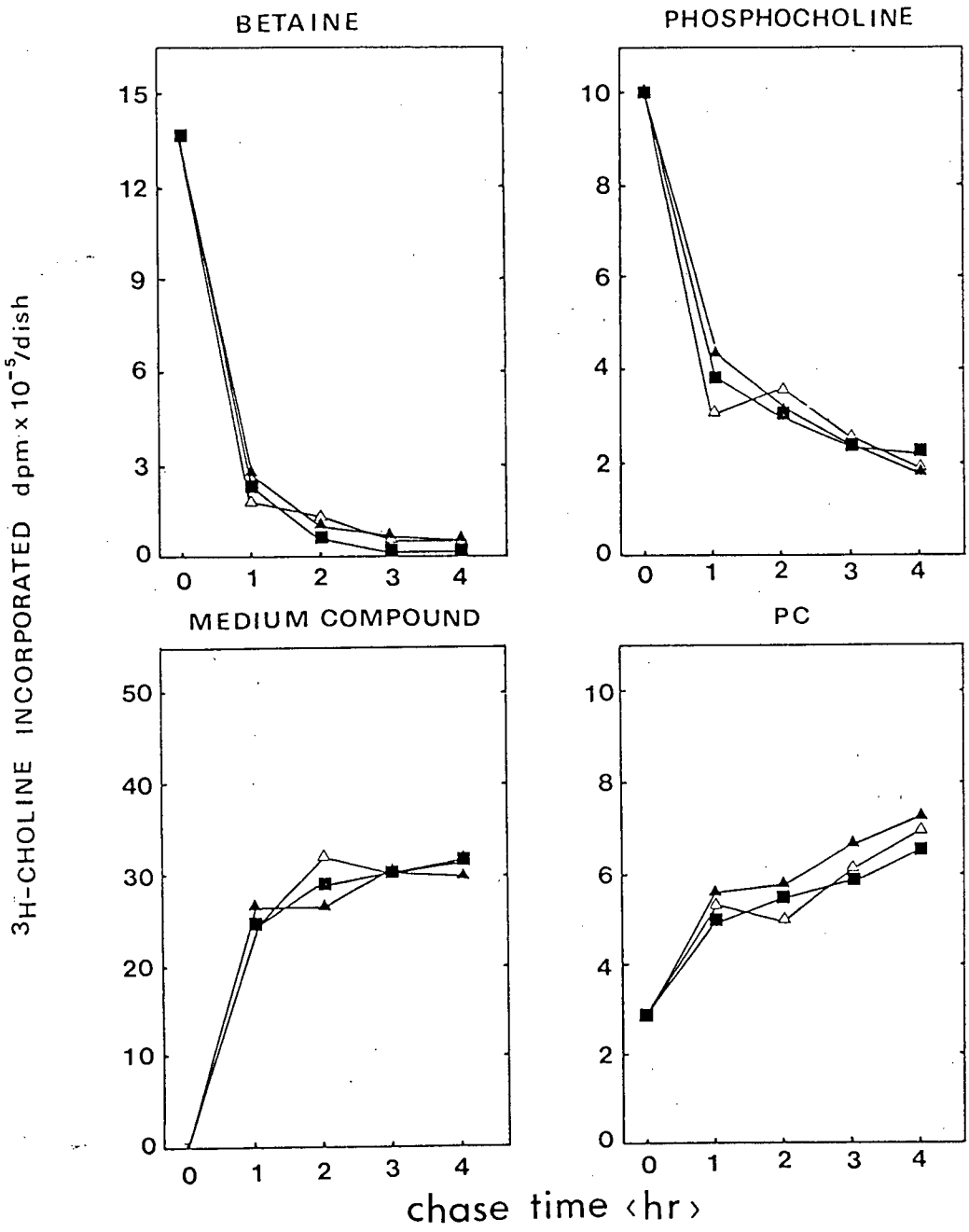

Fig.11. Influence of Dapsone or II on The Metabolism of [methyl-^{3}H] Choline in Rat Hepatocytes. Monolayer cultures of rat hepatocytes in 60 mm dishes (3 x 10^6 cells/dish) were washed with serum-free MEM twice, and then pulsed with 10 uCi [methyl-^{3}H]choline (0.12 Ci/mmol) in the same medium for 1 hr. The cells were subsequently chased with 28 uM choline in the absence (Δ) or presence of 100 ug/ml dapsone (▲) or III (■) dissolved in 0.1% DMSO for up to 4 hr. All chase media contained 0.1% DMSO. Radioactivity incorporated into the cellular choline metabolites and medium compounds was determined as described under Materials and Methods. Each point represents the mean of two dishes. The experiment was repeated, and similar results were obtained.

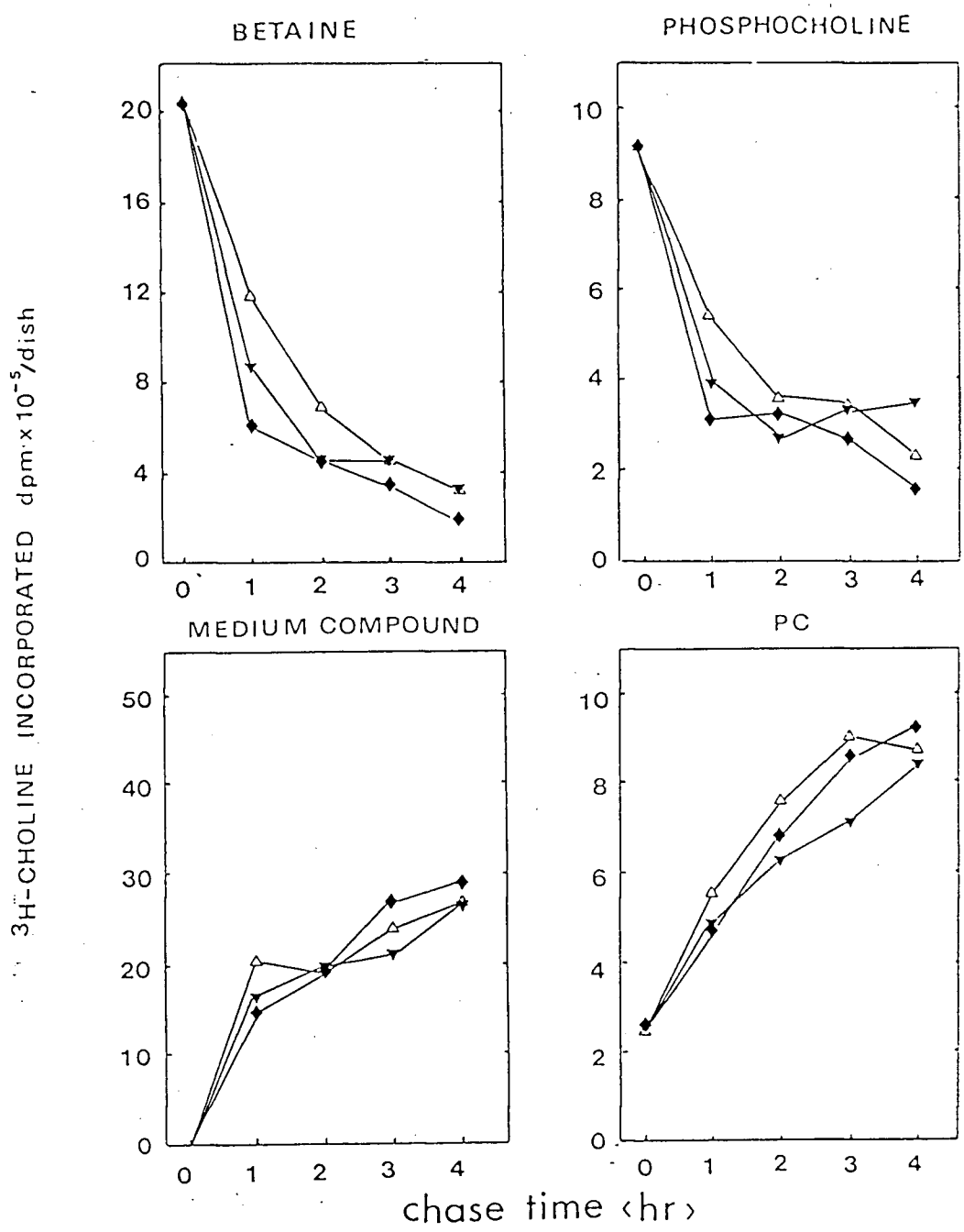

Fig.12. Influence of AUS and IV on The Metabolism of [methyl-^{3}H]Choline in Rat Hepatocytes. The experimental procedure was similar to that described in the legend of Fig.11. AUS (▼) and IV (♦) in the final concentration of 100 ug/ml were used here. Each point represents the average of two dishes. The experiment was repeated, and similar results were obtained.

DISCUSSION

There is no known inherited deficiency in PC biosynthesis, so the studies on the biological function of PC in lipoprotein secretion have been performed only in "experimental" diseases, such as choline deficiency in the diet (67). The situation has become more complicated since PC biosynthesis via the CDP-choline pathway seems to be highly resistant to the environmental variations and the quantitative amount of the rate-limiting enzyme CT is almost unchanged in any physiological condition. The report from Bonney *et al* (60) that diphenylsulfone compounds could specifically inhibit PC biosynthesis in macrophages in a non-toxic manner encouraged us to study PC biosynthesis and lipoprotein secretion in hepatocytes by using these compounds. Unfortunately, our results showed that there were no effects on [*methyl*-^{3}H]choline metabolism in hepatocytes administered these compounds (100 ug/ml). The simplest explanation of this phenomenon might be the detoxification ability of liver cells. However, the data obtained from the studies on cultured macrophages by using [^{14}C]dapsone or [^{14}C]AUS showed that the compounds were not accumulated by the cells (60), which implied that the diphenylsulfones did not penetrate the cell membrane. Thus it is unconvincing to explain the lack of effects in hepatocytes treated with the compounds by a cellular detoxification mechanism. On the other hand, it is still possible that we have not

found an effective dosage of these diphenysulfones on rat hepatocytes, since we only did experiments in which the compound concentration was 100 ug per ml.

The parent compound of this series diphenylsulfones, dapsone, is an established antimalarial and antileprotic agent (68), and leprosy is a disease which is charaterized by polymorphonuclear infiltration. Bonney et al (59) found that dapsone and its derivatives inhibited the release of the inflammatory mediators by macrophages which is stimulated by zymosan. The mechanism of the inhibition of PC biosynthesis by the diphenylsulfones in mouse and chick macrophage has been postulated by Bonney et al (60) and Shigeura et al (18) to be because sulfones acted at the site(s) of conversion of phosphocholine to PC. They found little or no inhibition of the choline transport, or of choline phosphorylation. The results from the experiments done on HeLa cells presented here does not agree with this previous conclusion. We found that all the diphenylsulfones, except dapsone, inhibited only the choline incorporation into the cells, but not the conversion of label from choline to PC. The radioactivity distribution in cellular intermediates of PC synthesis in HeLa cells from both pulse labeling (Table 2 and Table 3) and pulse-chase experiment (Fig.10) support this point of view.

The reason for reduced total [methyl-^{3}H]choline incorporation into HeLa cells was not examined here as the major purpose of the experiment was to verify the effect of the compounds on the conversion of choline to PC. It is possible that the reduced

choline incorporation is due to inhibited choline transport and there are several probabilities which might account for the result. It has been reported that there were two mechanisms involved in choline transport in rat liver, saturable and unsaturable uptake (69), and the apparent Km for choline for the saturable mechanism was 10 uM in rat hepatocytes (70). Since the concentration of choline in rat plasma normally ranges between 10 to 20 uM (71), the facilitated transport of choline by liver probably approaches saturation, although there seems no limit on choline entry by passive diffusion. The reduced choline incorporation into the HeLa cells treated with diphenylsulfones might be due to the inhibition of the membrane carriers which are specific for choline transport. On the other hand, studies on choline transport with Novikoff hepatoma cells argued that the choline transport served as the rate-limiting step in PC biosynthesis when the concentration of choline was below 20 uM, in which the choline incorporation into PC was limited by the rate of formation of phosphocholine catalyzed by choline kinase (72), while at concentrations above 20 uM, the rate of choline incorporation into PC was independent of medium choline concentration (72). Reduced choline phosphorylation in the treated HeLa cells might also be the cause of the observations of reduced choline incorporation. That is, the inhibition of choline kinase results in less conversion of choline to phosphocholine, therefore the medium [methyl-^{3}H]choline entering via passive diffusion into the diphenylsulfone treated cells is reduced due to an increase in cellular choline concentration. We do not know whether or not

the diphenolsulfones inhibit choline incorporation into rat hepatocytes. Pritchard and Vance (32) have studied the role of choline transport in PC biosynthesis in rat hepatocytes, and found the rate of hepatic PC biosynthesis was almost not influenced by the rate of choline transport when the concentration of choline in the culture medium ranged between 5-40 uM. The results from HeLa cells presented here confirmed this statement.

In conclution, the diphenylsulfone compounds appears not suitable for the studies on the inhibition of PC biosynthesis via CDP-choline pathway in rat hepatocytes or HeLa cells. Therefore, it is still of interest to find a specific inhibitor of PC biosynthesis via CDP-choline pathway in livers to study the relationship between PC biosynthesis and lipoprotein secretion.

CHAPTER IV. EFFECTS OF VASOPRESSIN ON CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE IN RAT HEPATOCYTES

The regulation of PC biosynthesis has been studied under a variety of developmental and physiological conditions (24,34,73, 74,75,76,76a) and by genetic manipulations (77,78). Based on analyses of the activities of enzymes in PC synthesis as well as levels of metabolic intermediates, it was concluded that the CT was regulatory in the CDP-choline pathway under the experimental conditions. The studies on the regulatory mechanisms of CT activity and the biosynthesis of PC have focused on the short term control. The effect of substrate concentration on PC synthesis varies in different cells. In polio virus-infected HeLa cells (79,80), the concentration of cytosolic CTP seems to be critical for regulation of the CT catalyzed reaction and PC anabolism, whereas in the cockerel treated with diethylstilboestrol (DES), it was shown that the concentration of cellular phosphocholine restricted the rate of PC synthesis in the liver (81,82). However, in BHK cells, both CTP and phosphocholine appeared to limit the rate of PC biosynthesis (83).

Since CT has a bimodal distribution within cells and its activity is phospholipid-dependent, this suggests that the enzyme is regulated via a translocation mechanism. The bulk of CT located in cytosol is inactive and functions as an enzyme reservoir. But once the enzyme becomes associated with membrane, it will be activated by phospholipids. Several different mechanisms have

been proposed to explain how cells control the relative distribution of CT. Studies done by Sleight and Kent on CHO cells (75) and embryonic chick muscle cells (76) demonstrated that the CT might be regulated by a membrane repairing mechanism. Exposure of the inactive cytosolic enzyme to membranes depleted of PC, which could be formed by phospholipase C treatment, results in both the binding and activation of the enzyme. One question associated with this proposol is whether the increased membrane-associated CT is caused directly by the depletion of PC in the membrane or by higher concentration of diacylglcerol (DG) which is a product of phospholipase. DG has been demonstrated to promote aggregation of CT *in vitro*, and this effect was mimicked by an exogenous phospholipase C from *Bacillus cereus* (84).

There are two other models of regulation. The first is the reversible phosphorylation hypothesis. Covalent modification of CT via a phosphorylation-dephosphorylation cycle correlated with changes in the distribution of CT activity between cytosol and microsome. Regulation of PC biosynthesis by this mechanism has been proposed in rat hepatocytes (61,74) and in *in vitro* studies with liver cytosol (27). In the presence of cAMP analogues and cAMP phosphodiesterase inhibitors, there is more soluble CT and less membrane-associated enzyme in hepaocytes (61,74). Similarly, incubation of rat liver cytosol *in vitro* with Mg-ATP or phosphoprotein phosphatase inhibitor, NaF, prevents the cytosolic CT from associating with membranes upon incubation at 37° C (27). This effect could be abolished by the addition of cAMP protein kinase inhibitors into the incubation system and results in

increased proportion of CT activity in cytosol (27). The dephosphorylated CT has a tendency to associate with membrane (generally E.R.) and to be activated by phospholipids, while the phosphorylated CT is inactive and located in the cytosol. The reversible phosphorylation mechanism in regulation of PC biosynthesis is assumed to be similar to the mechanisms involved in fatty acid and cholestrol biosynthesis (85). Phosphorylation of acetyl-CoA carboxylase (86) and HMG-CoA reductase (87) also render these regulatory enzymes inactive in their respective pathways. Pelech and Vance (88) speculated that the phosphorylation of CT might be hormonally controlled by glucacon, since cAMP is the established second messenger of this hormone. However, the direct incorporation of [^{32}P]phosphate into the enzyme remains to be demonstrated.

A second mechanism derived from the studies done on rat hepatocytes (28) and HeLa cells (66), suggested that the translocation of CT from cytosol to microsomes and the stimulation of PC synthesis is regulated by fatty acids and fatty acyl-CoA (28). It was shown in rat hepatocytes that the effect of unsaturated long chain fatty acids (1 mM palmitate or 3 mM oleate) on CT translocation is so strong that it will reverse the phosphorylation-mediated decrease in CT activity associated with microsomes (61). However, the mechanism of fatty acid-mediated translocation is poorly understood. It has been speculated that DG may account for some of the translocation of CT, since the pool size of DG in rat hepatocytes supplemented with 1 mM oleate was increased (28,15). Still, the question remaining is whether or not DG can

enhance the binding of the enzyme to microsomes *in vivo*.

Vasopressin, an alpha-adrenergic agonist, has been shown to cause a transient (within 4 min) increase of 50% in the total concentration of DG in rat hepatocytes (89). The DG formed in the presence of vasopressin is considered to be derived from the hydrolysis of PtdIns(4,5)P_2, PtdIns4P and PtdIns and/or perhaps the other phospholipids (89). This reaction has been shown to be catalyzed by phospholipase C which appears to be activated by vasopressin in cultures (90). The question relating to the effects of vasopressin on the PC biosynthesis is whether or not the transient increase of DG will promote the translocation of CT from cytosol to microsomes and hence activate the CDP-choline formation. To answer this question, hepatocytes were treated with vasopressin and the effects of the hormone on CT distribution and PC synthesis were determined in this paper.

A protocol for estimation of the subcellular location of cellular enzymes has been developed by MacKall *et al* (91). Cells are disrupted by treatment with digitonin, and the rate of release of soluble enzyme determined. The release of cytosolic enzymes from cultured cells is more rapid than membrane-associated enzymes. Thus, if the increase of DG, caused by the administration of vasopressin to the cells, results in the translocation of CT from cytosol to micrsomes, a reduction in the rate of release of cytosolic CT from the cells into the medium should be observed.

The results presented here shows that the addition of vasopressin (5-20 nM) to rat hepatocytes resulted in a reduced rate

of release of cytosolic CT into the culture medium. However, the increased rate of [methyl-^{3}H]choline incorporation into PC was not observed in either pulse-labeling or in pulse-chase experiments.

MATERIALS AND METHODS

Chemicals- Wistar rats (180 g) were supplied by the University of British Columbia Animal Unit. Digitonin (80%) and arginine vasopressin (Grade VIII) were obtained from Sigma. Vasopressin was dissolved in 0.9% NaCl as a stock solution of 5 ug/ml and kept at -20° C. Dulbecco's phosphate buffered saline (PBS) contains 2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl and 8.1 mM $NaHPO_4$, pH 7.4.

Incubation of Hepatocytes with Vasopressin- Rat hepatocytes were isolated from normally fed Wistar rats and maintained in 60 mm culture dishes (2×10^6 cells/dish) as previously described (in Chapter III, Materials and Methods). For the study on digitonin-mediated release of cytosolic CT, the cells were washed with serum-free MEM twice, and incubated in the same medium containing 5-20 nM vasopressin for 0-8 min. For the [methyl-^{3}H]choline incorporation study, each dish of hepatocytes was labeled with 10 uCi of [methyl-^{3}H]choline (0.12 Ci/mmol) in the same medium containing 10 nm vasopressin for 0-60 min. For a pulse-chase study, the cells were washed with serum-free MEM twice and then pulsed with 10 uCi of [methyl-^{3}H]choline (0.12 Ci/mmol) in the same medium for 30 min. The cells were washed with serum-free MEM containing 28 uM choline and chased in the same medium in the absence or presence of 10 nM vasopressin for up to 10 min. In order to avoid any effects of serum in the medium on the experiment, in some studies the medium was replaced by serum-free MEM

12 hr before the pulse-chase period.

Release of Cytosolic CT from Rat Hepatocytes- Digitonin-mediated release of cytosolic CT from cultured rat hepatocytes was performed essentially as described by Mackall et al. (91). After removal of culture medium, the cell monolayer was washed carefully with 2.5 ml of ice-cold PBS. Cold digitonin-release buffer (1.0 ml per 60 mm cluture dish) containing 10 mM Tris-HCl (pH 7.4), 250 mM sucrose, 0.5 mM PMSF and 0.5 mg/ml digitonin was carefully pipetted into each culture dish to initiate enzyme release. The dishes were placed on an ice-cold tray and the cells were incubated with digitonin up to 8 min. The dishes were occasionally gently swirled during the incubation period. At the times indicated the digitonin-release buffer was removed, and an aliquot of the sample was used for CT assay.

Other Methods- The assay of CT activity and the separation of choline metabolites by TLC are described under the previous Materials and Methods section. CDP-choline was separated from betaine by TLC with acetone/methanol/HCl (10:90:4; v:v:v) as described previously by Lim et al (92).

RESULTS

Effect of Vasopressin on The Digitonin-mediated Release of CT from Rat Hepatocytes Exposure of hepatocytes to 10 nM vasopressin led to a reduced rate of release of cytosolic CT into the culture medium (Fig.13). The maximal effect was observed when the cell was incubated with vasopressin for 3 min. After 3 min incubation, less than 50% (1 min) and 65% (2 min) of the cellular CT, compared with those of the controls, was released into the medium. The reduced release of CT was also observed in cells treated with vasopressin for only 1 min. Prolonged incubation (5 and 10 min) of the cells with vasopressin at the same concentration resulted in a return to normal rate of release of CT. This suggested that the effect of vasopressin on the translocation of CT was transient. The reduced release of CT from the cells treated with vasopressin was maintained up to 8 min.

Effect of Different Concentrations of Vasopressin on The Digitonin Mediated Release of CT from Rat Hepatocytes In order to determine the maximally effective concentration of vasopressin on the digitonin-mediated release of CT in rat hepatocytes, different concentrations (5-20 nM) of vasopressin were investigated (Fig. 14). Preliminary studies showed that the maximal effect of vasopressin was obtained with a concentration of 5 nM. The cells treated with 20 nM of vasopressin did not show a further inhibition in the rate of CT release compared with those treated

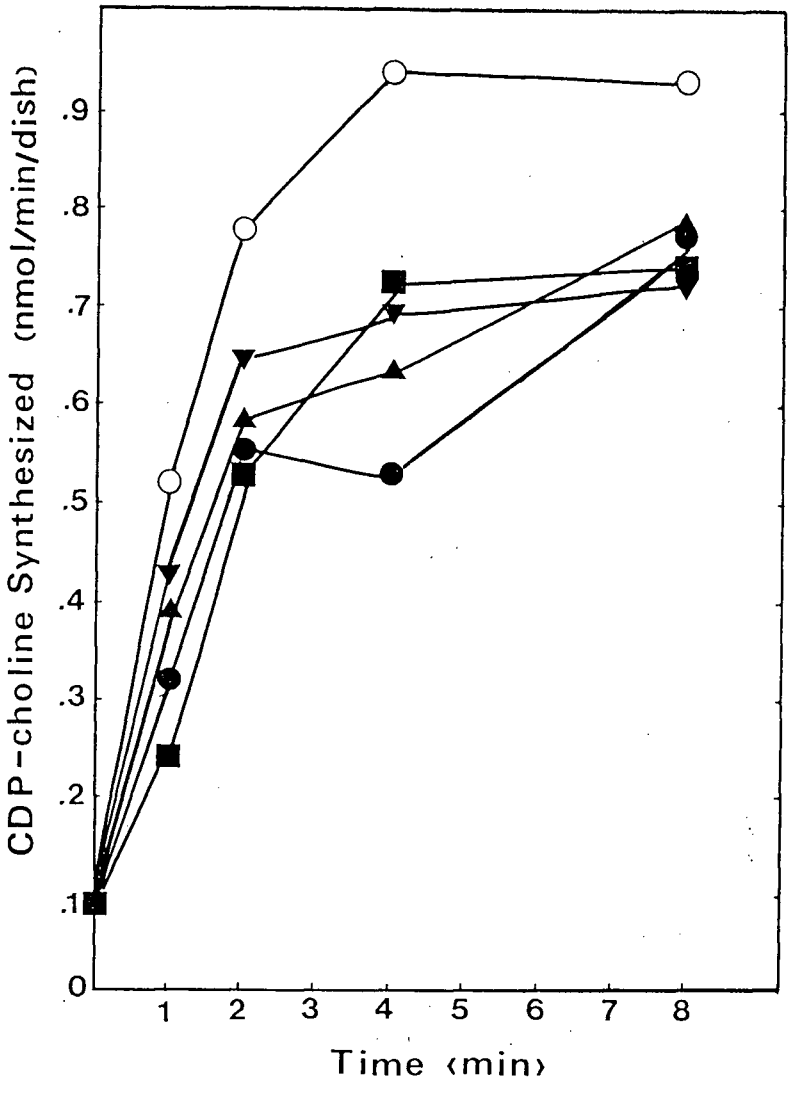

Fig 13. Digitonin-mediated Release of CT from Rat Hepatocytes. Rat hepatocytes in 60 mm dishes (2 x 10^6 cells) were incubated for 0 min (○), 1 min (▲), 3 min (■), 5 min (●) or 10 min (▼) in serum-free medium containing 10 nM vasopressin. The cell medium was replaced with 1 ml of ice-cold digitonin (0.5 mg/ml) after washing the cells with 2.5 ml of ice-cold PBS, and the incubation continued on an ice-cold metal tray for up to 8 min. CT activity in the digitonin extract was subsequently determined in the presence of 0.2 mg of total rat liver phospholipid and 10 nmol of oleate. Each point represents the average of two dishes. The experiment was repeated, and the same results were obtained.

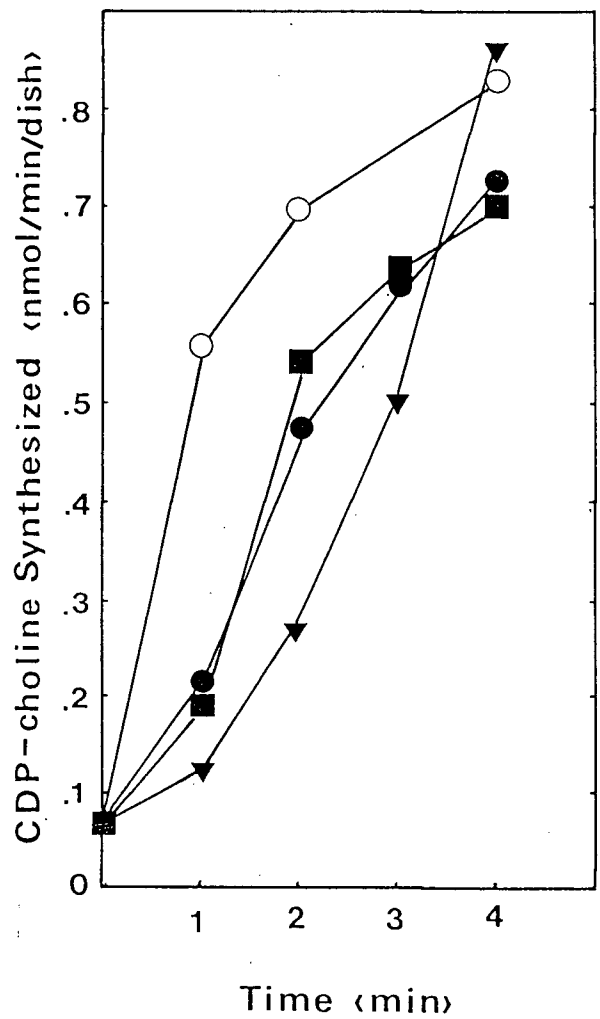

Fig.14. Effect of Different Concentrations of Vasopressin on The Digitonin-mediated Release of CT from Rat Hepatocytes. Rat hepatocytes in 60 mm dishes (2×10^6 cells) were incubated for 3 min in serum-free medium in the absence (○) or presence of 5 nM (▼), 10 nM (■) or 20 nM (●) of vasopressin. The cells were washed with 25 ml of ice-cold PBS and incubated in 1 ml of ice-cold digitonin (0.5 mg/ml) for up to 4 min. CT activity in the digitonin extract was measured as described under the legend of Fig.15. Each point represents the mean of two dishes.

with 10 nM of hormone. The release of CT returned to the control level by 4 min (Fig. 14).

Effect of Vasopressin on The Incorporation of [methyl-^{3}H]choline into Phospholipids : Pulse Labeling Studies- The reduced release of CT from the hepatocytes exposed to vasopressin indicated that part of the cytosolic CT became membrane-associated. Therefore, it was expected that PC biosynthesis in the cells incubated with vasopressin would be stimulated. However, hepatocytes labeled with [methyl-^{3}H]choline in the presence of 10 nM vasopressin did not exhibit an increase in the rate of the incorporation of choline into PC (Fig.15 A) during 60 min. The radioactive pool size of CDP-choline is too small to be examined (data not shown). The rate of total [methyl-^{3}H]choline incorporation into the cells in the presence of 10 nM vasopressin was not altered relative to the control in 60 min period (Fig.15 B).

Influence of Vasopressin on [Methyl-^{3}H]Choline Metabolism in Rat Hepatocytes : pulse-Chase Studies- Since the effect of vasopressin on CT translocation was transient (Fig. 13) and the amount of [methyl-^{3}H]choline incorporation into the cells was small in the first 5 min (Fig. 15A), the pulse labeling experiment might not be able to show the effect of vasopressin on stimulating CT activity. In this experiment, cells were prelabeled with [methyl-^{3}H]choline for 30 min in the absence of vasopressin and then chased in the medium containing 10 nM vasopressin for up to 10 min. The incorporation of labeled choline into PC was determined. The results from two seperate experiments were shown in

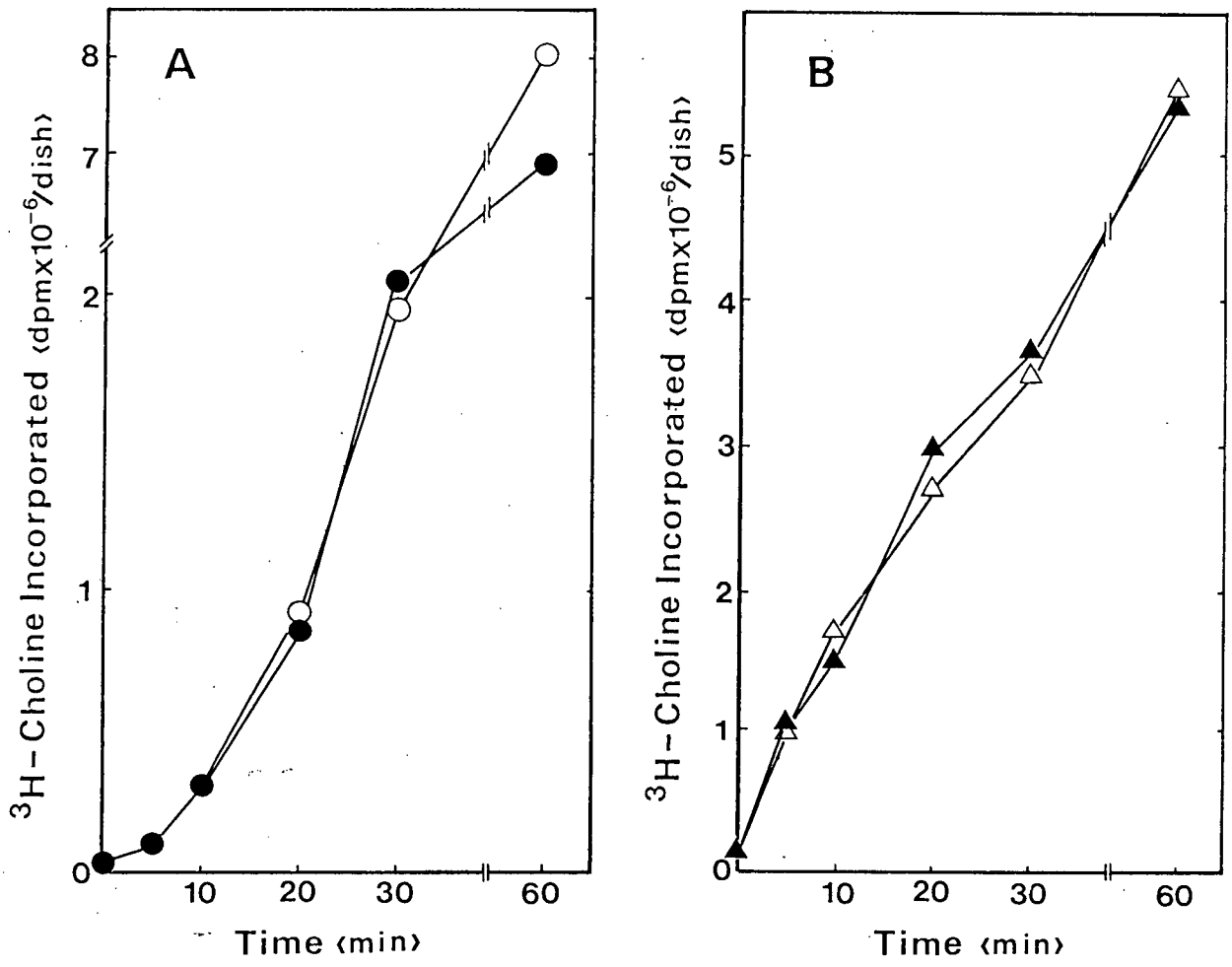

Fig.15. Effect of Vasopressin on The Incorporation of [methyl-^{3}H]Choline into Phospholipids. At zero time, rat hepatocytes in 60 mm dishes (2 x 10^6 cells) were labeled with 10 uCi of [methyl-H^3]choline (0.12 Ci/mmol) per dish in serum-free medium for up to 60 min in the absence (○, △) or presence of 10 nM vasopressin (●, ▲). Radioactivity incorporated into the cellular PC (A) and the total cellular choline metabolites (B) were determined as described under Materials and Methods. Each point represents the mean of three dishes.

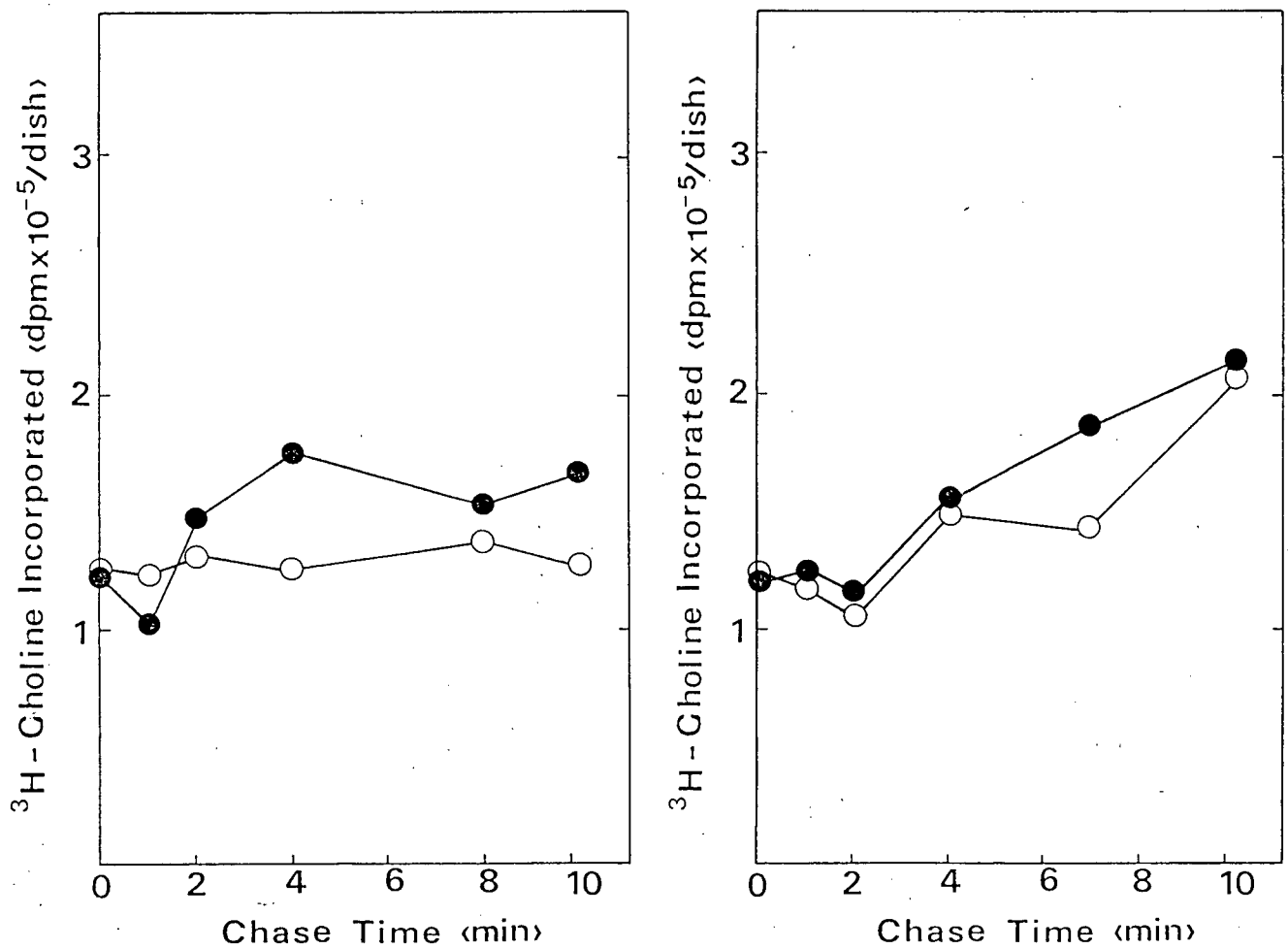

Fig. 16. Influence of Vasopressin on The Incorporation of [Methyl-^{3}H]Choline into PC in Rat Hepatocytes. Monolayer cultures of rat hepatocytes in 60 mm dishes (2 x 10^6 cells/dish) were washed with serum-free MEM twice, and then pulsed with 10 uCi [methyl-^{3}H]choline (0.12 Ci/mmol) in the same medium for 30 min. The cells were subsequently chased with 28 uM choline in the absence (○) or presence of 10 nM vasopressin (●) for up to 10 min. Radioactivity incorporated into cellular PC was determined as described under Materials and Methods. The points in panel A represent the mean of two dishes while in panel B represent the average of three dishes.

Fig. 16 A and B, respectively. The data from the first experiment showed that the cells chased in the presence of 10 nM vasopressin exhibited an increase by about 50% in labeled PC relative to the controls at 4 min chase time (Fig. 16A). However, the same result could not be obtained when the same experiment was repeated (Fig. 16B).

In order to substantiate the effect of vasopressin on PC biosynthesis, the hepatocytes were preincubated in serum-free MEM for 12 hr before starting the pulse-chase experiment to avoid any trace amount of vasopressin from serum in the culture medium which might interfere the cell response to the addition of vasopressin. The results summarized in Table 4 showed that within 4-min chase period there were no statisticlly significant alternations in radiactivity incorporation into PC between the cells treated with vasopressin and those of controls. Also, the disappearance of radiactivity from the methanol-water phase within 4-min chase period showed the same pattern in both hormone treated cells and controls (Table 4). The decreased cellular radioactivity recovery was due to the secretion of betaine, a product of choline oxidation, into culture medium by hepatocytes (see Chapter III).

Table 4. Effect of Vasopressin on The Incorporation of [Methyl-^{3}H]Choline into PC*

Addition	Chloroform Phase (dpm x 10^{-5}/dish)		Methanol-water Phase (dpm x 10^{-5}/dish)	
	0 min	4 min	0 min	4 min
Control	0.43±0.02	0.45±0.08	17.0±0.9	12.1±0.7
Vasopressin	0.49±0.04	0.43±0.07	17.5±0.7	12.1±0.8

* Rat hepatocytes were cultured as described in Materials and Methods. After monolayer of cells were formed in the culture dishes, the cells were incubated in serum-free medium for 12 hr before pulse labeling. Each dish of cells were labeled with 10 uCi of [methyl-^{3}H]choline (0.12 Ci/mmol) for 30 min in the absence of vasopressin and then chased for 4 min in the medium containing 10 nM vasopressin. The [methyl-^{3}H]choline incorporation into PC and the other choline metabolites was determined in both chloroform phase and methanol-water phase extracted by the Bligh and Dyer's method. The results are the average±S.D.; N=5.

DISCUSSION

The effects of vasopressin on the metabolism of carbohydrates and phospholipds have been studied. The hormone stimulates different membrane events including the degradation and resynthesis of PI, the so-called PI cycle (93), and Ca^{++} fluxes (94). The enhanced breakdown of PI has been suggested to be due to the hormonal stimulation of phospholipase C activity. The decrease in the concentration of PtdIns(4,5)P_2, PtdIns4P and PtdIns and the increase of DG formation in hepatocytes treated with vasopressin can be mimicked by exogenous phospholipase C (90,95). Our studies have demonstrated that vasopressin (10 nM) treatment will diminish the release of intracellular CT from rat hepatocytes, which implies that the cytosolic CT can be translocated to membrane within the cells exposed to this hormone. The reduced release of cellular CT caused by the administration of vasopressin observed in this experiment can be explained as the DG-dependent CT translocation, since it is well known that DG stimulates the aggregation of partially purified cytosolic CT from rat liver (84). Studies done in rat liver cytosol pretreated with phospholipase C exhibited a 2.5-fold increase of aggregated form of CT in the cytosol corresponding to a twofold elevation of the concentration of DG (84). Although the phospholipid precursor of DG in the reactions catalyzed by phospholipase C is generally accepted to be PI, some studies argue that the hydrolysis of other phospholipids (PC, PE) might also account for the increase

in DG (89). It would be worth further investigation to see whether or not there is a PC-cycle corresponding to the addition of vasopressin, since the majority of phospholipid is PC.

The reduced release of CT from hepatocytes treated with vasopressin appeared to depend on the time of incubation of cells with the hormone. The maximal effect on the translocation of CT was observed when the cells had been pretreated with the hormone for 3 min. This result is compatible with the observation of Hughes et al (89) that a transient increase in the total cellular concentration of DG in hepatocytes was induced by vasopressin, and the increase reached maximal levels at around 1 min and returned to basal value by 4 min. The transient increase in DG causes a transient translocation of CT to membranes, as prolonged incubation of the cells with vasopressin appeared to result in a restored fast release of CT (Fig.13). Thus, the translocation of CT within the cells treated with vasopressin seems reversible in vivo. The same reversibility of CT translocation has been observed in HeLa cells treated with oleate (Cornell, R.B., unpublished result). While the addition of oleate into the cell culture promotes association of cytosolic CT with membrane and causes the decreased release of CT, the binding of oleate from the cell culture by adding albumin will rapidly reverse this association between membrane and CT and restore the rapid release of CT from cytosol.

It has been proposed that the translocation of CT from cytosol to microsomes will stimulate the conversion of phosphocholine and CTP to CDP-choline and PPi and hence stimulate the formation of PC. This is because CT is a rate limiting enzyme in

PC synthesis via the CDP-choline pathway (88). However, in our vasopressin treatment experiment, an increased rate of PC biosynthesis was not observed in either pulse-labeling (Fig. 15A) or in pulse-chase (Fig. 16B, Table 4) experiments in spite of the decreased release of cytosolic CT. One explanation of this discrepancy might be that the increased cellular concentration of Ca^{++}, caused by the addition of vasopressin (94), inhibits the conversion of CDP-choline to PC catalyzed by cholinephosphotransferase (CPT), since Ca^{++} is a known inhibitor of this enzyme. Thus the mass effect of vasopressin on PC synthesis is not observable. A possible accumulation of CDP-choline in the vasopressin treated hepatocytes, due to the blockage of the conversion of CDP-choline to PC, might have been observed. Unfortunately, the radioactive pool size of CDP-choline in the hepatocytes is so small that I was unable to examine this possibility.

Studies on the effect of vasopressin on PC synthesis by using different precusors have been done in rat hepatocytes. Pollard and Brindley (97) found a 19% increase in the synthesis of total phospholipids (mainly PC) from [1-^{14}C] oleate in Wistar-rat hepatocytes after vasopressin treatment (100 nM, 30 min). On the other hand, Alemany et al (96) claimed the addition of 100 nM vasopressin to the same cells induced a rapid, transient (within 4 min) inhibition of the rate of incorporation of [methyl-^{3}H] choline into PC. These aspects were not investigated in the present work. In addition, I have not done the control experiment to show whether there is any effect of vasopressin on plasma membrane and hence interfere with the permeability of cellular

proteins in the digitonin-mediated release of CT experiment. The precise effect of vasopressin on PC synthesis, if any, remains unknown.

Another possible regulatory mechanism involved in the CT translocation is a reversible phosphorylation cycle (27,61, 74,85). Indirect evidence showed that dephosphorylation of the CT promotes binding of the enzyme to the E.R. where specific phospholipids activate this enzyme. This in turn leads to an increased rate of PC biosynthesis, while PC synthesis is inhibited after phosphorylation of CT by a putative cAMP-dependent protein kinase (85). Vasopressin is well recognized to stimulate hepatic carbohydrate metabolism through a Ca^{++}-requiring, cAMP-independent mechanism (98). Recent studies done by Garrison et al (99) have shown vasopressin can simulate the phosphorylation of 10 hepatic cytosolic proteins via a Ca^{++}-linked, cAMP-independent mechanism. Still, evidence showed that the PC biosynthesis via PE methylation pathway was also regulated by a phosphorylation-dephosphorylation mechanism (85) and vasopressin stimulated methyltransferase through a calmodulin-dependent protein kinase (100). So, whether the biosynthesis of PC in rat hepatocytes is controlled by a vasopressin stimulated phosphorylation or dephosphorylation mechanism will be an interesting topic for further studies.

In conclusion, the treatment of rat hepatocytes with vasopressin (5 nM) caused a diminished release of intracellular CT into the culture medium, implying translocation of the cytosolic enzyme to membrane. However, no altered rate of [methyl-^{3}H]

choline incorporation into PC was observed in the hormone treated cells.

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