PHOSPHATIDYLCHOLINE BIOSYNTHESIS AND LIPOPROTEIN SECRETION IN RAT HEPATOCYTES

by

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Date June 2, 1988
Young male rats fed a choline-deficient diet for three days accumulated triacylglycerol (TG) in the liver, and had reduced very low density lipoprotein (VLDL), but not high density lipoprotein (HDL), levels in the plasma. Cultured hepatocytes obtained from these rats were used as a model system to investigate how choline deficiency affected hepatic lipogenesis, apolipoprotein synthesis and lipoprotein secretion. When the cells were cultured in a medium free of choline and methionine, the secretion of TG and phosphatidylcholine (PC) was impaired. Supplementation of choline, methionine or lysophosphatidylcholine (lysoPC) to the culture medium increased the secretion of these lipids to normal levels, and stimulated PC biosynthesis. Fractionation of the secreted lipoproteins by ultracentrifugation revealed that the reduced secretion of TG and PC from choline-deficient cells was mainly due to impaired secretion of VLDL. The secretion of HDL and lipid-free proteins (for example albumin), however, was not affected by choline and methionine deficiency. Supplementation of betaine and homocysteine also stimulated PC biosynthesis and enhanced hepatic VLDL secretion. However, supplementation of ethanolamine, N-monomethyllethanolamine or N,N-dimethylethanolamine did not correct the impaired VLDL secretion from the hepatocytes, although an active synthesis of phosphatidylmonomethyllethanolamine (PMME) and phosphatidyl1dimethylethanolamine was observed. Choline deficiency had no effect on the rate of incorporation of $[^3]$H]leucine into cellular apolipoprotein B, E and C or on the rate of disappearance of radioactivity from the labeled apolipoproteins. These results suggest that biosynthesis of PC is specifically required for hepatic VLDL (but not HDL) secretion, and any one of the three synthetic pathways, the CDP-choline pathway, methylation of phosphatidylethanolamine (PE) or reacylation of lysoPC, is sufficient to provide the required PC.
The total activity of cytidylyltransferase in liver was unchanged in choline deficiency. However, choline deficiency caused an abnormal distribution of cytidylyltransferase activity between rat liver cytosol and microsomes (mainly endoplasmic reticulum), a decrease in the cytosolic enzyme activity and an increase in the microsomal enzyme activity. In cultured hepatocytes from the choline-deficient rat, the abnormally distributed cytidylyltransferase activity could be rapidly reversed by the addition of choline, but not lysoPC, to the culture medium. The stimulated microsomal activity of cytidylyltransferase during choline deficiency might be a mechanism whereby the cells could more effectively utilize phosphocholine to maintain a normal CDP-choline level in the choline-deficient liver.

Rat liver PE N-methyltransferase catalyzes all three transmethylation reactions in the conversion of PE to PC. The \textit{in vitro} activity of PE N-methyltransferase was increased in choline-deficient livers using endogenous PE as the methyl group acceptor. However, no significant changes were observed in the enzyme activity when exogenous PMME was used as the methyl group acceptor. Addition of methionine to the cultured hepatocytes obtained from choline-deficient rats resulted in a concomitant reduction in cellular PE levels and the specific activity of PE-dependent methyltransferase. However, the specific activity of PMME-dependent methyltransferase was not significantly altered upon the addition of methionine. No change in PE N-methyltransferase activity was observed in the cultured hepatocytes supplemented with choline. Immunoblotting of PE N-methyltransferase, in crude liver microsomes and in membrane fractions of cultured hepatocytes, revealed that the enzyme mass was not altered by choline and methionine deficiency. Thus, hepatic PE N-methyltransferase is preserved in choline deficiency, and its activity is probably dependent on the availability of metabolic substrates (i.e. methionine and PE).
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LIST OF ABBREVIATIONS

A  ampere  
AdoMet  5-adenosylmethionine  
AdoHcy  5'-adenosylhomocysteine  
ADP  adenosine 5'-diphosphate  
apo  (prefix) apoprotein  
ATP  adenosine 5'-triphosphate  
BF  bottom fraction of salt gradient (d>1.18 g/ml)  
BSA  bovine serum albumin  
cAMP  adenosine 3',5'-monophosphate  
CD  choline deficient  
CDP  cytidine 5'-diphosphate  
CE  cholesterol ester  
Ci  curie  
CoA  coenzyme A  
cpm  counts per minute  
CS  choline supplemented  
CTP  cytidine 5'-triphosphate  
d  density  
DG  diacylglycerol  
DMF  dimethyl formamide  
DMSO  dimethyl sulfoxide  
DNA  deoxyribonucleic acid  
DOC  deoxycholic acid  
dpm  disintergrations per minute  
DTT  dithiothreitol  
DZA  deazaadenosine  
EDTA  ethylenediaminetetraacetic acid  
EGTA  ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid  
ELISA  enzyme-linked immunosorbent assay  
ER  endoplasmic reticulum  
Fig.  figure  
g  gram  
g  gravity  
h  hour  
HDL  high density lipoprotein  
Hepes  4-(2-hydroxyethyl)-1-piperazineethane sulphonlic acid  
HMG-CoA  β-hydroxy-β-methyl glutaryl-CoA  
HPTLC  high performance thin-layer chromatography  
Ig  immunoglobulin  
l  liter  
Kd  kilodalton  
LCAT  lecithin cholesterol acyltransferase  
LDL  low density lipoprotein  
Lyso  (prefix) lysophospholipid  
M  molar  
min  minute  
mRNA  messenger ribonucleic acid  
n.s.  not significant  
PAGE  polyacrylamide gel electrophoresis  
p  statistical probability
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
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<td>PDME</td>
<td>phosphatidyl-$N,\text{N-}$dimethylethanolamine</td>
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INTRODUCTION

Coronary atherosclerosis, a common cardiovascular disease affecting the intima (the innermost layer) of the main and intermediary arteries, remains one of the chief causes of death in industrialized countries. Accumulated evidence has suggested that coronary atherosclerosis can result from disorders of lipid transport by lipoproteins in blood plasma, and the risk of this disease is positively related to an abnormally high ratio of plasma VLDL/LDL versus HDL, which are mainly synthesized in the liver [1]. A major component of plasma lipoproteins is phospholipids which form a monolayer surface surrounding lipoproteins. The role of biosynthesis of phospholipid, especially phosphatidylcholine (PC), in hepatic lipoprotein assembly and secretion has rarely been investigated. The present thesis is directed toward understanding the requirement of PC biosynthesis for hepatic lipoprotein secretion.

1. Liver as a Site for the Synthesis of Plasma Lipoproteins

The liver performs many important functions in plasma lipoprotein metabolism, including the biosynthesis of VLDL and HDL. In rats, approximately 80% of the protein components of lipoproteins (called apolipoproteins or apoproteins) in the plasma arise from the liver, and the remainder from intestine [2]. Hepatic biosynthesis and secretion of lipoproteins has been more directly demonstrated in \textit{ex vivo} studies using liver slices [3], perfused liver [4], and cultured hepatocytes [5] as experimental models. Subcellular fractionation studies [6] have demonstrated that the nascent lipoprotein particles isolated from the Golgi apparatus of rat liver possess similar properties to plasma VLDL. Furthermore, studies conducted \textit{in vivo} demonstrated a remarkable increase in hepatic neutral lipid content accompanied by severe hypolipoproteinemia (low levels of lipoproteins in blood) following, for example, feeding of orotic acid [7] or choline-deficient diet [8,9]
Cultured monolayer hepatocytes have been used extensively to study lipid metabolism and the secretion of plasma lipoproteins [10,11]. Advantages of cultured hepatocytes for studying regulation of plasma lipoprotein synthesis are several. First, the homogeneous population of cells obtained from a single animal minimizes or eliminates modifying effects introduced by dietary factors. Second, the requirement for a specific lipid or apoprotein synthetic pathway in the synthesis and secretion of lipoproteins can be studied in a chemically defined environment for a relatively long period. Third, cultured hepatocytes do not catabolize secreted VLDL at an appreciable rate, thus, the accumulated VLDL in the medium is a product solely of secretion [5].

2. Choline Phospholipid as a Component of Plasma Lipoproteins

2.1. General Structure of Plasma Lipoproteins

Plasma lipoprotein classes are operationally defined either according to centrifugal density as the high (HDL), low (LDL), intermediate (IDL), very low-density lipoproteins (VLDL) and the chylomicrons [12], or according to electrophoretic migration as the alpha-(α), beta-(β), slow pre-beta and prebeta-(pre-β) migrating lipoproteins [13]. Human HDL is usually subdivided into at least two subclasses, HDL₂ and HDL₃, because rate zonal ultracentrifugation produces a bimodal distribution of HDL [14]. In the plasma, lipoprotein particles are in a state of dynamic equilibrium with respect to each other, as well as with various tissue and membrane compartments within the body. All lipoproteins are large macromolecular structures containing a neutral lipid core of triacylglycerols (TG) and cholesterol esters (CE), with a monolayer of phospholipids and cholesterol in which various apolipoproteins are embedded [15]. Mature plasma lipoproteins are normally spherical (5-1200 nm in diameter), except newly secreted HDL from the liver, which appears as bilayer disks [16] that resemble the abnormal HDL found in humans with genetically-determined lecithin: cholesterol
acyltransferase (LCAT) deficiency [17]. A classification of the major human plasma lipoproteins and some of their physical properties is given in Table I.

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Electrophoretic definition</th>
<th>Particle size (nm)</th>
<th>Molecular weight</th>
<th>Density (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>Remains at origin(^b)</td>
<td>75-1200</td>
<td>~400,000,000</td>
<td>0.93</td>
</tr>
<tr>
<td>VLDL</td>
<td>Pre-β lipoproteins</td>
<td>30-80</td>
<td>10-80,000,000</td>
<td>0.93 - 1.006</td>
</tr>
<tr>
<td>IDL</td>
<td>Slow-pre-β lipoproteins(^c)</td>
<td>25-35</td>
<td>5-10,000,000</td>
<td>1.006- 1.019</td>
</tr>
<tr>
<td>LDL</td>
<td>β lipoproteins</td>
<td>18-35</td>
<td>2,300,000-10,000,000</td>
<td>1.019- 1.063</td>
</tr>
<tr>
<td>HDL(_2)</td>
<td>α lipoproteins</td>
<td>9-12</td>
<td>360,000</td>
<td>1.063- 1.125</td>
</tr>
<tr>
<td>HDL(_3)</td>
<td>α lipoproteins</td>
<td>5-9</td>
<td>175,000</td>
<td>1.125- 1.210</td>
</tr>
</tbody>
</table>

\(^a\) From Smith et al. [18].
\(^b\) On paper electrophoresis.
\(^c\) On agarose electrophoresis.

2.2. Choline Phospholipids in Plasma Lipoproteins

Phospholipids are major components of plasma lipoproteins. Phospholipids comprise about 20% of the total weight of human plasma VLDL, LDL and HDL, and 13%, 21% and 26% of the total weight of rat plasma VLDL, LDL and HDL, respectively (Table II). The smaller lipoprotein particles usually have higher contents of more dense surface components (such as phospholipids and proteins), and the larger particles have higher contents of core components (i.e. TG and CE). The protein content in plasma lipoproteins of human and rat are similar. However, human VLDL and LDL contain relatively higher CE than in the rat, while rat VLDL and LDL contain higher TG than that in human.
Table II. Chemical Composition of Human and Rat Plasma Lipoproteins*

<table>
<thead>
<tr>
<th>Components</th>
<th>VLDL (d&lt;1.006)</th>
<th>LDL (1.007&lt;d&lt;1.063)</th>
<th>HDL (1.063&lt;d&lt;1.21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Man&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Rat&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Man&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protein</td>
<td>7.7</td>
<td>9.4</td>
<td>20.9</td>
</tr>
<tr>
<td>Cholesterol ester</td>
<td>14.9</td>
<td>1.9</td>
<td>38.0</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>49.9</td>
<td>73.6</td>
<td>11.2</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>6.7</td>
<td>2.7</td>
<td>9.0</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>18.6</td>
<td>12.6</td>
<td>22.1</td>
</tr>
</tbody>
</table>

* Values are mean % by weight.

<sup>a</sup> From Mills and Taylaur [19].

<sup>b</sup> From Mjos <i>et al.</i> [20].

<sup>c</sup> From Pasquali-Ronchetti <i>et al.</i> [21].

Of the phospholipid subclasses, choline-containing phospholipids account for about 90% of the total in all plasma lipoproteins (Table III). The most striking feature is the great predominance of PC, which ranges from 50 to 75% of the total phospholipids. Sphingomyelin (SPM) contributes about 24% of phospholipids in human VLDL and LDL, but less in HDL. The SPM levels in young rat HDL are markedly lower than those in their human counterparts, while old rats have higher SPM content in VLDL and HDL (11%) [23]. The LDL and HDL in young rat (1.5 months) have distinguishably elevated contents of lysoPC, which constitutes the second prevalent phospholipid (>20%). In old rats (24 months), the lysoPC content in HDL is decreased to 3.2% of the total phospholipids [23]. The relevance of these variations in choline-containing phospholipid contents to the structure and physiology of their associated lipoprotein particles is presently unknown.
Table III. Phospholipid Distribution in Human and Rat Plasma Lipoproteins*

<table>
<thead>
<tr>
<th>Species</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Man(^a)</td>
<td>Rat(^b)</td>
<td>Man(^a)</td>
</tr>
<tr>
<td>PC</td>
<td>66.2</td>
<td>75.5</td>
<td>66.3</td>
</tr>
<tr>
<td>SPM</td>
<td>23.1</td>
<td>4.2</td>
<td>25.3</td>
</tr>
<tr>
<td>LysoPC</td>
<td>5.1</td>
<td>7.1</td>
<td>4.1</td>
</tr>
<tr>
<td>PE</td>
<td>({)</td>
<td>2.5</td>
<td>(})</td>
</tr>
<tr>
<td>PS</td>
<td>({)</td>
<td>5.7</td>
<td>5.5</td>
</tr>
<tr>
<td>PI</td>
<td>({)</td>
<td>4.0</td>
<td>(})</td>
</tr>
</tbody>
</table>

* Values are % by weight.
\(^a\) From Skipski [22].
\(^b\) From Malhotra and Kritchevsky [23]; data from young (1.5 months) Wistar rats.

A strong similarity usually exists between the fatty acid composition of PC in the different lipoprotein classes of human [24] and rat [9]. However, significant differences between the fatty acid compositions of PC, as well as SPM, present in LDL and HDL from freshly isolated human umbilical cord blood has recently been demonstrated [26]. The equilibration of PC species among the lipoproteins during circulation may simply result from spontaneous exchange or may be due to several lipid exchange proteins identified in plasma [27-29]. Massey et al. [30] have determined the rate of spontaneous phospholipid exchange and shown it to be too slow (compared to the plasma half-life of lipoproteins) to be of physiological significance.

Cellular phospholipids appeared to equilibrate quickly among the various subcellular membranes in hepatocytes [31]. The fatty acid compositions of PC in rat plasma, however, is different from those in the liver [32]. Vance's group [33] has demonstrated significant differences between the fatty acid profiles of PC, as well as PE, present in cultured rat hepatocytes and the secreted lipoproteins. Thus, despite the rapid equilibration of phospholipid within the cells, there may
be selection of particular PC pools synthesized for hepatic lipoprotein secretion [32,33]. The possible significance of the selective secretion of lipoprotein PC will be discussed later (see Selection of Phosphatidylcholine Pools in Lipoprotein Secretion).

2.3. Metabolic Functions of Choline Phospholipids in Plasma Lipoproteins

Aside from its role as a structural constituent of lipoproteins, PC also serves as donors of the unsaturated fatty acyl moiety from its sn-2 position for the synthesis of cholesterol ester by LCAT; a reaction thought to regulate cholesterol efflux from nonhepatic cells into the circulation [34,35]. Involvement of PC in the synthesis and secretion of hepatic and intestinal apoB-containing lipoproteins (VLDL and chylomicron) has been implicated in choline deficiency studies [36]. In rats deficient in choline or PC, there is a striking decrease in lipid levels in the plasma [9] and a tremendous accumulation of lipids (mainly TG) in the liver [8] as well as in the intestinal mucosa [37]. Direct studies on the role of active PC synthesis in the process of hepatic lipoprotein assembly and secretion is the main theme of this thesis.

The physiological and biochemical functions of sphingomyelin and lysoPC in plasma lipoproteins are less understood. An age-dependent variation of these phospholipids in plasma lipoproteins has been observed [23]. LysoPC in plasma is also bound to albumin, the most abundant circulating protein responsible for transporting long-chain fatty acids [38]. The plasma lysoPC originates either from the reaction of LCAT or from direct hepatic secretion [39]. Acquisition of the choline moiety from albumin-bound lysoPC (but not from serum-bound PC or sphingomyelin) for membrane PC synthesis has been reported in cultured Chinese hamster ovary cells (CHO cells) deprived of choline [40].
3. Hepatic Biosynthesis of Phosphatidylcholine and Other Phospholipids

3.1. Synthesis of Phosphatidylcholine

In mammals, liver is the major site for the biosynthesis of PC [41]. Hepatic synthesis of PC can be achieved via more than one pathway. Three important ones are discussed below.

3.1.1. Synthesis of Phosphatidylcholine from Choline

In the liver, approximately 60-80% of the PC synthesized is from choline [42] via the CDP-choline pathway, which is first described by Kennedy and his co-workers [43]. The dietary requirement of choline for animals [109] and choline as a vitamin for animal cell growth in culture [44] has been well established. Choline can not be synthesized directly in most of the animal cells which depend largely upon the CDP-choline pathway for de novo PC synthesis. Only liver cells can synthesize choline endogenously via the sequential methylation of PE (see Synthesis of PC from PE Methylation). The plasma choline concentration is higher in newborn rats than in adults (65 μM versus 10 μM) [45], and the same age-dependent changes are also found in other animals [46]. High choline concentrations may promote PC synthesis in the newborn for growth. There is an elevation in the activities of the enzymes involved in hepatic PC synthesis [47], and a rapid increase in the liver PC content after birth [48]. An active biosynthesis and increased secretion of PC is also observed in the lung of fetuses immediately prior to term and in the newborn [49,50]. This is crucial for the production of lung surfactant which prevents alveolar collapse during expiration.

The initial reaction in the CDP-choline pathway is Mg\(^{2+}\)-ATP-dependent phosphorylation of choline catalyzed by choline kinase (EC 2.7.1.32), which was discovered by Wittenberg and Kornberg [51]. Purification of choline kinase has been achieved in rat kidney and the purified kidney enzyme appears to be a
dimer of two subunits having a molecular weight of 42,000 daltons [52]. The purified kidney choline kinase also possesses ethanolamine kinase activity [53]. Choline and ethanolamine kinase activities are also co-purified from liver, lung and intestinal cytosols [75]. Polyclonal antibody against the purified kidney enzyme inhibits both choline and ethanolamine kinase activities, in a parallel manner, in crude enzyme preparations from kidney, lung, liver and intestinal cytosols [53]. These results strongly suggest that both kinase activities are represented on the same enzyme protein. The phosphorylation of choline appears not to be a rate-limiting step in the CDP-choline pathway in rat liver and some other cells [54].

Conversion of phosphocholine and CTP to CDP-choline and pyrophosphate is catalyzed by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15), an enzyme the activity of which is found in both cytosol and microsomal (mainly ER) membranes [55]. The in vitro activity of cytidylyltransferase requires phospholipid, suggesting that in cells the membrane-associated enzyme is the active species, while the cytosolic form is inactive. The subcellular localization of cytidylyltransferase between cytosol and microsomes is thought to be the key mechanism for enzyme regulation [59], and many factors affecting the subcellular distribution of cytidylyltransferase have been demonstrated (discussed in Regulation of PC Synthesis from Choline).

Cytidylyltransferase was first purified by Choy et al. [56] from rat liver cytosol 960-fold to a specific activity of 106 nmol/min/mg protein. This enzyme had an apparent molecular weight of 220,000 as analyzed by gel filtration, and gave a single protein and activity band on nondenaturing polyacrylamide electrophoresis [56]. However, the purified cytidylyltransferase was extremely unstable and would aggregate to form polymers at 4 °C [56]. These behaviors hampered further characterization studies of cytidylyltransferase. Recently,
Weinhold et al. [57] have achieved a 2,180-fold purification of cytidylyltransferase from rat liver cytosol to a specific activity of 12,500 nmol/min/mg protein. The purified enzyme appears to be stable at -70 °C in the presence of Triton X-100 and 0.2 M phosphate and has an apparent native molecular weight of 240,000 [57]. This is compatible with the previous molecular weight determination reported by Choy et al. [56]. Weinhold et al. [57] proposed a tetramer structure for the hepatic cytidylyltransferase, based on the molecular mass, of two 38,000-dalton and two 45,000-dalton subunits. They [58] have further demonstrated that the large subunit contains the catalytic activity and the 38,000 dalton subunit is a contaminant. The purified cytosolic enzyme requires phospholipids for maximal activity [57,58]; this is consistent with the view that the microsomal enzyme is the active species in the cell and the cytosolic enzyme acts as a reservoir [59,60].

The formation of PC synthesis is finally achieved by the condensation of CDP-choline and 1,2-diacylglycerol, a reaction catalyzed by CDP-choline:1,2-diacylglycerol phosphocholinetransferase (EC 2.7.8.2) [61]. The enzyme utilizes 1,2-diacylglycerols (DG), which originate either from triacylglycerol or from phosphatidic acid [62]. The D-1,2-DG appears more active than the L-enantiomorph [63]. Thus, the flux of DG to PC may regulate the balance between hepatic phospholipid synthesis and neutral lipid synthesis. Phosphocholine-transferase activity is located exclusively on the cytosolic side of the microsomal fraction of the tissue [64] and has not yet been purified.

3.1.2. Synthesis of Phosphatidylcholine by Phosphatidylethanolamine Methylation

Hepatic PC synthesis is also achieved by sequential methylation of PE as described by Bremer and Greenberg [65]. This pathway involves three stepwise transfers of methyl groups from S-adenosyl-L-methionine (AdoMet) to PE, catalyzed by PE N-methyltransferase (EC 2.1.1.17). Formation of PC through PE methylation appears only quantitatively important in the liver; it accounts for
20-40% of hepatic PC synthesis [42]. The methyltransferase has been successfully purified from rat liver microsomes by Ridgway and Vance [66], and is composed of a single subunit with a molecular weight of 18,000. The purified liver PE N-methyltransferase catalyzes all three transmethylation steps for the conversion of PE to PC [66], and does not display specificity for molecular species of PE, PMME or PDME [67]. In liver cells, it has been suggested that the rate of PE methylation is influenced by the concentration of the substrates, AdoMet and PE [68]. In addition, the activity of PE methyltransferase is strongly affected by the ratio of cellular AdoMet to S-adenosyl-L-homocysteine (AdoHcy) [69].

### 3.1.3. Formation of Phosphatidylcholine from Lysophosphatidylcholine

The formation of PC from lysoPC can be achieved by reacylation of lysoPC with acyl-CoA as described by Lands [70]. Two enzymes are responsible. Acyl-CoA:1-acylglycerol-3-phosphocholine O-acyltransferase (EC. 2.3.1.23) and acyl-CoA:2-acylglycerol-3-phosphocholine O-acyltransferase (EC. 2.3.1.62) transfer a fatty acid to the 2-position of 1-acylglycerol-3-phosphocholine and 1-position of 2-acylglycerol-3-phosphocholine, respectively. These reactions, together with the deacylation of PC to generate lysoPC catalyzed by phospholipase A₂ (EC. 3.1.1.4) and phospholipase A₁ (EC. 3.1.1.32), are of particular importance for the remodeling of the fatty acyl moieties of PC [61]. Since high concentrations of lysoPC causes perturbations of membrane structure [71], the concentration of lysoPC in rat liver microsomes is maintained at about 2% [72]. Although reacylation of lysoPC does not lead to synthesis of PC de novo, it may be an important pathway for PC synthesis in the intestine where lysoPC is readily absorbed from bile [73] (see Intestinal Lipoprotein Metabolism in Choline Deficiency).

Alternatively, formation of PC from two molecules of lysoPC [74] has been described. The importance of this reaction for PC formation in the liver has not
been demonstrated. Fig.1 summarizes the three major synthetic pathways for PC in the liver.

![Diagram of phosphatidylcholine synthesis in the liver]

Fig.1. Major pathways of phosphatidylcholine synthesis in the liver.
3.2. Synthesis of Phosphatidylethanolamine

Hepatic PE is the product of at least three synthetic pathways (Fig. 2): the CDP-ethanolamine pathway (which is similar to the CDP-choline pathway), the decarboxylation of PS and base-exchange between phospholipid and ethanolamine [60].

3.2.1. Formation of Phosphatidylethanolamine from Ethanolamine

The de novo synthesis of PE via the CDP-ethanolamine pathway was first described by Kennedy and Weiss [43]. Three enzymes are involved in the CDP-ethanolamine pathway: ethanolamine kinase (EC. 2.7.1.82), CTP:phosphoethanolamine cytidylyltransferase (EC. 2.7.7.14) and CDP-ethanolamine:1,2-diacylglycerol phosphoethanolaminetransferase (EC. 2.7.8.1). In rat kidney, lung, liver and intestine, the phosphorylation of ethanolamine is catalyzed by the kinase which phosphorylates choline (choline kinase) [53].

The cytidylyltransferase for the synthesis of CDP-ethanolamine has been purified 1,000-fold from rat liver cytosol, and may be a dimer of two subunits with a molecular weight of 49-50,000 [76]. The enzyme is distinct from its counterpart for CDP-choline synthesis by its exclusive cytosolic location, and its phospholipid-independent activity [76]. Phosphoethanolaminetransferase, like its counterpart for PC synthesis, is also an intrinsic protein in the microsomal membranes, and has not been purified. Characterization of phosphoethanolaminetransferase shows the enzyme is distinct from cholinephosphotransferase [77]. The activity of phosphoethanolaminetransferase, unlike cholinephosphotransferase which is strongly inhibited by manganese and trypsin treatment, is activated by manganese and less sensitive to trypsin treatment [78].

Although the CDP-ethanolamine pathway has been described in rat hepatocytes, in ordinary culture conditions the cells do not require ethanolamine in the medium [83]. The relative importance of this pathway to hepatic de novo
synthesis of PE has not been assessed. Sundler and Åkesson [42] have demonstrated that the formation of CDP-ethanolamine is the rate limiting step for PE synthesis, and the incorporation of \[^{3}H\]glycerol into PE in rat hepatocytes was stimulated by ethanolamine. However, in human Y79 retinoblastoma cells, ethanolamine has no effect on cellular PE levels nor on the activity of PE \(N\)-methyltransferase [79].

### 3.2.2. Formation of Phosphatidylethanolamine from Phosphatidylserine Decarboxylation

The formation of PE from PS is catalyzed by PS decarboxylase or PS carboxy-lyase (EC 4.1.1.65), a mitochondrial enzyme in the liver [81]. Since PS is synthesized predominantly in the microsomes by base-exchange reaction between PE and serine [82], the segregation of these two reactions implicates an intracellular transport of PS from microsomes to mitochondria for the PE synthesis. The significant contribution of PS decarboxylation for PE synthesis has been demonstrated in cultured baby hamster kidney cells [83]. However, the contribution of this pathway in the cultured rat hepatocytes has not been studied. In cultured rat mammary carcinoma cell lines, ethanolamine-responsive and ethanolamine-nonresponsive cells have been described [80]. Although the mechanism(s) involved are unknown, the different requirement of ethanolamine for cell growth may reflect a defect in production of PE from PS in those ethanolamine-responsive cells.

The major pathways for PE synthesis in the liver is summarized in Figure 2.
Fig. 2. Biosynthetic pathways of phosphatidylethanolamine.

3.3. Formation of Phosphatidyl-\( N \)-monomethylethanolamine and Phosphatidyl-\( N,N \)-dimethylethanolamine

Three stepwise methylations of hepatic PE catalyzed by PE \( N \)-methyltransferase lead to the sequential formation of two partially methylated intermediates, PMME and PDME, and PC. These metabolic intermediates, PMME and PDME, are normally present in very small amounts [42]. However, in the absence of choline and methionine, synthesis of phospholipids containing \( N \)-monomethyl- or \( N,N \)-dimethylethanolamine from the corresponding free bases has been observed in cultured rat hepatocytes [42]. Formation of these mono- or di-methylated
phospholipids from the polar bases has also been observed in brain [84], cultured mouse fibroblast cells (LM cells) [85,86] and CHO cells [86] where methylation of PE does not occur. The synthetic pathway for these partially methylated phospholipids is presumably the cytidine diphosphate pathway used for PC and PE synthesis. The in vitro formation of the CDP-esters of N-mono- and N,N-dimethylethanolamine from their phosphate esters and the synthesis of corresponding phospholipids from these CDP-esters has been demonstrated [84]. CDP-N-monomethylethanolamine and CDP-N,N-dimethylethanolamine are formed by different enzymes, probably phosphoethanolamine cytidylyltransferase and phosphocholine cytidylyltransferase, respectively [87]. The accumulation of these partially methylated phospholipids in LM cells has been demonstrated to have no effect on the activities of at least six membrane-bound enzymes: (Na\(^+\),K\(^+\))-ATPase, 5'-nucleotidase, NADH:cytochrome c reductase, glucose-6-phosphatase, inosine diphosphatase, and succinate:cytochrome c reductase [85]. In addition, the characteristic temperatures (transition temperatures) of the membranes, determined by measuring the quantum yield of different fluorescent membrane probes at various temperatures, is not changed in LM cells having an altered phospholipid composition [88]. This suggests that the general physical properties of plasma membranes and intracellular membranes are maintained despite large alterations in their phospholipid head groups.

### 3.4. Betaine Formation and Methyl Groups

The oxidation of choline to betaine is catalyzed by two enzymes. First, choline is oxidized to betaine aldehyde by choline dehydrogenase (EC. 1.1.99.1) which is found in mitochondria in a membrane-bound form. Betaine aldehyde is then oxidized to betaine by betaine aldehyde dehydrogenase (EC 1.2.1.8) which appears to be present both in mitochondria and the soluble fraction of liver [89]. The oxidation of choline in the liver has been suggested as a major mechanism for
diminishing free choline concentration [45]. In cultured hepatocytes, the majority of cellular tritium-labeled choline is rapidly oxidized to betaine and released into the culture medium [90]. In livers from choline-deficient rats, however, the enzyme activities involved in the formation of betaine were reduced by 40% [91].

On the other hand, betaine is an important methyl donor for the biosynthesis of methionine [92]. Betaine donates its methyl group directly to homocysteine to form methionine and dimethylglycine in a reaction catalyzed by betaine:homocysteine 5-methyltransferase (EC 2.1.1.5) [93]. Methionine is also formed from homocysteine and N5-methyltetrahydrofolate by vitamin B12-dependent methionine synthase or N5-methyltetrahydrofolate:homocysteine methyltransferase (EC 2.1.1.13) [94]. The one-carbon unit in N5-methyltetrahydrofolate can be generated from dimethylglycine and sarcosine, two demethylated products of betaine [95]. In the liver, the conservation of methionine is achieved mainly by the reactions catalyzed by these two methyltransferase. Fig.3 outlines the metabolic interrelationship among choline and methyl groups.

Fig.3. Metabolic interrelationship among choline, methyl groups and phospholipids.
3.5. Regulation of Hepatic Phosphatidylcholine Biosynthesis

3.5.1. Regulation of Phosphatidylcholine Synthesis from Choline

The synthesis of hepatic PC from choline is regulated by the activity of cytidylyltransferase, which catalyzes the formation of CDP-choline [41]. In many model systems examined, cytidylyltransferase activity is regulated by its subcellular localization between cytosol and microsomes (mainly ER) [59]. The enzyme activity translocates reversibly between cytosol, where it is inactive, and microsomes, where it is activated by phospholipids on the membrane. Several regulatory mechanisms have been proposed to explain how cells control the subcellular translocation of cytidylyltransferase activity.

Pelech et al. [96,97] have proposed that the subcellular localization of cytidylyltransferase is regulated by reversible phosphorylation/dephosphorylation. Treatment of cultured hepatocytes with cAMP analogues resulted in a decrease (about 30%) in the membrane-associated cytidylyltransferase activity and an increase (15%) in the cytosolic enzyme activity [96]. This phosphorylation-mediated enzyme translocation from membrane to cytosol was accompanied by a 35% decrease in hepatic PC synthesis from choline [96]. Pelech et al. [96,97] postulate that the cytosolic cytidylyltransferase is a phosphorylated and inactive form, whereas the microsomal enzyme is a dephosphorylated and active form. In very recent studies, the purified cytidylyltransferase from rat liver cytosol has been proved to be a substrate of the catalytic subunit of cAMP-dependent protein kinase, and a phosphorylation-mediated enzyme translocation has been demonstrated in vitro [98].

The other established mechanism for translocation of cytidylyltransferase is based on the observations in cultured rat hepatocytes [90] and HeLa cells [99] that free fatty acid promotes association of the enzyme to membranes and stimulates PC biosynthesis. The effect of fatty acid on cytidylyltransferase
translocation can be effectively reversed by addition of albumin into the culture medium [99]. The fatty acid-mediated translocation of cytidylyltransferase appears to be independent of the phosphorylation and dephosphorylation mechanism. In the cultured hepatocytes treated with cAMP analogues, the addition of 1 mM palmitate could effectively abolish the inhibitory effect of cAMP on PC biosynthesis, and convert cytidylyltransferase into the membrane-bound form [100]. Fig.4 presents the proposed models for regulation of cytidylyltransferase by phosphorylation and fatty acids.

![Diagram](image)

**Fig.4.** Proposed models for the regulation of subcellular localization and activity of cytidylyltransferase by phosphorylation and fatty acids in the liver.
3.5.2. Choline Deficiency and Cytidylyltransferase Activity

Since choline is a major building block for PC, the response of cytidylyltransferase to choline deficiency has been studied from the viewpoint of metabolic regulation of hepatic PC synthesis. The cytosolic activity of cytidylyltransferase in the liver after 2 days of choline deficiency was reduced by 40% [91], but the liver microsomal activity of cytidylyltransferase in choline deficiency was not determined. An increase in the microsomal activity of cytidylyltransferase, and a concomitant decrease in the cytosolic activity of the enzyme, has been observed in type II pneumonocytes (epithelial cells in lung which are responsible for surfactant synthesis and secretion) [101] and in CHO cells [102] during choline deficiency. The changes in cytidylyltransferase activity between cytosol and microsomes caused by choline deficiency can be reversed by the addition of choline to the culture medium [101,102]. Sleight and Kent [86] have examined the subcellular distribution of cytidylyltransferase in CHO and LM cells cultured in a medium with or without choline, N-monomethylethanolamine or N,N-dimethylethanolamine for 3 days. The specific activity of membrane-bound cytidylyltransferase in CHO cells was increased 2.3- and 3.6-fold in the presence of N,N-dimethylethanolamine and N-monomethyl-ethanolamine, respectively, compared to that in the presence of choline. The specific activity of cytosolic enzyme, however, was unchanged in the presence of these partially methylated head groups [86]. In choline-deficient LM cells, almost all cellular cytidylyltransferase activity (95% of the total) was associated with membranes, whereas the addition of N-monomethylethanolamine, N,N-dimethylethanolamine or choline reduced the membrane-bound enzyme activity to 80%, 56% or 40% of the total, respectively [86]. These findings suggest that the microsomal activity of cytidylyltransferase may be regulated by the concentration of choline and/or PC.
Involvement of diacylglycerol (DG) in the process of translocation of cytidylyltransferase must be considered (Fig.4), since choline deficiency caused accumulation of DG in livers [135]. In a series of experiments using phospholipase C to deplete membrane PC content, Sleight and Kent [103-105] have demonstrated that the decrease in membrane PC and increase in cellular DG levels created by phospholipase C treatment appeared to promote the translocation of cytidylyltransferase to the membranes. Cornell and Vance [99] have shown in vitro that the cytosolic cytidiyltransferase of HeLa cells binds to diolein/PC vesicles, and the binding results in enzyme activation. In cultured rat hepatocytes, supplementation of 1 mM oleate also results in a 2-fold increase in cellular DG levels [90]. Therefore, the changes in cytidiyltransferase activity, and its subcellular distribution observed in phospholipase C- or fatty acid-treated cells, may result from elevated cellular DG (probably more important is the membrane-associated DG) concentrations. However, in oleate-treated HeLa cells, while greater than 95% of cytidiyltransferase becomes associated with microsomes, the cellular DG concentration is not increased [59]. Thus, a clear answer to whether or not DG affects cytidiyltransferase activity and its subcellular location remains to be found.

3.5.3. Regulation Between the CDP-Choline Pathway and Phosphatidylethanolamine Methylation Pathway

Several lines of evidence suggest that hepatic synthesis of PC by the CDP-choline pathway and PE methylation is coordinately regulated. First, inhibition of PE methylation by 3-deazaadenosine (DZA) stimulated the synthesis of PC via the CDP-choline pathway [69]. Second, while fatty acids stimulated PC synthesis in hepatocytes via the CDP-choline pathway, the PE methylation pathway was inhibited [106]. Third, the in vitro activity of PE \(N\)-methyltransferase in choline deficiency is increased by two-fold using endogenous PE as a substrate [91].
Synthesis of PC via PE methylation during choline deficiency will be discussed later (see Hepatic Phosphatidylcholine Synthesis in Choline Deficiency).

4. Role of Choline Phospholipid Synthesis in Lipoprotein Secretion

4.1. Lipotropic Effect of Choline (Historical Retrospect)

The term lipotropic, first used by Best, Huntsman and Ridout [107], is derived from the Greek lipos meaning "fat" and the trope meaning "turning" (turning of fat). Lipotropic compounds were originally defined as those substances which "decreased the rate of deposition or accelerated the rate of removal of liver fat" [108].

The discovery of dietary choline as a lipotropic factor is attributed to Best and Hunstman in 1932 [109], which was a direct result of their studies on diabetes in the depancreatized dog. In 1924, Allan et al. [110] described how depancreatized dogs maintained with insulin could survive for long periods of time but developed fatty liver. Subsequently, Best and Hershey [111] showed that raw beef pancreas supplements in the diet of depancreatized dogs protected against fatty liver, and the active principle was lecithin. In a continuation of these studies, a test diet high in fat and low in protein was developed and the rat was used as the experimental animal. Best and Huntsman [109] found the animals rapidly developed enlarged fatty livers, and this was successfully prevented by adding lecithin, and later choline, to the diet. In 1937, Tucker and Eckstein [112] discovered that methionine could replace choline in preventing fatty livers in rats. Interestingly, later studies revealed that although the dietary supplementation of pancreas to the insulin-treated, depancreatized dog diminish liver fat by supplying cholinephospholipids, the pancreas functions in vivo by supplying proteolytic enzymes essential for the liberation of methionine from dietary protein [113]. After the development of radioisotopes, the transfer of the
methyl group (transmethylation) from methionine to choline was demonstrated [114], and later Cantoni [115] discovered that the methyl donor was AdoMet. Thus, in producing a choline deficiency in animals, a diet low in methionine should also be considered. Strictly speaking, the term "choline deficiency" should be altered to "choline and methionine deficiency".

The findings of the lipotropic effect of choline, and the biochemical relationship between choline and methionine, opened the way for many investigations on the role of specific dietary nutrients and their interrelationships in the control of fat content of the liver; these nutrients include not only choline and methionine, but also betaine [116], homocysteine [117], folic acid and vitamin B₁₂ [118,119].

4.2. Effect of Choline Deficiency on the Liver

4.2.1. Triacylglycerol Metabolism in Choline Deficiency

Triacylglycerols (TG) in animals are mostly stored in adipose tissue, and can also be deposited in liver and other organs [120]. The normal liver contains about 5% by weight as fat including TG, fatty acids, phospholipids and cholesterol (free and esterified). When fat accumulates in the liver, it is almost all TG; 40-50% of the liver weight may be due to fat [121]. Hepatic TG is mainly synthesized from acyl-CoA and dihydroxyacetone phosphate or glycerol 3-phosphate, and is directly affected by the availability of free fatty acids. The fatty acids used for TG synthesis in the liver are derived from endogenous synthesis, from dietary fatty acids and from adipose tissue. The rate of mitochondrial fatty acid oxidation and ketone body formation also affects the available concentration of fatty acids, and therefore TG synthesis [122,123]. In a fasting state, hepatocytes as well as the small intestine synthesize and secrete TG-rich lipoproteins. The liver accounts for 80% of the total TG-rich lipoproteins secreted [124]. Therefore, hepatic TG levels are influenced by the availability of precursors, by the synthetic rate and
by the efficiency of export [125]. Derangement in one or more of the metabolic regulatory steps will lead to an excessive accumulation of TG within hepatocytes, which is clinically termed "fatty liver", "fatty degeneration" or "fatty metamorphosis" [121].

Two metabolically distinct pools of TG have been identified in rat liver [126-128]. One is a storage pool containing the bulk of hepatic TG, associated with the cytoplasm of hepatocytes. The TG accumulated in liver during choline deficiency is mainly associated with this storage pool [130]. The second is an active pool (the precursor pool of plasma TG), associated with ER where its synthesis takes place [126,127,129]. Lombardi et al. [8] reported that there was no difference in TG content of microsomal membranes from CD rat livers compared to those of normal [8]. Failure to assemble the TG synthesized in microsomes into TG-rich lipoprotein particles in the lumen of ER probably leads to the TG shunt into the storage area of cytoplasm [131].

It is well established that as early as 24 hours after feeding rats a diet deficient in choline, there is a characteristic increase in the levels of neutral fats (mainly TG) in the liver [8,132-134]. Choline deficiency also produced hypolipidemia in rats; the concentrations of plasma cholesterol ester, unesterified cholesterol, phospholipids and TG were all reduced [135]. The concept that the impairment of TG release from the liver was the major contributing factor to the development of fatty liver in choline deficiency emerged in the 1930s. Best's group [136] showed in experiments using deuterium labeling of fatty acids that the source of fat accumulated in the liver upon choline deficiency was de novo synthesis and not mobilization from the depots (adipose tissue). Stetten and Salcedo [137], using similar techniques, found that the content of radiolabeled fatty acids in the liver was greater than that of the depots in the CD animals, indicating the export of newly made fatty acids from liver was impaired. More
direct evidence in support of this concept was obtained from a series of
experiments using the isolated perfused liver described by Mookerjea [138]. In
addition, it has been shown that choline deficiency does not increase
significantly hepatic synthesis of fatty acids [139,140].

The metabolic interrelationship between hepatic TG synthesis and choline-
phospholipid synthesis was suggested to explain the increase in TG and lowering
of PC in CD livers [63], since 1,2-DG is an intermediate for both TG and PC
synthesis. However, Lombardi et al. [8] demonstrated in experiments using a
labeled-palmitate injection technique that the rate of incorporation of labeled-
fatty acid into liver TG in CD rats did not differ from that in CS animals. The same
findings were reported by Blumenstein using similar techniques [134]. The effect
of choline deficiency on the activities of enzymes involved in TG synthesis, such
as phosphatidate phosphohydrolase or diacylglycerol acyltransferase, has not
been determined.

It is known that hepatic DG levels, like TG, are increased during choline
deficiency [135]. Tinoco et al. [135] reported a 6-fold increase in rat hepatic DG
concentration after 7 days of choline deficiency. Whether this extra DG results
from elevated hepatic TG storage in choline deficiency, from insufficient PC
synthesis due to a lack of choline or both is not clear.

4.2.2. Phospholipid Metabolism in Choline Deficiency

4.2.2.1. Changes in Precursor Pools of Phospholipids
in Choline Deficiency

A decrease in PC and an increase in PE, therefore lowering the ratio of PC
to PE, is another early characteristic feature in CD liver [132-134]. From the
viewpoint that choline is the precursor for de novo synthesis of cholinephospho-
lipids, changes in metabolic precursor pools of PC and PE in choline deficiency
have been extensively studied.
In rat plasma, choline concentrations normally range between 7-10 μM; after 5-8 days of choline deficiency, the choline level is reduced to 5 μM [141]. Haines and Rose [142] reported that choline and phosphocholine levels in the liver were decreased by 12- and 5-fold, respectively, after 2 to 3 days of choline deficiency. The decrease in hepatic choline was also reported by other groups [143-145]. Kennedy’s group [146] using an isotope-dilution method have determined CDP-choline levels in rat livers after 9-13 days of choline deficiency. The amount of CDP-choline in CD livers was about 30% lower than the control (0.85% (w/w) choline in the diet), if calculated on wet liver weight bases (control, 5.0±0.2; deficient, 3.6±0.2 μmol/100 g liver). However, the difference disappeared if the data were calculated on a protein basis (control, 31.9; deficient, 32.5 μmol/mg protein), or if the total amounts of CDP-choline in each liver were compared (control, 0.32±0.03; deficient, 0.36±0.04 μmoles per liver) [146]. Kennedy [146b] has further reported that 8-week choline deficiency had no effect on hepatic CDP-choline levels (control, 6.5±0.5; deficient, 6.2±0.6 μmol/100 g liver, n=3). It is noteworthy that the control levels of CDP-choline observed by Kennedy was higher than the data presented by Schneider (3.38 μmol/100 g liver) [146c] and by Åkesson’s group (0.87 μmol/100 g liver) [146d] using a liquid N₂ freezing technique. Normal CDP-choline levels during choline deficiency are also observed in lung [177]. A mechanism whereby the CD lung maintains normal CDP-choline levels will be discussed later (see section 4.3.2.).

The concentrations of hepatic ethanolamine, phosphoethanolamine and CDP-ethanolamine, on the other hand, are increased by 4.6-, 4.8- and 2.0-fold, respectively, in CD rats [142,147]. The sources of the PE precursors increased in the CD liver were not clear. The low ratio of PC to PE in choline deficiency presumably arose as the results of the decreased metabolic precursor pools of PC and the increased precursor pools of PE. Tinoco et al. [135] reported that in CD
livers, the increased amount of cellular PE was due largely to a disproportionate increase in molecular species that contain palmitic acid (16:0), stearic acid (18:0), arachidonic acid (20:4 n-6) and cervonic acid (22:6 n-3), whereas the reduction in cellular PC was due mainly to decreased palmitic acid (16:0), oleic acid (18:1 n-9) and linoleic acid (18:2 n-6). A selective formation of PE from DG in choline deficiency has been suggested [135].

4.2.2.2. Phosphatidylcholine Synthesis from Choline in Choline Deficiency

Since hepatic PC is normally synthesized from choline, the effect of choline deficiency on the activities of enzymes involved in the CDP-choline pathway has received considerable attention.

In rat liver, the activity of choline kinase in vitro is not significantly altered by 18 days of choline deficiency, except that there is a slight transient increase after 1 day [91]. On the other hand, the in vitro oxidation of choline by mitochondria to betaine is decreased by 30-40% in the liver after 4 days of choline deficiency [91]. These results suggest that hepatic utilization of choline may be regulated by its oxidation rather than its phosphorylation. This is consistent with the viewpoint that the main biological function of choline oxidation is to reduce free choline in the liver [45]. However, assay of choline dehydrogenase and betaine aldehyde dehydrogenase in vitro showed their activities were unaffected at early stages of choline deficiency [144]. The variance in these two results may result from the difference in the assays performed by the different laboratories.

The in vitro activity of cytidylyltransferase was decreased by about 40% in liver cytosol after 2 to 3 days of choline deficiency [91]. However, immunotitration of the cytosolic cytidylyltransferase using chicken antiserum to the enzyme showed the amount of cytosolic enzyme was not changed for at least 18 days of choline deficiency [148]. Therefore, the change in the specific activity of cytosolic cytidylyltransferase requires an explanation other than an adaptive
change in enzyme mass. Choy et al. [148] speculated that the enzyme activity might be regulated by an unidentified cellular modulator(s). The activity of membrane-associated cytidylyltransferase, the active species of the enzyme, has not been determined in CD livers.

The effect of choline deficiency on phosphocholinetransferase is controversial. Cornatzer's group [149-151] reported that hepatic phosphocholine- transferase activity was reduced by 74% and 86% after 7 and 14 days, respectively, of choline deficiency. However, Schneider and Vance [91] found that the activity of phosphocholinetransferase activity was not altered after 4 days of choline deficiency, and reduced by only 15% after 11 days. The reason for this discrepancy is unclear.

4.2.2.3. Phosphatidylcholine Synthesis from Phosphatidylethanolamine Methylation in Choline Deficiency

The study of PE methylation in choline deficiency is complicated, partly owing to the difficulties in measuring the activity of PE methyltransferase 

\textit{in vitro}. When the activity of PE methyltransferase was assayed using endogenous microsomal PE as a substrate, the rate of incorporation of methyl groups from AdoMet to phospholipids was 65 to 100% higher in CD livers compared to normal [91,150-152]. However, when exogenous PDME was used as the methyl group acceptor in the assay, the measured PE methyltransferase activity was 50% less in CD livers than that in normal [149,151]. These apparent discrepancies may reflect a variance in the presence of endogenous substrate for each, since PE is present in large quantities \textit{in vivo} (especially in CD liver) whereas the concentration of PDME is low [42]. The change, if there were any, in the amount of PE methyltransferase during choline and methionine deficiency has not been determined.
4.2.2.4. Phosphatidylcholine Degradation in Choline Deficiency

The degradation or turnover of PC in choline deficiency has rarely been studied in rat liver. Pulse-chase studies using $^{32}$Pi and $[^{14}$C]choline in cultured CHO cells demonstrate that the turnover of lipid choline is suppressed by choline deprivation, and the free choline generated during phospholipid turnover is salvaged very efficiently by the cells [102]. However, the utilization of phosphate esters of choline is not affected by the deficiency [102].

4.2.2.5. Phosphatidylethanolamine Synthesis in Choline Deficiency

The effect of choline deficiency on hepatic de novo synthesis of PE via the CDP-ethanolamine pathway has also been investigated. Choline deficiency does not alter PE synthesis from ethanolamine; the in vitro activities of ethanolamine kinase, phosphoethanolamine cytidylyltransferase and phosphoethanolamine-transferase are not changed in the livers of CD rats for at least 18 days [91,135]. The lack of change in these enzyme activities implies that the observed increase in the concentration of hepatic PE [132-134] probably results from the increased pool sizes of precursors [142, 147], or from impaired PE methylation as a result of limited availability of substrate (i.e. methyl group) in choline and methionine deficiency.

The effect of choline deficiency on hepatic synthesis of PE by PS decarboxylation was studied in rat hepatoma cells. The PS and PE synthesized from serine was not changed during choline deficiency [153].

As a summary, the activities of the enzymes involved in hepatic phospholipid synthesis appear to be well preserved during choline deficiency. The altered hepatic PC/PE ratio in CD rats may result from the changed precursor pools for these lipids.
4.2.3. Protein Metabolism in Choline Deficiency

Choline deficiency also produces a striking decrease in the amount of circulating lipoproteins. Lombardi and Oler [154] reported that the concentrations of plasma VLDL, LDL and HDL proteins were reduced by 53%, 60% and 15% (p<0.05), respectively, in rats after 24 h of choline deficiency, whereas the concentrations of plasma chylomicrons and d>1.21 proteins were unchanged. The decrease in the amount of plasma lipoproteins can be corrected by choline supplementation [154]. The release of radiolabeled plasma proteins (lipoproteins and non-lipoproteins) was also impaired in CD rats, but oral administration of choline corrected this defect [154]. It was also demonstrated that a delay in secretion of albumin occurred in CD rats [155]. Secretion of glycoproteins from perfused livers was used to determine the effect of choline deficiency on plasma lipoprotein secretion, because plasma lipoproteins were rapidly labeled with $[^{14}\text{C}]$glucosamine [138]. Mookerjea [156] showed that the secretion of glycosylated VLDL, LDL and d>1.21 proteins were inhibited in CD livers, but release of the labeled HDL was not affected by choline deficiency. Since it has been demonstrated that apoA-I is not a glycoprotein [157], and the glycosylation of apolipoproteins is not essential to hepatic VLDL secretion [158], the significance of the inhibited glycosylation of lipoproteins during choline deficiency should be re-evaluated. The effect of choline deficiency on hepatic apolipoprotein (e.g. apoB and apoE) synthesis has not been reported.

Despite the result that the secretion of plasma lipoproteins was reduced, choline deficiency appears not to affect hepatic protein synthesis. The rate of incorporation of $[^{14}\text{C}]$leucine or $[^{14}\text{C}]$glucosamine into hepatic proteins was unchanged in rats after 1 day of choline deficiency [154,155]. Lombardi [159] has suggested that there may be a slowdown in the intracellular transport of plasma proteins in the CD rats, due to the depletion of PC in ER membranes. However, the
normal secretion of d>1.21 proteins [154] indicates that the secretion of lipid-free plasma proteins is not affected by choline deficiency.

4.2.4. Bile Synthesis in Choline Deficiency

One of the major activities performed by the liver is to synthesize and secrete bile. Bile contains phospholipids, cholesterol, bile acids which are derived from cholesterol, and some proteins (e.g. albumin) [160]. Biliary phospholipid is predominantly PC (80-95%) with a unique fatty acid pattern, i.e. C16:0-C18:2 and C16:0-C18:1 [161]. The PC secreted in bile appears to be synthesized principally by the CDP-choline pathway [162]. In rats deficient in choline for 5-7 days, the amount of total cholesterol, conjugated bile acids and phospholipids in secreted bile is reduced by more than 60% [163]. Mookerjea's group [164] demonstrated in the pefused livers of CD rats that the secretion of total bile acids was decreased by 20% compared to the fistula bile of normal rats. Robins and Armstrong [165] have shown that the reduction in bile secretion results from an inhibited hepatic PC synthesis, and adequate dietary choline is necessary to maintain normal biliary PC and cholesterol secretion. Thus, a link between the supply of PC and bile secretion appears to be established.

4.2.5. Liver Cancer in Choline Deficiency

Feeding a choline-deficient diet markedly influences the process of chemical hepatocarcinogenesis in the rats. Lombardi's group [166,167] has demonstrated that whereas the diet has no effect on initiation, it strongly promotes carcinogenesis after administration of a single initiator dose of, for example, diethylnitrosamine to rats. Recently, researchers [168-170] observed that feeding rats a CD diet without any added carcinogens could lead to the development of liver cancer. Feeding a choline-deficient, methionine-low diet for 1 or 2 weeks effectively substituted for partial hepatectomy in the initiation of liver carcinogenesis [168]. The mechanisms whereby choline deficiency exerts
such hepatic mitogenic effects are not known. It has been suggested that the induction of DNA damage in a proliferating liver by the CD diet may be an early important event leading to initiation of liver carcinogenesis [171].

4.3. Effect of Choline Deficiency on Other Organs

4.3.1. Kidney Hemorrhagic Degeneration in Choline Deficiency

A renal lesion called "hemorrhagic degeneration", a direct effect of choline deficiency, was first recognized by Griffith and Wade in 1939 [172,173]. Feeding weanling rats a low-choline diet for 8 days led to the characteristic deposition of liver lipids, as well as the renal pathology [174]. The hemorrhagic degeneration is an important criteria for the severity of the choline-deficient diet, and it can be wholly prevented by supplementation with low choline levels which do not affect the deposition of liver lipids [174]. How hemorrhagic degeneration occurs in choline deficiency remains unanswered. The concentration of free choline and choline phospholipid in the kidney are virtually unchanged during choline deficiency [175]. However, a higher concentration of sphingomyelin and a lower level of PI is observed in the CD kidney shortly before necrosis [175]. Whether there is any relevance to the changes in these phospholipid levels to the kidney pathology is not understood.

4.3.2. Phosphatidylcholine Synthesis by Lung in Choline Deficiency

Since PC is of major physiological significance in lung function, the effect of choline deficiency on PC synthesis by lung has also received considerable attention. Yost et al. [176] first described that in rats fed a choline- and methionine-deficient diet for 2-3 weeks, the PC content of the whole lung homogenate is unchanged (control, 1.54±0.05; deficient, 1.46±0.06 mg PI/g dry weight), although the concentration of free choline is decreased by 40%. In addition, they have observed that PC content in lung lavage is slightly increased,
and the major species of the lavage PC is disaturated PC. These results indicated that, unlike the liver, the lung could maintain its normal PC levels during choline deficiency. Subsequently, the authors [177] have shown that choline deficiency results in a decrease in choline and phosphocholine content in the lung, but has no effect on CDP-choline or PC content. They [177] have demonstrated in the CD perfused lung that the utilization of choline and fatty acid for PC synthesis is largely stimulated. Consistent with these results, Tesan et al. [101] have demonstrated that in cultured type II pneumocytes depleted in choline, the activity of cytidylyltransferase is increased. Thus, the observed normal CDP-choline pools in the CD lung may result from stimulated cytidylyltransferase activity. Tesan et al. [101] proposed that the stimulated cytidylyltransferase in lung during choline deficiency was caused by the translocation of enzyme activity from cytosol to membrane. The activated cytidylyltransferase in choline deficiency probably compensates for decreased choline availability in order to maintain the normal PC content of lungs. Although the translocation of cytidylyltransferase activity from cytosol to microsomes in choline deficiency has been shown, a clear demonstration of the transport of cytidylyltransferase protein between cytosol and microsomes has not been presented. The PE methylation pathway does not contribute in a major way to PC synthesis in normal lungs nor even in CD lungs [178].

4.3.3. Intestinal Lipoprotein Metabolism in Choline Deficiency

An important role for PC in the release of intestinal lipoproteins has been demonstrated by O'Doherty et al. [37] in in vivo studies with 24 h bile-fistula rats. The rats failed to secrete chylomicrons and accumulated fat in the mucosa unless PC, lysoPC or choline was present in the intestinal lumen. Subsequently, they [179] demonstrated in cultured epithelial cells isolated from the intestinal mucosa of 24-h bile-fistula rats that supplementation of either choline or lysoPC to the
culture medium resulted in increased PC synthesis and effective release of chylomicrons. Cells maintained in a choline-free medium were unable to secrete chylomicrons. Thus, intestinal reacylation of absorbed lysoPC (after hydrolysis of biliary PC by pancreatic phospholipase [182]) or \textit{de novo} synthesis of PC from choline is an essential requirement for the transport of absorbed fat into lymph [73].

Rat intestine also secretes HDL [181]. Bearnot \textit{et al.} [182] have shown that although the secretion of lymph chylomicrons and VLDL was reduced by bile diversion, the secretion of apoA-I is maintained (basal, 119±15 \(\mu\)g/h; diverted, 140±20 \(\mu\)g/h) from the intestinal lumen. In addition, the secretion of lymph HDL was not affected by bile diversion, except that newly secreted HDL from the diverted rats had a reduced size (basal, 165±7 Å; diverted, 126±5 Å) and a depleted cholesterol content [182]. Therefore, the supply of PC appears to be only important for the secretion of lymph apoB-containing lipoproteins (chylomicron and VLDL), but not to apoA-I-containing lipoproteins.

4.4. Is Phosphatidylcholine Synthesis Required for Hepatic Lipoprotein Secretion?

4.4.1. Correlation Between Synthesis of Phosphatidylcholine and Secretion of Core Components of Lipoproteins

Plasma lipoprotein phospholipids are composed mainly of PC and SPM. During choline deficiency, rat hepatocytes appear to be unable to utilize pre-existing PC or other phospholipids for lipoprotein secretion, since hepatic PC levels in rat liver after 2-3 days of choline deficiency are not dramatically diminished (control, 93±12 \(\mu\)moles per 100 g body weight; CD, 77±9 \(\mu\)moles per 100 g body weight), and hepatic PE levels are increased [142]. Thus, the potential involvement of PC biosynthesis in hepatic lipoprotein secretion has been strongly implicated. In addition, many observations demonstrated that there is a
good correlation between hepatic synthesis and secretion of core components (TG and CE) of lipoproteins and hepatic synthesis PC (described below).

Fatty acids have been shown to stimulate TG synthesis and secretion in perfused rat livers [183-185]. Davis and Boogaerts [186], working on cultured rat hepatocytes, found that 1 mM oleate stimulated TG secretion by 2-fold. Similarly, Pelech et al. [79] showed that 1 mM oleate stimulated by 2-fold [$^{3}$H]choline incorporation from phosphocholine into PC in hepatocytes, due to an increased activity of the membrane-associated cytidylyltransferase. However, the rate of hepatic apolipoprotein (apoB, E and A-I) synthesis or secretion was not altered by fatty acid supplementation [186].

Cholesterol feeding also stimulates the secretion of VLDL, with a relatively higher ratio of CE to TG, by cultured rat hepatocytes [187]. Lim et al. [188] observed that feeding rats a diet containing 5% cholesterol and 2% cholate caused a 6-fold increase in plasma cholesterol (from 99±18 to 631±78 mg/dl) and a 3-fold increase in plasma PC (from 53.5±17 to 163±30 mg/dl). The increased plasma PC in cholesterol feeding appears to be correlated with an increased rate of hepatic synthesis of PC (3-fold) and an enhanced activity of membrane-associated cytidylyltransferase [188]. Whether the elevated plasma PC and cholesterol is due to stimulated secretion or to inhibited clearance of VLDL in those animals was not explained.

4.4.2. Sites of Phosphatidylcholine Synthesis and Assembly into Lipoproteins

In the liver, PC as well as other phospholipids are mainly synthesized and incorporated into microsomal membranes [189]. While the ER is the principal location of phospholipid biosynthetic activity in the liver, the Golgi apparatus also appears to possess the capacity for making membrane phospholipids [78,190,191]. In rat liver, the activities of cytidylyltransferase, cholinephosphotransferase and PE N-methyltransferase are found in ER as well as in Golgi
[78,190], indicating the Golgi can effectively synthesize PC via both the CDP-choline pathway and PE methylation pathway.

Topology studies of the requisite enzymes for PC synthesis suggest that the enzymes reside primarily in the outer leaflet of the membranes of ER and the Golgi [64,78,192]. For the assembly of lipoproteins, the newly synthesized phospholipids on the cytosolic surface must be transported into the cisternal space of these organelles where the nascent lipoprotein particles appear to be segregated. It is well known that the rate of phospholipid movement across artificial membranes is extremely slow, with a half-time of more than 12 days [193,194]. However, the movement of phospholipids across microsomal membranes is rapid with a measured half-life of less than 5 min [195,196]. These results indicate that there may be a phospholipid transport protein in the microsomal membrane. Bretscher [197] first proposed that a "flippase" might be located in the microsomal membrane and facilitate the transbilayer movement of phospholipids. Recently, Bishop and Bell [198] provided evidence that PC translocation across microsomal membranes was facilitated by a specific transport protein; PC "flippase". The transport of lysoPC in microsomes may also be a protein-mediated process [199]. A functional phospholipid "flippase" from rat liver microsomes has been reconstituted into lipid vesicles by Backer and Dawidowicz [200] recently, and a complete transbilayer movement of PC in this reconstituted system achieved within 15 min. The protein-facilitated transbilayer movement of other phospholipids (i.e. PE, PI and PS) in microsomal membranes has not been demonstrated.

The asymmetric distribution of phospholipids across the plane of the bilayer has been documented in the red blood cell membranes [201] and other biological membranes [202]. Owing to technical difficulties, whether there is an asymmetric distribution of the lipids in microsomal membranes is unknown.
Despite this, striking differences between the phospholipid compositions present in the membranes and contents of the isolated microsomes as well as the Golgi apparatus have been reported from different laboratories (Table IV) [191,203]. While PE contributes about 20% of total phospholipids in the membranes of microsomes and the Golgi apparatus, there was barely detectable PE in the content of these organelles [191]. A similar result was obtained by Howell and Palade [203] in the Golgi apparatus using $^{32}$P$_i$ to label the phospholipids. Apparently, PE is a poorly utilized phospholipid species for plasma lipoprotein assembly. PC and SPM, on the other hand, are the predominant phospholipids present in the content of microsomes as well as the Golgi. The Golgi content also contains relatively high concentrations of lysoPC compared to its membranes [203]. Currently, whether this compositional difference of phospholipids between the content and membranes reflects the asymmetry of microsomal and Golgi membranes is not clear.

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<td>Golgi content</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Micosome Membrane</td>
</tr>
<tr>
<td></td>
<td>Micosome Content</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>PA$^c$</td>
<td>0.11 0.15 n.d. n.d.</td>
</tr>
<tr>
<td>PS</td>
<td>2.74 3.04 8.9 10.8</td>
</tr>
<tr>
<td>PI</td>
<td>4.59 4.32 8.6 6.8</td>
</tr>
<tr>
<td>PE</td>
<td>16.97 20.8 19.8 20.7</td>
</tr>
<tr>
<td>PC</td>
<td>59.98 58.1 51.1 48.8</td>
</tr>
<tr>
<td>LysoPC</td>
<td>6.90 5.40 n.d. n.d.</td>
</tr>
<tr>
<td>SPM</td>
<td>9.34 8.17 11.5 13.0</td>
</tr>
</tbody>
</table>

* Data are % of total phospholipids in each fraction or subfraction.

** Data are calculated from Howell and Palade [203]; phospholipids are labeled with $^{32}$Pi.

$^b$ Data are calculated from Higgins and Fieldsend [191]; n.d., not detectable.

$^c$ PA, phosphatidic acid.
Like any secretory proteins, lipoproteins undergo direct transit within the hepatocyte through a series of membrane-bound intracellular compartments including ER and Golgi that constitute the so-called "secretory pathway" [205]. Several lines of evidence suggest that PC and other phospholipids are introduced into the nascent lipoprotein particles in both ER and Golgi during the course of assembly and transit. Howell and Palade [6] have shown that 30 min after injection of radio-labeled choline, inositol or phosphate, phospholipids in the Golgi membrane and content are both labeled to the same specific radioactivity. Janero and Lane's group [205] have demonstrated, in cultured hepatocytes isolated from estrogenized chick liver, a biphasic pattern of incorporation of radioactive glycerol into phospholipid secreted with VLDL: a sharp burst of secretion in the early phase (<15 min after the pulse), followed by a plateau (between 15-30 min), and then further release of radioactivity in the second phase. In the early phase, the incorporation of radioactive glycerol into the secreted VLDL phospholipids was faster than into secreted TG, although cellular phospholipids were not labeled preferentially as compared to cellular TG. The authors [205] proposed that the rapid incorporation of glycerol into VLDL phospholipids occurred in the Golgi, where the synthesized lipids were introduced into VLDL just prior to secretion. The delayed labeling of phospholipids and TG in secreted VLDL represented the time required for the newly made particles (acquiring phospholipids in the ER) to transit from the ER through the Golgi into the medium. Recently, Higgins and Huston [206], using radioactive palmitic acid to label phospholipids in the ER and the Golgi of rats, have provided additional evidence of rapid labeling of phospholipids in both the content and membrane subfractions of these organelles.
4.4.3. Selection of Phosphatidylcholine Pools in Lipoprotein Secretion

There was little knowledge about the selection of PC pools for hepatic lipoprotein secretion until Vance et al. [207] tackled the problem recently. From the initial studies in cultured hepatocytes treated with a specific inhibitor of transmethylation, 3-deazaadenosine (DZA), they found that the secretion of all classes of lipoproteins were normal even though the methylation of PE was inhibited by greater than 95%. This result suggested to the authors that the synthesis of PC from ethanolamine was not specifically required for hepatic lipoprotein secretion, although the liver possesses a uniquely high activity of PE methylation [207]. Subsequently, Vance and Vance [33, 208] discovered a DZA-insensitive methylation of PE involved in hepatic lipoprotein secretion. A rapid secretion of PC labeled by $[^3H]$serine from the hepatocytes was observed regardless of the presence of DZA, whereas the amount of $[^3H]$ethanolamine-labeled PC was depressed by the inhibitor. These data imply a selection among the PC synthetic pathways during the course of lipoprotein assembly and secretion.

Although PE methylation may not be important for hepatic lipoprotein secretion when the CDP-choline pathway is functional [207], in choline deficiency, methylation of PE may compensate for the defect in PC synthesis. Evidence from a preliminary experiment showed that supplementation of AdoMet to hepatocytes isolated from CD rat liver stimulated the secretion of radiolabeled TG, and enhanced the incorporation of labeled ethanolamine into cellular PC and PE [209]. However, AdoMet had no effect on TG secretion from the hepatocytes obtained from normal rat liver [209]. These results suggested that transmethylation might be stimulated by AdoMet in choline deficiency and compensate for the inhibited CDP-choline pathway.
5. Hepatic Synthesis of Apolipoproteins and Lipoprotein Secretion

5.1. Distribution of Plasma Apolipoproteins

Each ultracentrifugal class of plasma lipoproteins contains multiple apolipoprotein components (Table V). At least nine apolipoproteins (A-I, A-II, A-IV, B\textsubscript{100}, B\textsubscript{48}, C-I, C-II, C-III, E) have been identified, isolated and characterized over the past 20 years owing to rapid advances in the techniques of protein chemistry and molecular biology. The task of structure analysis of human lipoproteins, including the large molecular weight form of apoprotein B or apoB\textsubscript{100} [210-213] and its derivative apoB\textsubscript{48} [214], has been almost entirely fulfilled, except that the structure of human apoA-IV has not been revealed [157]. The structure of rat apoA-IV cDNA, however, has been determined [215]. Amino acid sequences of the apolipoproteins are either derived from direct amino acid sequencing or deduced from the corresponding cDNA and in some cases from genomic DNA clones. The locations of most of the apolipoprotein genes have been mapped in human chromosomes [157].

Hepatocytes and the absorptive cells of the small intestine are sources of virtually all circulating plasma apolipoproteins [2]. Interestingly, synthesis of apoE has been documented in a wide variety of other tissues including brain and nerve tissue, kidney, adrenal gland and reticuloendothelial cells [217-219].

ApoA-I and A-II are the major constituents of human and rat HDL, accounting for 90% of the total HDL apoproteins. In all species studied, only human and chimpanzee plasma apoA-II exists as a dimer of two identical subunits linked by a disulfide bridge [220,221], while in the others (including rat) apoA-II is a monomer [222]. The significance of this difference is unknown. ApoA-IV is a prominent component of newly secreted lymph chylomicrons, but is only a minor component of human HDL. However, it is a major apolipoprotein constituent of rat HDL [223]. The significance of this difference is not known. Unlike most
other apolipoproteins, a significant fraction of apoA-IV in human as well as rat plasma is present unassociated with lipoproteins [224].

The B apolipoproteins are by far the largest polypeptides known to be synthesized in mammalian cells. Two forms of apoB exist in human, the large one has a molecular weight of 512,937, as calculated from the amino acid sequence [211], while the small one has a molecular weight 48% of that large one [238]. Therefore, the two human apoBs are termed apoB\textsubscript{100} and apoB\textsubscript{48}, respectively. Stoichiometry studies using an immunochemical approach have shown that only one apoB molecule is present on each lipoprotein particles [225]. In humans, apoB\textsubscript{100} is the predominant (98%) protein in LDL and the major apoB protein found in VLDL, while the apoB\textsubscript{48} is exclusively found in chylomicrons and their remnants. Accordingly, human apoB\textsubscript{100} and apoB\textsubscript{48} are believed to be synthesized and secreted from the liver and the intestine, respectively. It is now clear that human apoB\textsubscript{48} is the amino-terminal half of apoB\textsubscript{100} [214] (see below).

Rat plasma B apoproteins differ from their human counterparts by their apparent molecular weights, distributions and synthetic sites. Since there still exists some doubt as to the exact molecular weights of rat B apoproteins [216a,226,228,229], the term apoB\textsubscript{H} is used to designate the high molecular weight form (335-590 Kd) and apoB\textsubscript{L} to designate the low molecular weight form (240-260 Kd). Unlike humans, rat livers possess the capacity to synthesize and secrete both forms of B apoproteins, and the rat plasma VLDL and LDL contain both apoB\textsubscript{H} and apoB\textsubscript{L} [216]. The clear relationship between rat apoB\textsubscript{H} and apoB\textsubscript{L} is not understood. Studies in rat hepatocytes suggest that apoB\textsubscript{H} and apoB\textsubscript{L} may be structurally and metabolically distinct proteins, the circulating half-life of apoB\textsubscript{H} is more than two times longer than that of apoB\textsubscript{L} [216,226b,229].

The C apoproteins are represented by three low molecular weight proteins, designated as C-I, C-II and C-III. They range in molecular weight from 6,600 to
Table V  Characteristics of Apolipoproteins in Human and Rat Plasma

<table>
<thead>
<tr>
<th></th>
<th>Molecular weight</th>
<th>Major tissue source</th>
<th>% of Lipoprotein protein content</th>
<th>Human</th>
<th>Rat</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Human</td>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>apoA-I</td>
<td>28,100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27,000&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Liver &amp; intestine</td>
<td>60%HDL</td>
<td>60%HDL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7%chylos*</td>
<td></td>
</tr>
<tr>
<td>apoA-II</td>
<td>17,400&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8,000&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Liver &amp; intestine</td>
<td>25%HDL</td>
<td>&lt;20%HDL</td>
</tr>
<tr>
<td></td>
<td>(dimer)</td>
<td>(monomer)</td>
<td></td>
<td>4%chylos</td>
<td></td>
</tr>
<tr>
<td>apoA-IV</td>
<td>46,000</td>
<td>46,000&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Liver &amp; intestine</td>
<td>chylos, VLD</td>
<td>HDL, chylos</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HDL, d&gt;1.21</td>
<td>d&gt;1.21</td>
</tr>
<tr>
<td>apoB-100</td>
<td>512,937&lt;sup&gt;h&lt;/sup&gt;</td>
<td>335,000&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Liver</td>
<td>98%LDL</td>
<td>40-60%VLDL</td>
</tr>
<tr>
<td></td>
<td>(apoB&lt;sub&gt;H&lt;/sub&gt;)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>apoB-48</td>
<td>264,000&lt;sup&gt;h&lt;/sup&gt;</td>
<td>240,000&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Intestine &amp; intestine</td>
<td>chylos</td>
<td>90%chylos</td>
</tr>
<tr>
<td></td>
<td>(apoB&lt;sub&gt;L&lt;/sub&gt;)</td>
<td></td>
<td></td>
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<tr>
<td>apoC-I</td>
<td>6,600&lt;sup&gt;j&lt;/sup&gt;</td>
<td>7,000&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Liver</td>
<td>14%chylos</td>
<td>VLDL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4%VLDL</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-5%HDL</td>
<td></td>
</tr>
<tr>
<td>apoC-II</td>
<td>8,800&lt;sup&gt;k&lt;/sup&gt;</td>
<td>8,000&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Liver</td>
<td>14%chylos</td>
<td>VLDL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7-10%VLDL</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1-3%HDL</td>
<td></td>
</tr>
<tr>
<td>apoC-III</td>
<td>8,700&lt;sup&gt;l&lt;/sup&gt;</td>
<td>10,000-11,000&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Liver</td>
<td>34%chylos</td>
<td>VLDL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35-40%VLDL</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3-5%HDL</td>
<td></td>
</tr>
<tr>
<td>apoE</td>
<td>34,200&lt;sup&gt;m&lt;/sup&gt;</td>
<td>35,000-37,000&lt;sup&gt;n&lt;/sup&gt;</td>
<td>Liver</td>
<td>VLDL</td>
<td>VLDL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LDL, HDL</td>
<td>LDL, HDL</td>
</tr>
</tbody>
</table>

* chylos, chylomicrons.
a Data on apolipoprotein distribution in man taken from Schaefer et al. [232].
b From Brewer et al. [233].
c From Swaney et al. [234].
d From Brewer et al. [220].
e From Herbert et al. [222] and Swaney and Gidez [235].
f From Parks and Rudel [236].
g From Zannis and Breslow [237].
h From Kane et al. [238]; a calculated molecular mass of 512,937 daltons for apoB<sub>100</sub> was found by Yang et al. [211].
i From Krishnaiah et al. [216a].
j From Shulman et al. [239].
Footnotes to Table V (continued)

k From Jackson et al. [240].
l From Karathanasis et al. [241].
m From Rall et al. [242].
n From Weisgraber and Assmann [243] and McLean et al. [244].

8,800, and have the common property of redistribution among lipoprotein classes. In the fasting state, the C apolipoproteins are mainly associated with HDL [227]. During absorption of dietary fat by intestine and production of chylomicrons or during the active synthesis of VLDL by liver, the C apolipoproteins preferentially redistribute to the surface of the TG-rich lipoproteins [227]. The physiological significance of this will be discussed later.

The E apolipoprotein is a component of chylomicrons and their remnants, VLDL and HDL. ApoE is enriched in concentration in the plasma of many animals after feeding diets high in fat and cholesterol, and becomes a major protein constituent of two cholesterol-rich lipoproteins, β-VLDL (intestinal and hepatic remnants) and HDL_c (cholesterol-rich HDL-with apoE) [231].

5.2. Metabolic Functions of Plasma Apolipoproteins

Metabolic functions of plasma apolipoproteins, unlike phospholipid components in lipoproteins, are better understood. The major function of apolipoproteins is in the transport and metabolism of plasma lipids.

In humans, the B apoproteins are essential for the formation and secretion of TG-rich lipoproteins: apoB_{100} for hepatic VLDL and apoB_{48} for intestinal chylomicrons. In puromycin- [245] or cycloheximide-treated hepatocytes [246], apoB synthesis and VLDL secretion was blocked. Absence of plasma apoB and the virtual lack of the B-containing lipoproteins (chylomicrons, VLDL and LDL) are characteristic features of abetalipoproteinemia, an autosomal recessive disease [247]. Recent analysis of hepatic mRNA and gene coding for apoB_{100} in
abetalipoproteinemia subjects have revealed that one patient has normal apoB genes and mRNA [248]. Thus, a defect in translation or post-translational processing of apoB\textsubscript{100}, rather than a defect in gene expression or mRNA processing, has been suggested [248]. ApoB\textsubscript{100} also serves as a ligand for the LDL (B/E) receptor, which mediates the clearance of plasma LDL as well as IDL in humans [249] and in rats [250]. The binding domain for B/E receptors is located within the C-terminal portion of apoB\textsubscript{100} [210], which shows homology with the binding domain in apoE for the same receptor [251,252]. ApoB\textsubscript{48} does not contain the B/E receptor binding domain, thus, the clearance of chylomicron remnants is mainly mediated by apoE via the B/E receptor or via the hepatic apoE receptor [253]. Mahley's group [254-256] has shown that the hepatic apoE receptor is distinct from the B/E receptor, and these two receptors are regulated independently.

ApoE also plays numerous functions. ApoE is required for the formation of cholesterol ester-rich particles in the plasma, and the apoE-containing lipoproteins play a major role in cholesterol transport from peripheral tissues to the liver through a receptor-mediated process [231]. Numerous other tissues have been shown to possess significant quantities of apoE mRNA, and it has been estimated that 10-20% of the circulating apoE may derive from synthesis by peripheral tissues [231]. Mahley \textit{et al.} [256] have suggested that the apoE from peripheral origin may play important roles in lipid transport as well as in the peripheral nerve system.

The activities of three important plasma lipolytic enzymes, lipoprotein lipase, hepatic triglyceride lipase and LCAT, are regulated by the A and C apolipoproteins. Lipoprotein lipase (EC 3.1.1.34) catalyses the hydrolysis of circulating plasma TG transported in chylomicrons and VLDL. For maximal activity lipoprotein lipase requires the presence of apoC-II [257], and the absence
of this apolipoprotein results in an acute hypertriglyceridemia [258]. Another C apolipoprotein, apoC-III, appears to inhibit the action of lipoprotein lipase [259]. Thus, the rate of lipolysis may be regulated by the ratio of apoC-II/C-III on the lipoprotein surface. Hepatic triglyceride lipase (EC 3.1.1.3) catalyzes the hydrolysis of TG as well as phospholipids (e.g. PC) [260], and its activity is enhanced by apoA-II [261]. The activity of LCAT (EC 2.3.1.43) is greatly activated by apoA-I [262] and to a lesser extent by apoC-I, apoE and apoA-IV [263,264]. The transfer and exchange of these C and A apolipoproteins among lipoprotein particles plays an important role in plasma lipoprotein metabolism [227]. In addition, the C apolipoproteins inhibit the interaction of plasma lipoproteins with hepatic receptors, and thus hepatic removal [265,266].

5.3. Hepatic Biosynthesis and Secretion of Apolipoproteins

5.3.1. The B Apolipoproteins and VLDL Secretion

Human apoB\textsubscript{100} is composed of 4563 amino acids, its mRNA is about 14,000 nucleotides in length and the corresponding gene comprises 29 exons (43 kilobases), of which exon 26 (about 7.0 kilobase) carries about half of the coding sequence [214]. The time needed for the synthesis of apoB\textsubscript{100} has been assessed to be 14 min in HepG2 cells [267] and approximately 10 min in cultured hepatocytes from the estrogen-treated chicken [268]. A signal sequence of 27 amino acid residues is found in the amino-terminal of human apoB\textsubscript{100} and is cotranslationally removed [210-214]. It is well-known that apoB\textsubscript{100} is an N-linked glycoprotein, and the carbohydrate accounts for 4-5% of its weight [269]. However, the secretion of VLDL appears to be independent of the glycosylation of apolipoproteins as demonstrated in the tunicamycin-treated hepatocytes [158,205]. Brewer's group [270] recently found that apoB\textsubscript{100} in HepG2 cells was covalently acylated by palmitic acid, but the function of this acylation was unknown. In rats, the liver has the capacity to synthesize both apoB\textsubscript{H} and apoB\textsubscript{L}, and the VLDL
secreted from rat hepatocytes contain both form of the B apoproteins. Whether one rat VLDL particle (like human VLDL) contains one molecule of apoB has not been determined.

It is now clear that in humans, intestinal apoB_{48} mRNA and hepatic apoB_{100} mRNA are transcripts of the same apoB gene, and the intestinal apoB_{48} contains the amino-terminal 2152 amino acids of apoB_{100}. A co- or post-transcriptional modification of a single nucleotide (conversion of cytosine to uracil) at the middle of apoB_{100} mRNA, which was thought specifically occurred in the intestine, led to an in-frame stop codon in the mRNA and gave apoB_{48} mRNA in the intestine [214]. Recently, Brewer's group [214b] have demonstrated in human that the in-frame stop codon in apoB_{100} mRNA is not tissue specific and apoB_{48} mRNA is present in both liver and intestine. Thus, apoB_{100} and apoB_{48} equivalent apolipoproteins secreted from the rat liver are probably translated from the two different apoB mRNAs. However, why rat liver produces both forms of apoB but human liver only produces apoB_{100} is not clear.

ApoB_{100} is essential for human hepatic VLDL assembly and secretion as manifested by studies on patients with genetic defects [247] and from experiments using protein synthesis inhibitors [245,246]. In rats, however, hepatic TG-rich lipoprotein secretion appears to be linked with the low molecular form of apoB, apoB_{L}, as demonstrated in developmental [271,272] and dietary studies [272,273]. In the suckling rat, infusion with [^{3}H]lysine showed that apoB_{H} was the only species labeled in the liver. Consistent with this, Coleman et al. [272] found that hepatic apoB_{L} secretion from suckling rats was only 50% of that from adults, whereas the secretion of apoB_{H} was normal compared to that of adults. Fatty acid stimulates hepatic TG synthesis but not TG secretion in the suckling rats [272], whereas fasting decreases the secretion of TG and apoB_{L} but not apoB_{H} [273]. Thus, the rat hepatic synthesis of apoB_{L} may limit the rate of TG secretion. ApoB_{L} in rat
hepatocytes is reported to be phosphorylated [274], but the functional significance of this post-translational modification is unknown.

Electron micrographic studies and immunocytochemical analysis revealed the presence of apoB in the rough ER, on VLDL particles located in the lumen of the ER at the junction of rough and smooth ER, and on VLDL particles of the Golgi complex, secretory vesicles, and the space of Disse [204,275]. Pulse-chase studies in rat hepatocytes [230], human HepG2 cells [267] and estrogen-treated chicken hepatocytes [205] have demonstrated a precursor-product relationship between apoB100 in the ER and the Golgi apparatus. These results unambiguously indicate that hepatic secretion of apoB-containing lipoproteins is through the classical "secretory pathway" [204]. Several lines of evidence suggest that the newly synthesized apoB is cotranslationally associated with the membrane in the ER, from which it is subsequently transferred to a lipoprotein form in the ER lumen and in transit to the Golgi [267,276]. ApoB100 contains regions through the molecule with a pattern of continuous alterations between hydrophobic and hydrophilic sequences, and computer analysis predicts high probability for beta-sheet structure for most of the hydrophobic sequences [210,211]. Thus, a model of apoB-membrane interaction has been suggested: apoB is woven in and out of the inner leaflet of the ER membranes during the course of protein translation [276b].

In human Hep G2 cells [267] and chick hepatocytes [205], the time needed for the secretion of newly synthesized apoB100 has been estimated to be approximately 30 min; 10 min is needed for transfer between ER and Golgi, while 20 min for the transfer through the Golgi to the extracellular space. The requirement of 10 min for the B apoprotein to transit from ER to Golgi was also found in cultured rat hepatocytes [230]. Impairment of the intracellular movement of nascent VLDL particles will lead to a defect in secretion. In the
livers of orotic acid-fed rats, the translocation of nascent VLDL particles from the ER to the cis Golgi compartment was found to be blocked [277]. The mechanism for this impairment has not been explained. Chloroquine [278] and monensin [279] have also been reported to inhibit the intracellular transport of VLDL; the former appears to block the transit between the ER and the Golgi, while the latter inhibits the discharge of VLDL particles from the Golgi.

Is the active synthesis of apoB essential for hepatic TG-rich lipoprotein synthesis and secretion? Janero and Lane [246] showed, in cycloheximide-treated chicken hepatocytes, that the apoB nascent chains were arrested intracellularly and \([^3H]\)glycerol-labeled TG secretion was depressed. However, in puromycin-treated cells, the truncated nascent apoBs (molecular weight ranged from 20 Kd to 350 Kd) discharged from the polysomes were still quantitatively secreted as VLDL constituents, although the rate of \([^3H]\)glycerol-labeled TG secretion was impaired [246]. These results suggest the assembly of apolipoproteins and lipid to form VLDL particles does not entirely depend on ongoing apoB production.

Once made, is secretion the only metabolic destination for hepatic apoB? Pulse-chase experiments in chick hepatocytes showed that the apoB (chick VLDL contains only one form of apoB) synthesized in the cells was quantitatively recovered in the medium [268]. However, Borchardt and Davis [230] showed, in cultured rat hepatocytes, that the recovery of the de novo synthesized apoB$_H$ and apoB$_L$ in the culture medium was only 36% and 60%, respectively, while the secretion of albumin was essentially quantitative. Apparently, some of the newly synthesized B apoproteins were degraded within the cells. The mechanism by which the liver cells govern this intracellular degradation of apoBs is unknown. In chick heptocytes, however, it has been found that the apoB nascent chains trapped intracellularly on polysomes by cycloheximide are degraded, whereas
their integrity is preserved when they are discharged by puromycin and secreted as constituents of VLDL [246].

ApoE is also a quantitatively major apolipoprotein component of VLDL secreted by rat hepatocytes. Mature apoE is a single polypeptide containing 299 amino acids in humans [242] and 293 amino acids in rats [244]. Both human and rat apoE initially contain an 18 amino acid signal sequence which is cleaved during translation. ApoE is also a glycoprotein, containing 2-2.5% carbohydrate with sialic acid residues [280]. Glycosylation of apoE is not inhibited by tunicamycin, suggesting that oligosaccharides are O-linked rather than N-linked [280,281]. Zannis et al. [281b] have shown that human apoE is initially secreted from hepatocytes as a sialic acid-containing protein, but over 80% of plasma apoE is desialylated. The relevance of apoE synthesis and the post-translational modification to hepatic VLDL secretion has not been clearly demonstrated.

5.3.2. The A Apolipoproteins and HDL Secretion

Much less is known about the intracellular mechanisms involved in HDL synthesis and secretion. HDL secretion has been described in cultured hepatocytes and by perfused liver [16,282,283]. The newly secreted HDL from the perfused liver differs in important respects from circulating HDL isolated from the plasma. The newly formed HDL has a discoidal conformation, particularly when the activity of LCAT is inhibited during the liver perfusion [16]. This result demonstrates clearly that the transformation of HDL precursors to spherical HDL is dependent on LCAT activity. It does not, however, provide evidence that discoidal HDL particles are exclusively secreted from the liver cells. It has not been possible as yet to visualize HDL particles within subcellular compartments, or to isolate them from Golgi-rich fractions in hepatocytes. The intracellular pathway for HDL biosynthesis and secretion remains unknown. Howell and Palade [6] observed, in the isolated rat liver Golgi apparatus, nascent VLDL
particles and other lipoproteins of various sizes, some small enough to represent HDL precursors [6]. The conversion of these putative HDL precursors to secreted HDL has not been demonstrated. Although the origin of nascent discoidal HDL particles observed in the liver perfusate is poorly defined, it is clear that their major proteins (apoE and A-I) are synthesized in the liver. The apoproteins are thought to be associated mainly with the margin of the discoidal particle, while the disk is composed of a phospholipid (mainly PC) bilayer [284]. Eisenberg [285] has suggested that the formation of HDL precursors (discoidal lipoproteins) may occur in body fluids by the association of various apolipoprotein and lipid components. The sources of apolipoproteins are hepatic and intestinal synthesis or from VLDL and LDL during their catabolism, while the sources of phospholipids and cholesterol derive from the lipolysis of TG-rich lipoproteins or from membrane lipids.

In both human and rat, apo A-I is synthesized with a signal sequence of 18 amino acid residues and a hexapeptide prosegment. The signal peptide is removed during translation, while the prosegment is cleaved extracellularly after the pro-apoprotein is secreted into the plasma [286]. Apo A-II, like apo A-I, is synthesized as a preproapolipoprotein [286]. Involvement of the A apolipoproteins in hepatic de novo lipoprotein secretion is not clear. ApoA-I secretion by hepatocytes is unaffected by adding fatty acids to the medium [286b]. In liver perfusates of orotic acid-fed rats, the typical discoidal HDL accumulated and VLDL was absent [277]. These results suggest that hepatic VLDL and HDL may be formed and secreted independently. ApoA-I is thought to occupy a surface position on HDL particles [287]. An HDL-like lipid/apoprotein complex was observed in vitro by incubating human apoA-I with microsomal membranes of rat liver, but not with erythrocyte plasma membranes [288]. The newly secreted HDL from rat liver also contains an apoB₄₈-like apoprotein and apoE. Interestingly, when the secretion of
apoB_H is inhibited from the perfused liver of orotic acid-fed rats, the apoB_L-like protein is still released into the perfusate together with HDL [277].

6. The Thesis Investigations

The main goal of this thesis is to understand the role of PC synthesis in the genesis of hepatic lipoproteins. Although the characteristic features of choline deficiency can be readily observed in young rats fed a CD diet, it is difficult to investigate the mechanisms in the whole animals. In the present research, cultured rat hepatocytes obtained from a single liver of the CD rats were used as an experimental model. The questions to be answered in this research are:

1. Is the accumulation of TG in the livers and the decrease in lipoprotein levels in the plasma during choline deficiency due to impaired secretion of hepatic lipoproteins?

2. Does the impaired hepatic lipoprotein secretion result from a defect in PC synthesis? If it does, which pathway for PC synthesis is required for hepatic lipoprotein secretion? Will PE methylation or reacylation of lysoPC compensate for the CDP-choline pathway? Is PC synthesis required for both VLDL and HDL secretion from hepatocytes? Is there a specific requirement of PC synthesis for hepatic lipoprotein secretion? Can synthesis of PE, PMME or PDME substitute for PC?

3. What is the effect of choline deficiency on cytidylyltransferase activity and its subcellular location? What is the effect of choline deficiency on PE N-methyltransferase activity and enzyme mass?
EXPERIMENTAL PROCEDURES

1. Materials

Choline-deficient diet was obtained from ICN Biochemicals, Canada. Dulbecco's modified Eagle's medium (Formula #79-5141, containing no arginine, choline or methionone), Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hanks' balanced salt solution, Ca\(^{2+}\)- and Mg\(^{2+}\)-containing Hanks' balanced salt solution, penicillin G (10,000 units/ml) and streptomycin (10,000 µg/ml) were all obtained from Gibco Laboratories, Grand Island, NY. Primaria culture dishes (60 mm in diameter) were obtained from Becton Dickinson & Co., Oxnard, CA. Fetal bovine serum, pentobarbitone, Silica Gel 60 TLC plates (20x20 cm), HPTLC plates (10x20 cm), L-ascorbic acid, D-glucose, sodium azide, sodium chloride, EDTA, iodine, sucrose, glycerol, diethylether, di-iso-propylether, 1-butanol, methanol, perchloric acid (70%), glacial acetic acid, hydrochloric acid and formic acid (98-100%) were obtained from BDH Chemicals. Insulin, BSA (fatty acid free), collagenase (type I, 300 units/mg and containing less than 0.4 units/mg trypsic activity), choline oxidase (15 units/mg protein), catalase (from bovine liver, 45,000 units per mg protein), phospholipase A\(_2\) (from Crotalus adamanteus venom), leupeptin, L-ornithine, cytochrome C (type III), uridine-5'-'diphosphogalactose, N-acetylglucosamine, glutathione, dextran, L-leucine, L-methionine, D,L-homocysteine, pyruvic acid (sodium salt), triolein, L-α-lysophosphatidylcholine (Type I, from egg yolk), ε-aminocaproic acid, EGTA, DMSO, PMSF, Tris base, Hepes, deoxycholic acid, β-mercaptoethanol, ATP, phosphocholine chloride, CDP-choline, choline chloride, ethanolamine, N-monomethylethanolamine, N,N-dimethylethanolamine, betaine, hydroxylamine, guanidine hydrochloride, N-lauroyl-sarcosine (sodium salt), Nonidet P40, Triton X-100, Tween 20, digitonin (approx. 50%), laurysarcosine (sodium salt), Cab-O-Sil (fumed silica), boron trifluoride-methanol (14% BF\(_3\) in methanol), Folin & Ciocalteu's phenol reagent (2 N), Sudan black B, 4-chloro-1-napthol and reagents
for ELISA were all obtained from Sigma Chemical Co., St. Louis, Mo. L-[4,5-3H]leucine (45 Ci/mmol), [9,10-3H]oleic acid (4.2 Ci/mmol), UDP-D-[6-3H]galactose (14.5 Ci/mmol), 125I-labeled Protein A, [methyl-3H]choline (15 Ci/mmol) and aqueous counting scintillant were obtained from Amersham International, UK. [32P]PO4 (carrier free, 5 mCi/ml) and EN3HANCE (without 2-ethoxyethanol) were obtained from New England Nuclear, Boston, MA. Chloroform (HPLC grade), ammonium hydroxide (28%, w/v), ammonium molybdate, urea, sodium bicarbonate, sodium hydroxide, sodium bromide and magnesium chloride were obtained from Fisher Scientific. NADH, NADPH and trypsin-inhibitor (from soybean) were obtained from Boehringer-Mannheim, GmbH, West Germany. Ferric perchlorate was obtained from Fluka, Switzerland. Phosphoric acid, cupric acetate and TCA was obtained from J. T. Baker Chemical Co., Phillipsburg, NJ. Dimethyl formamide was obtained from Caledon Laboratories Ltd., Canada. Nalgene filters were obtained from Nalge Co., Rochester, NY. Soluene-350 and Hionic-Fluor scintillation cocktail were products of Packard Instrument Co., Downers Grove, IL. Reagents for gel electrophoresis and immunoblotting were all purchased from Bio-Rad Laboratories, Richmond, CA and Schwarz/Mann Biotech, Cleveland, OH. Protein A-Sepharose CL-4B was obtained from Pharmacia Fine Chemicals, Sweden. Rabbit anti-chicken IgG and the horse radish peroxidase conjugate were purchased from Organon Teknika Co., West Chester, PA.

2. Preparation of [methyl-3H]Betaine, Phospho[methyl-3H]choline and [methyl-3H]Lysophosphatidylcholine

[Methyl-3H]betaine (0.67 mM, 100 μCi/μmol) was prepared enzymatically from [methyl-3H]choline by treatment with choline oxidase and catalase [289]. Choline oxidase (1 mg of protein) and catalase (50 μg of protein) were incubated with 200 μCi [methyl-3H]choline at 37 °C for 1 h. The reaction was terminated by boiling the mixture for 1 min. [Methyl-3H]betaine, plus 2 μmole betaine as
carrier, was separated by TLC. The betaine was visualized with iodine, scraped, and eluted 15 times with 2 ml methanol. The methanol was evaporated and the sample was redissolved in 3 ml H$_2$O and stored at -20 °C.

Phospho[\textit{methyl}-$^3$H]choline (1.5 mM, 7.2 μCi/μmol) was synthesized enzymatically from [\textit{methyl}-$^3$H]choline and ATP with choline kinase based on the method of Vance \textit{et al.} [290].

For preparation of [\textit{methyl}-$^3$H]lysoPC, hepatocytes obtained from choline-deficient rats were incubated with [\textit{methyl}-$^3$H]choline (12 μCi/dish) in 2 ml choline-free medium for 2 h and PC was separated from the cell lipid extract by TLC. The purified [\textit{methyl}-$^3$H]PC (14,000 dpm/nmol) was treated with phospholipase A$_2$ as described by Tarn \textit{et al.} [291] and [\textit{methyl}-$^3$H]lysoPC was separated by TLC. The [\textit{methyl}-$^3$H]lysoPC was dissolved in water and egg yoke lysoPC was added to the sample to give a specific radioactivity of 250 dpm/nmol.

3. Animals and Diet

Male Sprague-Dawley rats, initially weighing 45-50 g, were used for all experiments. However, similar results were obtained when experiments were performed on male Wistar rats. The animals were fed a choline-deficient diet for three days. The diet consisted of 10% vitamin-free casein, 10% alpha protein, 20% lard, 56% sucrose, 4% salt mixture (Wesson) and ICN vitamin fortification mixture, but without choline chloride. The control animals were fed the same diet supplemented with choline chloride (0.4%, w/w). The body weights gained during this period were identical in both CD and CS groups, but the rats fed with the CD diet sometimes developed lustreless coats after two days.

4. Delipidation of Fetal Bovine Serum

Fetal bovine serum was delipidated with butanol/di-\textit{iso}-propyl ether (40:60; v/v) by the method of Cham and Knowles [292] and dialysed thoroughly against 0.9% NaCl (w/v) at 4 °C to remove residual organic solvent and choline or choline-
containing compounds. An adequate amount of water was added to the delipidated serum to obtain the initial volume. The extent of delipidation was examined by thin-layer chromatography of the chloroform/methanol (1:1, v/v) extract from the delipidated serum. Choline-containing phospholipids (PC, lysoPC and SPM) and other polar lipids (PI, PE and PS) were entirely removed after the delipidation. Triacylglycerol and free cholesterol were completely eliminated from the serum, but there were residual amounts of cholesterol ester and free fatty acids in the delipidated serum. The recovery of serum proteins after delipidation was 85-90%. Separation of the serum proteins by one-dimensional SDS-PAGE showed no loss of any particular protein component. The delipidated serum was sterilized by passing through a Nalgene filter with pore size of 0.2 μm and stored at -20 °C.

5. Preparation of Hepatocytes

Hepatocytes were routinely prepared from a single rat weighing about 60 g after feeding a CD diet for 3 days. The animal was anesthetized lightly with diethylether and then by intraperitoneal injection of pentobarbitone (5 mg/100g body weight). Hepatocytes were isolated by a collagenase perfusion technique essentially as described previously [293,294] with modifications. Since the rat used for preparing hepatocytes was rather small, the perfusion required patience and practice. The liver was infused through the portal vein with a Ca$^{2+}$- and Mg$^{2+}$-free Hanks' solution supplemented with 20 mM glucose, 25 mM Hepes, 0.5 mM EGTA and 10 μg/ml insulin at 5 ml/min. The perfusate was drained through the lower vena cava (below renal vein) and heart artery to clean out blood. After the liver was clearly blanched, the lower vena cava (above renal vein) and heart artery (close to diaphragm) were closed to allow the liver to swell. Once the liver was swollen, the perfusate was changed to a Ca$^{2+}$- and Mg$^{2+}$-containing Hanks' solution supplementated with 6 mg/ml of collagenase, 20 mM glucose, 25 mM
Hepes, 0.5 mM EGTA and 10 µg/ml insulin. After 10 min (depending on the particular liver and the collagenase used), the digested liver was excised, chopped with scissors in a culture dish, and gently shaken (by hand) with 10 ml collagenase solution in a centrifuge tube at 37 °C for about 2 min to break cell clumps. The cell suspension was filtered through a metal mesh and 40 ml of plating medium (Dulbecco's modified Eagle's medium containing 17% delipidated serum, 0.4 mM ornithine, 25 mM glucose, 10 mM Hepes buffer, 10 µg/ml insulin, 80 units/ml penicillin G and 80 µg/ml streptomycin but without arginine, choline or methionine) was quickly added to the cell suspension to stop the collagenase digestion. Undigested liver tissue was agitated in another 3 ml collagenase solution for 1-2 min, and the cell suspension was filtered as before. Hepatocytes were separated from other cell types by low-speed centrifugation at 500 rpm for 2 min with 40 ml plating medium in an IEC clinical centrifuge at room temperature, and this step was repeated three times. The cells were resuspended in plating medium and brought to a concentration of 1×10^6 cell/ml. After being filtered through a fine metal mesh to remove any aggregated cells, the hepatocytes were plated on culture dishes (3×10^6 cells/dish), dispersed evenly by gentle shaking and incubated at 37 °C in a 5% CO₂ atmosphere. Usually, 40-50 dishes of cells were obtained from one liver. The criterion of a good cell preparation is the ability of hepatocytes to attach evenly onto the culture dish within 2 h of plating. After 4-5 h, the cells were washed once with a serum-free medium prior to experiments. The incubation conditions for various experiments are indicated in the figure legends. Viability of the cells incubated with various polar head groups or methyl group donors (choline, methionine, betaine, ethanolamine, N-monomethyl-ethanolamine, N,N-dimethyl ethanolamine, etc.) in the serum-free medium was identical, as examined by Trypan blue exclusion. There was 10-15% of cellular protein lost at the end of a 24 h incubation.
In some experiments, 100 \( \mu M \) choline, 200 \( \mu M \) methionine or 100 \( \mu M \) choline + 200 \( \mu M \) methionine were added to the plating medium from 50 mM choline or 100 mM methionine stocks prepared in water and sterilized by filtration (0.2 \( \mu m \)). The stocks (50 mM) of betaine, homocysteine, ethanolamine, \( N \)-monomethylethanolamine and \( N,N \)-dimethylethanolamine were all prepared in water and stored at -20 \( ^\circ C \) before use. The purities of these chemicals were checked by TLC as described later (see Normal TLC and HPTLC).

6. Preparation of Lipoproteins from Rat Plasma

Rats (300-350 g) were fasted overnight and anesthetized lightly by diethyl ether. Blood was withdrawn by cardiac puncture using a 20-cc syringe with a 18G needle. The samples were immediately brought to 1 mg/ml EDTA, and centrifuged at 500xg for 20 min. The plasma was subjected to sequential flotation [295] to obtain VLDL (d<1.006 g/ml) and HDL (1.06<d<1.21 g/ml). The VLDL and HDL samples were re-centrifuged in NaBr solutions of density 1.006 g/ml and 1.21 g/ml, respectively, and the purified samples were used for raising antibodies or as apolipoprotein standards on SDS-PAGE.

In experiments when small amounts of plasma (0.3 to 0.5 ml) were obtained from young rats (50 g), the plasma lipoproteins were centrifuged in a salt gradient by single-spin centrifugation [296]. The plasma samples were diluted to 1 ml with NaCl solution (d=1.006 g/ml) which contained 0.01% EDTA. The salt gradient was prepared in a Beckman Quick-Seal centrifuge tube (16x76 mm). The tube was first loaded with 0.5 g sucrose, then 5 ml 4M NaCl, 1 ml diluted plasma sample, and finally filled with 0.67 M NaCl containing 0.05% (w/v) EDTA. The gradient was centrifuged at 50,000 rpm in a Ti 70.1 rotor for 34.5 h at 10 \( ^\circ C \). Sixteen fractions (about 0.8 ml each) were collected from the bottom of the tube after centrifugation and densities of these fractions were measured gravimetrically. The fractions were pooled as follows: fraction 1, bottom fraction
(d>1.18 g/ml); fractions 2-9, HDL (1.06<d<1.18 g/ml); fractions 10-13, LDL (1.03<d<1.06 g/ml); fractions 14-16, VLDL (d<1.03 g/ml). Lipoproteins in each of the four fractions were concentrated by absorption onto 10 mg of fumed silica (Cab-O-Sil). The fumed silica was washed with 5 ml H$_2$O twice by centrifugation at 10,000xg for 15 min to remove salt and sucrose. Five ml chloroform/methanol (2:1, v/v) was mixed with the silica gel to extract lipids from the absorbed lipoproteins. The silica was pelleted by centrifugation, the organic solvent was transferred into a capped glass tube and mixed with 1.5 ml H$_2$O to separate two phases. The chloroform phase was washed with a theoretical upper phase (chloroform/methanol/H$_2$O, 3:48:47, v/v) twice, dried under nitrogen and stored in capped glass tubes at -20 °C before lipid analysis. Residual organic solvent in the Cab-O-Sil after delipidation was evaporated under vacuum, and the absorbed apoproteins were solubilized with 1 ml sample solution [containing 2% (w/v) SDS, 6 M urea, 0.05 M Tris-HCl, pH 9.0, 0.1 % (w/v) DTT, 0.05 % (w/v) glutathione, 0.1 % (w/v) EDTA and 0.13 % (w/v) ε-aminocaproic acid] at 95 °C for 15 min.

7. Fractionation of Secreted Lipoproteins from Culture Medium

Lipoproteins in the culture medium were fractionated in a salt gradient by single-spin centrifugation [297]. The gradient was prepared in a Beckman pollyallomer Quick-Seal centrifuge tube (25 x89 mm). Medium (16 ml) combined from 8 dishes was mixed with 3.76 g NaCl, and was then loaded onto 1.6 g sucrose at the bottom of the tube. The tube was then loaded with 3.2 ml 3% (w/v) NaBr solution (d=1.020 g/ml) which contained 0.5% (w/v) glutathione and 1.3 % (w/v) ε-aminocaproic acid and finally filled with 0.67 M NaCl containing 0.05% (w/v) EDTA. The gradient was centrifuged at 50,000 rpm in a Ti 70 rotor for 42 h at 10°C. After centrifugation, 2-ml fractions were collected from the bottom of tubes. The density cut-off points for the medium lipoproteins were chosen the same as those for separating plasma lipoproteins. VLDL (d<1.02 g/ml), LDL (1.02<d<1.06 g/ml),
HDL (1.06<d<1.18 g/ml) and BF (d>1.18 g/ml), were pooled and concentrated by absorption onto fumed silica (Cab-O-Sil). Lipids were extracted from lipoproteins absorbed onto the Cab-O-Sil with chloroform/methanol (2:1, v/v), and apoproteins were solubilized with 1 ml 2% (w/v) SDS/6 M urea solution as described in the previous section (6. Preparation of Lipoproteins from Rat Plasma).

The medium lipoproteins were also fractionated by sequential flotation for comparison.

8. Preparation of Rabbit Antiserum Against Rat VLDL Apolipoproteins

VLDL was delipidated with methanol/diethylether, and resolubilized in a buffer containing 10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 1 mM NaN₃, 1 mM EDTA and 2 M guanidine hydrochloride, as described by Osborne [298]. The delipidated VLDL was dialysed against PBS over night and stored at 4°C until use. The purity of the VLDL sample was examined by SDS-PAGE which showed the sample contained two forms of apoB (B_H and B_L), apoE and apoC. The VLDL apoprotein (100 µg of protein) was injected subcutaneously at 5 to 6 sites on the back of the neck of a New Zealand white rabbit, initially with complete Freund's adjuvant followed 5 to 6 weeks later by antigen in incomplete Freund's adjuvant [299]. Two weeks after the boost, blood was drawn from the ear vein, incubated at 37 °C for 2 h and the serum was isolated by centrifugation at 50 x g for 20 min. Antisera were stored at -20°C in 0.5-ml aliquots containing 0.1% (w/v) NaN₃. Specificity of the antisem was evaluated by immunoblotting using alkaline phosphatase-conjugated goat-anti-rabbit IgG (described later). The antiserum was specific for apoB_H, apoB_L, apoE and apoC, but did not react with apoA-I.

9. Preparation of Microsomes and Cytosol from Rat Liver and Hepatocytes

The livers of anesthetized rats were perfused (through the portal vein) with a Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution as described previously,
and a 25% (w/v) liver homogenate was prepared in ice-cold homogenization buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM DTT, 1 mM PMSF and 0.25% NaN$_3$ by 5 strokes in a Potter-Elvehjem homogenizer. The cytosol was isolated by sequential centrifugation at 10,000×g (in a JA-20 rotor) for 10 min and at 125,000×g (in a Ti 70 rotor) for 60 min, and clear fat-free cytosol was collected. Microsomes were resuspended in a buffer containing 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose and 0.1 mM PMSF by 10 strokes in a small Potter-Elvehjem homogenizer.

Cultured hepatocytes were washed once with ice-cold PBS, and 4 dishes of the cells were scraped into 2 ml of the homogenization buffer. The cells were homogenized in a Potter-Elvehjem homogenizer by 40 strokes at 0 °C. Cytosol and microsomes were isolated by sequential centrifugation as described above except that a Ti 70.1 rotor was used for the centrifugation at 125,000×g. Microsomes were resuspended in 0.5 ml of 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose and 0.1 mM PMSF. In some experiments, cytosol and whole membranes (instead of microsomal membranes) were isolated by a single centrifugation at 125,000×g.

For the measurement of microsomal PE $N$-methyltransferase activity, 4 dishes of hepatocytes were homogenized in 2 ml of 20 mM potassium phosphate, (pH 7.9), 1 mM EDTA, 1 mM DTT and 250 mM sucrose as described above. After centrifugation at 125,000×g, the membranes were resuspended in 0.5 ml of the homogenization buffer.

10. Preparation of Endoplasmic Reticulum and Golgi Apparatus from Rat Liver

The livers of anesthetized rats were perfused with Hanks' balanced salt solution. In the [³H]leucine-labeling experiment, the livers were injected with 500 μCi [³H]leucine through the portal vein and labeled for 15-30 min. The livers were immediately excised and homogenized in 4 volumes of 37.5 mM Tris-HCl, pH6.5, 0.5 M sucrose, 5 mM MgCl$_2$, 1% (w/v) dextran, 0.1 mM PMSF, 100 μM
leupeptin and 1 μg/ml trypsin-inhibitor using a Polytron homogenizer for 30 seconds set at 2.5. The Golgi apparatus and ER were isolated from the livers essentially according to Croze and Morré [300], except that leupeptin and trypsin-inhibitor were added to all the solutions (except the sucrose solutions) during the purification procedure. There were two fractions of ER: ER I was located at the interface of 1.5M-2.0M sucrose, and ER II at the interface of 1.3M-1.5M sucrose after centrifugation. Proteins, 400, 100 and 300 μg from liver homogenates, Golgi and ER, respectively, were separated by SDS-PAGE and subjected to immunoblotting.

11. SDS-Polyacrylamide Gel Electrophoresis and Fluorography

Liver proteins and apolipoproteins were separated by SDS-PAGE on slab gels with a linear 3-15% or 3-20% acrylamide gradient as a separating gel and 3% acrylamide as a stacking gel in the presence of 0.1% SDS. When apolipoproteins were separated, the rat plasma lipoproteins prepared by sequential flotation [295] were used as a source of protein standards. Gels were stained with 0.12% (w/v) Coomassie Blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid, and destained with 7.5% (v/v) acetic acid and 5% (v/v) methanol to visualize proteins. After soaking with 10% (v/v) glycerol for 2-3 h, the gels were dried between two pieces of cellophane membrane backing using a Bio-Rad slab dryer. The relative intensities of stained protein bands were measured densitometrically using a CAMAG thin-layer chromatography Scanner at 550 nm and the area under each peak was integrated using a CAMAG SP4290 integrator.

For fluorography, the destained gel was first impregnated with EN^HANCE for 1 h, and then soaked with cold 10% (v/v) glycerol solution (4 °C) for 2-3 h. The gel was dried on a piece of filter paper, and subjected to fluorography at -70°C using a Kodak-XAR-5 x-ray film.
12. Immunoblotting of Apolipoproteins

Apolipoprotein samples were first separated by SDS-PAGE on a 3-15% gradient gel, and then electrophoretically transferred onto a nitrocellulose membrane in a buffer containing 25 mM Tris, 192 mM glycine, pH 8.3 and 20% (v/v) methanol [301]. The transfer was run at 64V/0.20A for 14 h at room temperature, which was stabilized using a cooling coil. The following procedures were all performed at 37°C. The nitrocellulose membrane was incubated with rabbit anti-apoVLDL antiserum in solution A (1:500 dilution) which contained 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.25% (w/v) gelatin and 0.05% (v/v) Nonidet P40. After 5 h incubation, the membrane was washed with solution A for 2 h, and then incubated with 10 μCi $^{125}$I-labeled Protein A (a second probe) in the same solution for another 2 h. Finally, the membrane was washed with a solution containing 1 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.25% (w/v) gelatin and 0.4% (w/v) N-lauroylsarcosine for 1 h. After being dried at 70°C for 15 min, the membrane was subjected to autoradiography at -70°C using a Kodak-XAR-5 x-ray film.

For the determination of antiserum specificity, immunoblotting was performed using goat-anti-rabbit IgG alkaline phosphatase conjugate as a second probe. The following procedures were performed at room temperature. After transfer, the nitrocellulose membrane was incubated with 100 ml solution T (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 3% (w/v) gelatin for 2 h. The membrane was rinsed with 150 ml solution T containing 0.05% (v/v) Tween 20 twice and incubated with rabbit anti-apoVLDL antiserum in 100 ml of the same solution (1:500 dilution) for at least 5 h. The membrane was rinsed again as described before and then incubated with the second probe (Bio-Rad's goat anti-rabbit IgG alkaline phosphatase conjugate) in 100 ml of the same solution (1:3000 dilution) for 2 h. After being washed with Tween-containing solution T twice and
with the same solution but without Tween one time, the membrane was incubated with 100 ml of the color development solution containing 30 mg nitro blue tetrazolium (in 1 ml 70% DMF), 15 mg 5-bromo-4-chloro-3-indolyl phosphate (in 1 ml DMF), 0.1 M NaHCO₃ and 1 mM MgCl₂, pH 9.8. The color development was stopped by rinsing the membrane with a large amount of water.

13. Radioimmunoprecipitation of [³H]Apolipoproteins

The metabolism of VLDL apoproteins was examined in hepatocytes labeled with [³H]leucine (100 μCi/dish). One dish of cells was scraped in 0.5 ml hot buffer R [1mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) deoxycholic acid, 1 mM DTT, 0.15 M NaCl, 0.015% (w/v) PMSF and 0.05 M Tris-HCl, pH 8.0] containing 1% (w/v) SDS. The sample was boiled for 2-3 min, and added to 4.5 ml SDS-free buffer R to reduce the concentration of SDS to 0.1%. The culture medium from two dishes was combined and added to 0.4 ml of 10 x-concentrated buffer R (containing 1% SDS) to give a final concentration of SDS of 0.1% (w/v). One ml of the prepared cell samples or the total volume of the prepared medium samples were incubated with sufficient rabbit anti-apoVLDL antiserum (30 μl in this work) to precipitate quantitatively the apolipoproteins. After 24 h incubation at room temperature, 40 μl of protein A-Sepharose CL-4B (a 1:1 suspension in buffer R containing 0.1% SDS) was added for 2 h to bind the immune-complexes. The affinity beads were washed six times by centrifugation with buffer R containing 0.1% (w/v) SDS, and the washed beads were boiled in 150 μl of sample buffer containing 8 M urea, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 10 mM Tris/glycine, pH 8.3 [274]. The eluted protein samples were separated by SDS-PAGE as described above, except that the rat plasma VLDL apolipoproteins were used as carriers. After being visualized with Coomassie Blue R-250, the apoprotein bands were cut from the gel, and the gel slices were incubated with 1 ml of Soluene-350 at 50°C over night. The
amount of radioactivity was determined after incubating the samples with 10 ml of Hionic-Fluor scintillation cocktail for 3 days.

14. Immunoblotting of Phosphatidylethanolamine N-Methyltransferase

PE N-methyltransferase was purified from rat liver microsomes [66]. Rabbit antiserum against the purified enzyme was kindly provided and the immunoblotting was skillfully performed by Mr. Neale Ridgway, a graduate student in this laboratory. The rabbit polyclonal antibody was specific to PE N-methyltransferase (18.3 Kd) from crude liver microsomal preparations, and the microsomal protein comigrated with an authentic PE N-methyltransferase standard on SDS-PAGE.

15. Digitonin Permeabilization of Hepatocytes

Digitonin-mediated release of cytosolic enzymes from hepatocytes was performed essentially as described by Mackall et al. [302]. After removal of culture medium, hepatocytes were washed carefully with 2.5 ml ice-cold PBS and 0.8 or 1.0 ml of ice-cold digitonin solution (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 dish to initiate enzyme release. The dishes were placed on an ice-cold glass plate for up to 8 min and were occasionally gently swirled during the incubation. The digitonin solution was collected at the indicated times and cell debris was removed by centrifugation (in a microcentrifuge) at 14,000 rpm for 2 min. Aliquots of the samples were used for enzyme activity assay (60 μl for cytidylyltransferase and 50 μl for lactate dehydrogenase).

16. Lipid Extraction

For quantitative analysis, lipids were extracted from one dish of cells or from cultured medium combined from three dishes with chloroform/methanol (1:1; v/v) essentially according to Bligh and Dyer [303]. Before extraction, the cells were washed once with 2 ml of cold PBS and the medium was centrifuged at
10,000 rpm (JA 20 rotor) for 10 min to remove cell debris. One dish of the cells was harvested in 2 ml \( \text{H}_2\text{O} \) and sonicated for 10 min. An aliquot of the sample was saved for protein determination and the remainder was mixed with 0.7 ml \( \text{H}_2\text{O} \), 6 ml chloroform/methanol (1:1; v/v) and 0.06 ml acetic acid and vortexed vigorously for 1 min. The samples were centrifuged to separate two phases and the chloroform phase was washed with 3 ml of the theoretical upper phase two times. The chloroform was evaporated under nitrogen and the lipid samples were stored in a capped tubes at -20 °C.

In radioisotope labeling experiments, cells were rapidly harvested (after washing) in 1 ml methanol and mixed with 1 ml chloroform and 1 ml \( \text{H}_2\text{O} \). After centrifugation, an aliquot of the upper phase was subjected to TLC, and the chloroform phase was washed with the theoretical upper phase as described. Medium sample (6 ml) was mixed with 12 ml chloroform/methanol (1:1; v/v) and 0.12 ml acetic acid, and the chloroform phase was washed with 6 ml of theoretical upper phase. In radioisotope labeling experiments, lipids were extracted from one dish of the culture medium.

17. Normal Thin-Layer Chromatography and High Performance Thin-Layer Chromatography

For quantitative analysis of phospholipids and TG, the separation of lipids was performed on normal Silica Gel 60 TLC plates (20×20 cm). Phospholipids (lysoPC, SPM, PC, PI, PS and PE) were separated by developing the plates in chloroform/methanol/acetic acid/formic acid/\( \text{H}_2\text{O} \) (70:30:12.4:2; v/v). For the separation of PMME and PDME, two-dimensional TLC was performed. The plates were developed in chloroform/methanol/28% (w/v) \( \text{NH}_4\text{OH} \) (65:35:5; v/v) in the first direction and butanol/acetic acid/\( \text{H}_2\text{O} \) (60:20:20; v/v) in the second direction. Neutral lipids (free cholesterol, free fatty acids, TG and CE) were separated by developing the plates in hexane/di-iso-propyl ether/acetic acid (65:35:2; v/v). For the separation of 1,2-DG and 1,3-DG, the plates were developed in diethyl
ether/benzene/ethanol/acetic acid (40:50:2:0.2; v/v) [304]. All lipid spots were visualized with iodine vapor.

For qualitative analysis of lipids from secreted lipoproteins, both phospholipids and neutral lipids were separated on a HPTLC plate (10x20 cm) in one dimension [305]. The HPTLC plates were prewashed by developing the plates in chloroform/methanol/H$_2$O (60:35:8; v/v) and activated at 100 °C for 15 min. Phospholipids were separated by developing the plates halfway in chloroform/methanol/acetic acid/formic acid/H$_2$O (70:30:12:4:2; v/v). The plate was removed from the tank, dried and the neutral lipids were separated by development in hexane/di-iso-propyl ether/acetic acid (65:35:2; v/v) to the top of the plate. Lipid bands were visualized with cupric acetate (3%, w/v) in 8% (v/v) phosphoric acid [305].

In $[^3]$Holeate- or $[^{14}]$Acetate-labeling experiments, the radiolabeled phospholipids and neutral lipids were separated on normal TLC plates and the lipid bands were scraped into scintillation vials. Each sample was mixed with 0.5 ml H$_2$O and 10 ml of aqueous counting scintillant, and the radioactivities were determined after 2 days.

Choline and choline derivatives (betaine, phosphocholine and CDP-choline) were separated on normal silica Gel 60 TLC plates (20x20 cm) developed in methanol/0.6% (w/v) NaCl/28% (w/v) NH$_4$OH (10:10:1; v/v) [290]. In cytidylyltransferase assay, the separation of CDP-choline from phosphocholine was performed on plastic silica Gel 60 TLC plates (10x20 cm) developed in the same solvent.

18. Quantitative Analysis

18.1. Protein Assay

Protein was determined according to Lowry et al. [306] in the presence of 0.04% (w/v) DOC. BSA was used as a standard.
18.2. Phosphorus Assay

Phospholipid was digested with perchloric acid (70%) at 180 °C for 1 h, then the phosphorus content was quantitated in the presence of ammonium molybdate and ascorbic acid [307].

18.3 Triacylglycerol Assay

TG was treated with alkaline hydroxylamine and the content of fatty acyl groups was determined in the presence of ferric perchlorate [308]. Trilinolein was used as a standard.

18.4. Analysis of Fatty Acids and 1,2-Diacylglycerol in Liver Homogenate

Fatty acids were separated from the lipid extract of liver homogenate by TLC using a known amount of heptadecanoic acid as an internal standard. Fatty acid samples together with the internal standards were eluted with chloroform and subjected to gas chromatography as described below.

1,2-DG was separated by TLC and eluted from the silica gel with chloroform. A known amount of heptadecanoic acid was added to the sample as an internal standard.

Chloroform in fatty acid and 1,2-DG samples was evaporated under nitrogen. Methyl esters were prepared by boiling the lipid sample with 200 μl BF₃-methanol (Sigma) at 100 °C for 5-10 min. Reaction was stopped by adding 1 ml H₂O, and the methyl esters were extracted with 2 ml hexane. Aliquots (2-3 μl) of the methyl esters were injected onto a 6'x1/8" stainless steel column packed with 5% diethylene glycol succinate on 100-120 mesh Supelcoport (Supelco Inc., Bellefonte, PA, USA) in a Perkin-Elmer 8420 gas chromatograph. Temperature was programmed from 145 °C to 195 °C and helium was used as a carrier gas at a pressure of 170 kPa. The amount of each fatty acid methyl ester was determined by comparison of the area of each peak to that of the known amount of internal standard.
19. Enzyme Assays

19.1. CTP:Phosphocholine Cytidylyltransferase Assay

Cytidylyltransferase activity was determined by the method of Weinhold et al. [57], except that phospho[methyl-³H]choline (1.5 mM; 7.2 μCi/μmole) was used as a substrate. Each reaction contained 50-75 μg of proteins, and the production of CDP-choline was linear for 15 min. CDP-choline was separated from phospho-choline by TLC [290] as described above.

19.2. Phosphatidylethanolamine N-Methyltransferase Assay

Microsomal PE N-methyltransferase activity was determined according to Ridgway and Vance [66] using either endogenous PE or exogenous PMME as a substrate.

19.3. Lactate Dehydrogenase Assay

Lactate dehydrogenase activity was determined by the method of Bergmeyer and Bernt [309]. The rate of formation of NAD⁺ was monitored at 340 nm in a Shimadzu spectrophotometer.

19.4. NADPH:Cytochrome C Reductase Assay

NADPH:cytochrome C reductase was determined [310] using 25 μg protein from homogenate or microsomes or 75 μg protein from Golgi apparatus. The reaction was performed in a Shimadzu spectrophotometer at room temperature for 3 min and the kinetic measurement of the production of reduced cytochrome C was monitored at 550 nm.

19.5. UDP-Galactose:N-Acetylglucosamine Galactosyltransferase Assay

Galactosyltransferase activity was determined according to Ernster et al. [311] using N-acetylglucosamine as an acceptor for galactose. The amounts of proteins used for the assay were Golgi, 30 μg; ER, 100 μg; and homogenate, 200 μg.
RESULTS

1. Reduction in VLDL, but not HDL, in Plasma of Rats Deficient in Choline

In order to understand the role of PC synthesis in hepatic lipoprotein secretion, we adopted the well-established choline-deficient rat as a model. In the present work, the acute hypolipidemia and accumulation of fat in the liver after a short period of choline deficiency were confirmed. In addition, an elaborate analysis of plasma lipoprotein compositions and the VLDL apolipoprotein levels in the livers in choline deficiency was performed.

1.1. Lipid Contents in the Livers and Plasma of Choline-Deficient and Choline-Supplemented Rats

As reported by others [8,142], feeding rats a choline-deficient (CD) diet for three days produced two characteristic responses in the liver: (1) accumulation of TG (CD, 23.9; CS, 3.7 µmole/g liver) and (2) decrease in the ratio of PC to PE (CD, 1.22; CS, 1.88 µmole/g liver) (Table VI). The body weights gained during the 3-day period were identical, and there was no significant changes in the liver weight in the two dietary groups. Choline deficiency-produced hypolipidemia was also observed. After 3 days of choline deficiency, plasma TG and PC levels in the CD rats were about 40% and 30%, respectively, of those in the choline-supplemented (CS) rats (Table VI).

The levels of other lipid components in the liver and plasma were qualitatively analysed by HPTLC. Separation of both phospholipids and neutral lipids by one dimensional TLC allows us to compare the overall lipid profiles on one plate. The chromatogram (Fig.5) clearly demonstrated hypolipidemia in rats deficient in choline, with significant decreases in TG and PC levels. A tremendous accumulation of TG in the liver was also observed. The chromatogram showed that phospholipid components in the livers were not altered dramatically by choline deficiency.
Table VI. Major Lipid Contents in Liver and Plasma of Choline-Deficient and Choline-Supplemented Rats

Plasma and liver samples were taken from rats fed with CD or CS diets for 3 days, and lipids were extracted from the samples with chloroform/methanol (2:1; v/v). The amounts of PC, PE and TG were quantitated as described under Experimental Procedures. The data are presented as mean ± standard deviation, n=4. The significance of difference was determined by t-test.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body weight</th>
<th>Liver weight</th>
<th>Liver lipids (μmole/g liver)</th>
<th>Plasma lipids (μmole/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>g</td>
<td>PC</td>
<td>PE</td>
</tr>
<tr>
<td>CD</td>
<td>51.8±4.6</td>
<td>2.4±0.4</td>
<td>11.4±2.1 9.4±0.8 23.9±6.0</td>
<td>1.2±0.2 0.18±0.03 0.17±0.04</td>
</tr>
<tr>
<td>CS</td>
<td>54.5±8.7</td>
<td>2.5±0.7</td>
<td>15.3±1.0 8.1±0.8 3.7±0.9</td>
<td>1.7±0.1 0.16±0.05 0.46±0.10</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td>n.s</td>
<td>p&lt;0.02 p&lt;0.1 p&lt;0.001</td>
<td>p&lt;0.01 n.s. p&lt;0.01</td>
</tr>
</tbody>
</table>

n.s. : not significant.

Fig.5. High performance thin-layer chromatography (HPTLC) of major lipid components of plasma and livers from choline-deficient and choline-supplemented rats. Lipids were extracted from plasma and livers of two CS (CS_1, CS_2) and two CD (CD_1, CD_2) rats. The TLC plate was loaded with lipid samples from 3.5 μl of plasma and 1.25 mg of liver and chromatographed in one dimension using two solvent systems. Abbreviations: FC, free cholesterol; CL, cardiolipin.
1.2. Agarose Gel Electrophoresis of Plasma Lipoproteins from Choline-Deficient and Choline-Supplemented Rats

Comparative analysis of plasma lipoprotein levels was conducted using agarose gel electrophoresis as described by Eklund and Sjoblom [312]. The VLDL and LDL standards isolated by sequential flotation migrated faster than the corresponding lipoprotein fraction from a whole plasma sample on the agarose gel (Fig. 6). The band that corresponded to VLDL in the CD rat plasma was remarkably smaller than that from CS rats, whereas the band that corresponded to HDL in CD rat plasma was not visibly reduced (Fig. 6). This is the first indication that choline deficiency caused reduction in VLDL, but not HDL, levels in plasma. Further evidence was sought by measurement of the lipid and apolipoprotein content of the fractionated plasma lipoproteins.

Fig. 6. Agarose gel electrophoresis of plasma lipoproteins from choline-deficient and choline-supplemented rats. Plasma samples were prepared from two CS (CS₁, CS₂) and two CD (CD₁, CD₂) rats, and 5 μl of the samples were resolved by agarose gel electrophoresis in phosphate buffer at pH 7.0. Lipoproteins (VLDL, LDL and HDL) prepared from CS rat plasma by sequential flotation were used as standards. The gel was stained with Sudan Black B. The experiment was repeated and an identical result was obtained.
1.3. Lipid Composition of Fractionated Plasma Lipoproteins from Choline-Deficient and Choline-Supplemented Rats

Lipids of the fractionated plasma lipoproteins were qualitatively analyzed by HPTLC. The chromatogram (Fig.7) shows that all lipid components of VLDL (CE, TG, cholesterol and PC) were proportionally decreased in choline deficiency, indicating the number of plasma VLDL particles was reduced. LDL lipid levels were also reduced in CD plasma, and most significantly was PC. There was no difference in lipid profiles of HDL from CD and CS plasma, suggesting that choline deficiency did not affect plasma HDL lipid levels. In addition, the chromatogram demonstrated that the observed reduction in PC and TG levels in the whole plasma of CD rats (Fig.5) was due largely to the decreased VLDL levels. Only trace amounts of lipids were extracted from the dense protein fractions (d>1.18 g/ml) in the plasma (Fig.7).

Fig.7. High performance thin-layer chromatography (HPTLC) of major lipid components of fractionated plasma lipoproteins from choline-deficient and choline-supplemented rats. Lipoproteins were fractionated into VLDL, LDL HDL and BF (d>1.18 g/ml) in a salt gradient by centrifugation, and lipids were extracted from each of the four fractions with chloroform/methanol (2:1, v/v). The TLC plate was loaded with lipid samples derived from 30 μl of plasma. FC, free cholesterol.
1.4. Apolipoprotein Profiles of Fractionated Plasma Lipoproteins from Choline-Deficient and Choline-Supplemented Rats

Apolipoproteins in the fractionated plasma lipoproteins were semi-quantitatively analysed on SDS-PAGE stained with Coomassie Blue (Fig.8). The relative intensities of the apolipoprotein bands determined densitometrically are summarized in Table VII. The most significant change caused by choline deficiency was in apoproteins of VLDL; the intensities of apo-B_H, -B_L and -E in VLDL were only 37.1%, 11.0% and 37.2%, respectively, of the normal levels. The proportional decrease in the VLDL apolipoproteins suggests that the number of plasma VLDL particles is decreased in choline deficiency. The contents of apo-B_L, -E and -A-IV in LDL from CD plasma were also reduced to 34%, 11.1% and 64.3%, respectively, of normal levels, but the apoB_H level was unchanged. ApoA-I is the major apolipoprotein component of rat HD, and accounts for 60% (by weight) of the total apolipoproteins [222,235]. The density of apoA-I in HDL from CD plasma was 13.3% higher than the normal level, although the densities of two minor apolipoprotein components of HDL, apoA-IV and apoE, were only 35.6% and 63.3% respectively of the control levels (Table VII). Similarly, the level of apoA-I was increased in LDL and in the d>1.18 g/ml fraction of CD plasma (13.3% and 131.7%, respectively, higher than normal). There was also more apoA-IV (7.6%), apoE (24.4%) and apoA-I (131%) unassociated with lipoproteins (d>1.18 g/ml) in CD plasma.

Taken together, these results confirm the previous observations that choline deficiency produces hypolipidemia. While there is a striking decrease in plasma VLDL/LDL levels (apoBs and TG) in choline deficiency, the levels of HDL and other plasma proteins (unassociated with lipoproteins) are not altered or even increased (i.e. apoA-I).
Table VII. Integrated Densities of Apolipoprotein Bands from Gel Electrophoresis of Plasma Lipoproteins

The experiment was performed as described under the legend of Fig. 8. The intensities of the stained protein bands were scanned at 550 nm and the area under each peak was integrated as described under Experimental Procedures. The integrated densities of the apolipoproteins from CD samples are given as % of the integrated densities of those apolipoproteins from CS samples.

<table>
<thead>
<tr>
<th>Apoproteins</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
<th>d&gt;1.18 g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoB&lt;sub&gt;H&lt;/sub&gt;</td>
<td>37.1</td>
<td>101.4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>apoB&lt;sub&gt;L&lt;/sub&gt;</td>
<td>11.0</td>
<td>34.0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>apoA-IV</td>
<td>n.d.</td>
<td>11.1</td>
<td>35.6</td>
<td>107.6</td>
</tr>
<tr>
<td>apoE</td>
<td>37.2</td>
<td>64.3</td>
<td>63.3</td>
<td>124.4</td>
</tr>
<tr>
<td>apoA-I</td>
<td>n.d.</td>
<td>119.0</td>
<td>113.3</td>
<td>231.7</td>
</tr>
</tbody>
</table>

n.d.: not determined.

Fig. 8. SDS-polyacrylamide gel electrophoresis of plasma lipoproteins from choline-deficient and choline-supplemented rats. Lipoproteins were fractionated by ultracentrifugation. A polyacrylamide gel (3 to 20% gradient) was loaded with VLDL, LDL, HDL and BF (d>1.18 g/ml), which were derived from 75, 75, 25 and 12.5 µl of plasma, respectively. The gel was stained with Coomassie Blue R. Lanes 1, 4 and 7, VLDL, LDL and HDL standards obtained by sequential flotation; Lanes 2 and 3, VLDL of CD and CS; Lanes 5 and 6, LDL of CD and CS; Lanes 8 and 9, HDL of CD and CS; Lanes 10 and 11, BF of CD and CS. Apoprotein standards are indicated on the left. Alb, albumin.
1.5. VLDL Apolipoprotein Levels in the Livers of Choline-Deficient and Choline-Supplemented Rats

The impaired secretion of lipoproteins (mainly VLDL) by the liver in choline deficiency has been suggested [154]. Thus, it was of interest to know whether or not there is an accumulation of VLDL apolipoproteins in the CD liver. In answering this question, we performed immunoblotting of the proteins in whole liver homogenates using antiserum against rat VLDL apolipoproteins. The levels of cellular apoB\(_L\), apoE and apoC were nearly identical in the livers of both normal and CD rats (Fig.9). The high molecular weight form of apoB, apoB\(_H\), was not detected in whole liver homogenates (0.4 mg protein was loaded on the gel).

![Fig.9. Immunoblotting of VLDL apolipoproteins in the livers from choline-deficient and choline-supplemented rats.](image-url)
Knowing that choline deficiency had no effect on cellular VLDL apoprotein levels, we isolated ER and the Golgi apparatus from the livers of CS and CD rats and examined whether or not there was an impairment of intracellular transit of VLDL between these two organelles. The recovery of ER and Golgi proteins in CD and CS livers were identical (Table VIII). There was about 10-fold enrichment of NADPH:cytochrome c reductase activity in the ER, and 43- and 62-fold enrichment of galactosyl transferase activity in CD and CS Golgi, respectively, from the total homogenates (Table VIII). There was also 3-fold enrichment of NADPH:cytochrome c reductase activity in the Golgi fractions and 3-fold enrichments of galactosyl transferase activity in the ER fractions. The cross-contamination between these two subcellular fractions was estimated by the two marker enzymes; 44% of the protein in the Golgi fraction was from the ER, and 7-10% of the ER proteins were contaminated by the Golgi.

**Table VIII. Analysis of Microsomal Subfractions from the Liver**

Preparations of isolated microsomal subfractions, protein analysis and enzyme assays were performed as described under Experimental Procedures. For the enzyme assays, two ER fractions (ER I and ER II) were pooled. The specific activity of NADPH:cytochrome c reductase is expressed as nmole NADP⁺ produced per min per mg microsomal protein. The specific activity of galactosyl transferase is expressed as nmole galactose transferred per hour per mg microsomal protein. The number in parentheses gives the numbers of independent experiments.

<table>
<thead>
<tr>
<th>Microsomal protein</th>
<th>NADPH:cytochrome c reductase</th>
<th>Galactosyl transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/g liver</td>
<td>nmol/min/mg</td>
</tr>
<tr>
<td>Homogenate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>3.80 ± 2.20 (4)</td>
<td>19.7 ± 13.0 (4)</td>
</tr>
<tr>
<td>CS</td>
<td>4.30 ± 2.30 (4)</td>
<td>12.7 ± 11.1 (4)</td>
</tr>
<tr>
<td>Golgi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>0.37 ± 0.10 (8)</td>
<td>14.5 ± 7.70 (4)</td>
</tr>
<tr>
<td>CS</td>
<td>0.37 ± 0.13 (8)</td>
<td>12.4 ± 4.95 (4)</td>
</tr>
<tr>
<td>ER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>3.98 ± 1.24 (9)</td>
<td>32.0 ± 16.9 (4)</td>
</tr>
<tr>
<td>CS</td>
<td>4.39 ± 1.07 (9)</td>
<td>31.0 ± 17.4 (4)</td>
</tr>
</tbody>
</table>
The immunoblot of microsomal proteins (Fig. 10) showed that all four VLDL apoprotein components (apoB\textsubscript{H} and B\textsubscript{L}, apoE and apoC) could be detected in the Golgi apparatus, while as noted for total liver homogenates, apoB\textsubscript{H} was not found in the two ER fractions (0.3 mg protein on the gel). The apoE and apoC levels were slightly increased in the two CD ER fractions and were reduced in the CD Golgi fractions compared to those from CS livers (Fig. 10). However, the distribution of the B apolipoproteins (especially apoB\textsubscript{L}) between the two organelles appeared normal. Possibly there is a slightly impaired transport of apoE and apoC from ER to Golgi in choline deficiency, but the apparent transit of apoB was not altered. However, care has to be taken in the interpretation of these data because of possible contamination of the Golgi fractions by multivesicular bodies (endosomes) [313].

Fig. 10. Immunoblotting of VLDL apolipoproteins in the Golgi apparatus and endoplasmic reticulum. Golgi and ER were isolated from a single liver of a CS rat (CS) and a CD rat (CD). Two fractions of ER (I, located at the interface of 1.5M-2.0M sucrose and II, located at the interface of 1.3M-1.5M sucrose) were obtained as described in reference 300. Proteins (100 μg for the Golgi and 300 μg for the ER) were separated by SDS-PAGE. Immunoblotting of VLDL apoproteins was performed as described in the legend for Figure 9. The experiments were repeated four times and similar results were obtained.
2. The Active Biosynthesis of Phosphatidylcholine is Required for Hepatic VLDL Secretion

The results described previously demonstrated that choline deficiency perturbed hepatic lipid metabolism and produced low levels of VLDL in blood of the experimental animals. In order to explore the possibility that the defect of hepatic PC synthesis and VLDL secretion in choline deficiency is the primary cause for the reduced plasma VLDL levels, we have exploited the use of monolayer culture of hepatocytes from a single liver of CD rats.

2.1. Accumulation of Triacylglycerol in Hepatocytes from Choline-Deficient Rats

Hepatocytes isolated from rats fed a CD diet for 3 days contained TG about 6-fold higher than that of hepatocytes from choline-supplemented rats (432.9 versus 74.5 nmol/mg protein). This result is consistent with our previous data that TG content in CD livers is 6.5 times higher than normal (Table VI). Thus, the increased TG levels observed in CD livers are mainly due to TG accumulation in hepatocytes rather than in fat storage cells in the liver. In addition, there appears to be no loss of TG due to cell rupture during isolation from CD rats. Lipid droplets were clearly observed under the phase contrast microscope in the CD hepatocytes after being plated onto culture dishes. However, there was no other morphological abnormality of these TG-rich hepatocytes. The cells adhered to the surface of the culture dishes after 2-3 h like cells obtained from rats fed a normal diet. The choline and/or methionine supplementation experiments were performed in serum-free medium, and viability of the different group of cells incubated in the serum-free medium was identical, as examined by Trypan blue exclusion. There was about 15% of cellular protein lost at the end of a 24 h incubation period regardless of the addition of choline or methionine.
2.2. Effect of Choline and Methionine on the Secretion of Phosphatidylcholine and Triacylglycerol from Hepatocytes

Hepatic secretion of PC and TG mass was determined in hepatocytes cultured in a medium ± choline or methionine. During a 24 h incubation period, there were 3- and 2.4-fold increase in the secretion of TG and PC, respectively, when the cells were supplemented with either choline or methionine (Fig.11). The elevated rate of TG secretion (3.6 nmol/h/mg cell protein) in the presence of choline or methionine was similar to the rate observed in hepatocytes isolated from rats fed the CD diet supplemented with 0.4% (w/w) choline (data not shown), and agreed with the TG secretion rate observed in hepatocytes isolated from rats fed with Purina chow (3.09 µg/h/mg cell protein) reported by Steinberg's group [314]. These data indicate that the rate of TG secretion by hepatocytes in choline deficiency was reduced by almost 67%. The stimulatory effect of choline and methionine on hepatic TG and PC secretion was almost identical.

The increased rates of TG and PC secretion in hepatocytes supplemented with choline or methionine was probably a consequence of stimulated lipid synthesis, as demonstrated by the incorporation of [4,5-\(^{3}\)H]oleate into these secreted lipids (Fig.12). The cells were first cultured in the presence of choline or methionine for 18 h then incubated with [4,5-\(^{3}\)H]oleate. Similar to the secretion of mass, the secretion of \(^{[3}\)H]TG and \(^{[3}\)H]PC from the cells was elevated about 3.5-fold or 3.0-fold in an 8 h period in the presence of choline or methionine, respectively.

Taken together, these data demonstrate that hepatic secretion of TG and PC is decreased in choline deficiency and the impaired secretions can be rapidly corrected by the supplementation with either choline or methionine.
Fig. 11. Effect of choline or methionine on the secretion of triacylglycerol and phosphatidylcholine from hepatocytes. Hepatocytes from CD rats were plated in a choline- and methionine-free medium containing 17% delipidated serum. After 7-8 h, the medium was replaced with serum-free medium supplemented with one of: no addition (X), 100 µM choline (■), or 200 µM methionine (▲). At the indicated times, lipids were extracted from culture medium and separated by TLC, and TG and PC were quantitated. Each point is the average of two dishes, which do not differ by more than 10%. The experiment was repeated three times. The fold-difference for TG secretion was 2.8±0.3.

Fig. 12. Effect of choline or methionine on the secretion of [3H]triacylglycerol and [3H]phosphatidylcholine from hepatocytes. Hepatocytes from CD rats were plated in serum-containing media in the absence (X) or presence of choline (100 µM, ■) or methionine (200 µM, ▲). After an overnight incubation, the attached cells were incubated with [9,10-3H]oleate (4 µCi/dish) in a serum-free medium. At the indicated times, lipids were extracted from the culture medium and separated by TLC, and the radioactivity in TG, PC and PE was determined. Each point represents the average of two individual dishes which differ by less than 5%.
2.3. Effect of Choline and Methionine on the Secretion of Lipoproteins from Hepatocytes

Whether or not the increased secretion of TG and PC was due to stimulated lipoprotein secretion was determined by labeling the secreted proteins from hepatocytes with $[^3\text{H}]$leucine. The culture medium was separated into four fractions with densities equivalent to those of plasma VLDL, LDL, HDL and dense protein (BF, d>1.18 g/ml) in a salt gradient by single-spin centrifugation [297]. Lipids in each of the four fractions were analysed by HPTLC, while apolipoproteins were examined by SDS-PAGE followed by fluorography. Chromatography of lipids in lipoprotein fractions showed that the amount of VLDL and LDL lipids secreted (mainly TG and PC) from CD hepatocytes were reduced, while the secretion of HDL lipids (mainly PC) was not altered (Fig. 13). Supplementation with either choline or methionine stimulated the secretion of VLDL lipids, but not HDL lipids, from the hepatocytes. Compared with the amounts secreted when either choline or methionine were added, the secretion of VLDL lipids could not be further stimulated when both choline and methionine were added (Fig.13).

Table IX summarizes the secretion of $[^3\text{H}]$leucine-labeled apolipoproteins from hepatocytes. The radioactivity in VLDL secreted from CD cells was reduced by more than 50% compared to that from choline- and/or methionine-supplemented cells. In contrast, the tritium in LDL, HDL and BF secreted from CD cells was not altered. The radioactivity observed in the LDL fraction at the end of 12 h labeling was small, suggesting that minimal LDL was directly secreted from hepatocytes. Fluorography of $[^3\text{H}]$leucine-labeled apolipoproteins demonstrated that the secretion of the three major components of VLDL (apoB$_H$, apoB$_L$ and apoE) from the CD cells was reduced (Fig.14). However, the secretion of labeled-apolipoproteins in HDL (mainly apoE and apoA-I) and BF (such as albumin) from CD cells was not changed (Fig.14). The VLDL proteins were recovered from SDS-
Fig. 13. Lipids of fractionated lipoproteins secreted by hepatocytes supplemented with choline or methionine. Hepatocytes were plated in medium containing 17% delipidated serum and one of: no addition (-), 100 μM choline, (C), 200 μM methionine (M), or 100 μM choline + 200 μM methionine (C/M). After 7-8 h plating, the medium was replaced with serum-free medium and the cells were further cultured for 12 h. Medium combined from 8 dishes was fractionated into VLDL, LDL, HDL and BF (d>1.18 g/ml) in a salt gradient by ultracentrifugation, and lipoproteins in each of the four fractions were concentrated by absorption onto Cab-O-Sil. Lipids were extracted from Cab-O-Sil and aliquots of samples (VLDL lipid was derived from 1.6 ml of medium, and LDL, HDL and BF lipids were derived from 3.2 ml of medium) were analyzed by HPTLC. TG in VLDL lipids was overloaded onto the TLC plate in order to show phospholipids. The lipid standards are indicated on the right. FC, free cholesterol.
PAGE gels and the radioactivity was determined. The radioactivities in VLDL apoproteins from CD cells were about 24% of those from the cells supplemented with choline and/or methionine (data not shown). However, a small increase in the amount of radioactivity in apoE was consistently observed in HDL and BF from CD cells. The SDS-PAGE of secreted lipoproteins stained with Coomassie blue (data not shown) revealed a pattern similar to that shown by fluorography in Fig. 14, indicating the secretion of VLDL apoprotein mass from CD cells was also reduced, whereas the mass of HDL and BF proteins was not altered. There were no significant changes in the total uptake or the incorporation of \([^3\text{H}]\text{leucine}\) into cellular TCA-precipitable materials on the addition of choline or methionine (data not shown). Similar results were also observed when the medium lipoproteins were fractionated by a sequential flotation procedure [15].

**Table IX. Secretion of L-[4,5-\(^3\text{H}\)]\text{Leucine-Labeled Lipoproteins by Hepatocytes Supplemented with Choline and/or Methionine**

The experiment was performed as described in the legend to Figure 13, except that the cells were incubated with \([^3\text{H}]\text{leucine} (15 \mu\text{Ci/dish})\) in a serum-free medium for 12 h. Lipoproteins in the fractionated medium were concentrated by absorption onto Cab-O-Sil and apoproteins were solubilized from Cab-O-Sil with SDS/urea solution. Aliquots of the total solubilized apoproteins in each fraction were counted in aqueous counting scintillant. Data represent the mean of two measurements which do not differ by more than 10%. The experiment was repeated three times and similar results were obtained.

<table>
<thead>
<tr>
<th>Additions</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
<th>d&gt;1.18 g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>62</td>
<td>21</td>
<td>375</td>
<td>993</td>
</tr>
<tr>
<td>2. Choline (100 (\mu\text{M})</td>
<td>155</td>
<td>19</td>
<td>342</td>
<td>1003</td>
</tr>
<tr>
<td>3. Methionine (200 (\mu\text{M})</td>
<td>160</td>
<td>23</td>
<td>424</td>
<td>961</td>
</tr>
<tr>
<td>4. 2 + 3</td>
<td>153</td>
<td>21</td>
<td>336</td>
<td>970</td>
</tr>
</tbody>
</table>
Fig. 14. Fluorography of $[^3H]$leucine-labeled apolipoproteins secreted by hepatocytes supplemented with choline or methionine. The experiment was performed as described in the legend of Table IX. The protein samples derived from equal volumes of culture medium were separated by SDS-PAGE. After electrophoresis, the gel was dried and subjected to fluorography using EN$^3$HANCE. In this experiment, the x-ray film was exposed for 36 h for VLDL and HDL and 12 h for BF. There were no visible bands in LDL samples after 36 h exposure. Alb, albumin.
2.4. Effect of Choline and Methionine on Lipogenesis in Hepatocytes

Since the secretion of TG and PC from choline-deficient hepatocytes was stimulated within 4-5 h after the addition of choline or methionine (Fig.11), we examined the cellular TG, PC and PE levels before and after supplementation. When hepatocytes from CD rats were maintained in a choline- and methionine-free medium, the levels of PC and PE were 75±5 and 65±5 nmol/mg cell protein, respectively. Choline stimulated PC synthesis in these cells. The cellular PC level increased rapidly within 4-5 h of supplementation and remained at about 110 nmol/mg cell protein (Fig.15), which was similar to the PC concentration in hepatocytes isolated from normal rats. However, the addition of choline to the CD cells had little effect on the cellular PE level (Fig.15). Methionine also stimulated PC synthesis in CD cells and a normal PC level was observed after about 12 h (Fig.15). A concurrent decrease in the cellular PE level upon the addition of methionine (Fig.15) implied that the cells compensated for the choline deficiency by methylation of PE. TG levels in these cells remained high (from 400 to 550 nmol/mg cell protein) during this 24-h period; only a slight reduction in cellular TG concentration was observed in the cells supplemented with choline or methionine (Fig.15).

Stimulation of PC synthesis by the addition of choline or methionine was further demonstrated in [³H]oleate-labeling experiments (Fig.16). Labeling of PC with [³H]oleate was elevated by 3.1-fold in the presence of choline and 1.6-fold in the presence of methionine during the first 2 h incubation (Fig.16). Of the total tritium incorporated into phospholipids (mainly PC and PE), the proportion of radioactivity incorporated into PE in the first 2 h was 11% in the CS cells, 15% in the methionine-supplemented cells and 33% in the deficient cells (Fig.16). The radioactivity incorporated into neutral lipids was almost exclusively associated with TG. Surprisingly, there was still active TG synthesis in the deficient
hepatocytes, in spite of a grossly elevated TG level in these cells. The rates of \[^3\text{H}\]oleate incorporation into TG in the first 2 h were in the following order: deficient cells > methionine-supplemented cells > CS cells (Fig.16).

Fig.15. Effect of choline or methionine on the concentrations of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol in hepatocytes. The experiment was performed as described in the legend to Figure 11. Lipids were extracted from cells and separated by TLC, and PC, PE and TG were quantitated. Each point is the average of two dishes, which do not differ by more than 10%. \(\times\), no addition; ■, + choline (100 µM); ▲, + methionine (200 µM).
2.5. Effect of Choline and Methionine on the Synthesis of VLDL Apolipoproteins in Hepatocytes

To examine the effect of choline deficiency on VLDL apolipoprotein synthesis, we performed pulse-labeling studies with \[^3H\]leucine in cultured hepatocytes and immunoprecipitated cellular VLDL apolipoproteins using antiserum against apoVLDL. The rates of \[^3H\]leucine incorporation into cellular apoB\(_H\), -B\(_L\), -E and -C were independent of the addition of choline or methionine (Fig.17). In pulse-chase studies, the rates of disappearance of radioactivity from the \[^3H\]leucine-labeled proteins were not affected by choline deficiency (Fig.18). Hence, the metabolic rate of the cellular apolipoprotein precursors of VLDL appears not to be affected by reduced PC synthesis.
Fig. 17. Time course of $[^3$H]leucine incorporation into cellular apoB$_H$, apoB$_L$, apoE and apoC of hepatocytes. Five h after plating, hepatocytes were incubated with $[4,5-^3$H]leucine (100 µCi/dish) in serum-free medium in the absence (X) or presence of choline (100 µM, ■) or methionine (200 µM, △). At the indicated times, cells were harvested and lysed by boiling in SDS-containing buffer. Rabbit anti-VLDL apoproteins antiserum was added in an amount that precipitated quantitatively apoB, apoE and apoC in each sample. After a 24 h incubation, the immune-complexes were bound to protein A-Sepharose. Apolipoproteins were separated by SDS-PAGE and the amount of radioactivity in the gel containing apolipoprotein was quantitated. Each point represents the mean of two samples. The experiment was repeated and a similar result was observed.
Fig. 18. Disappearance of radioactivity from $[^3H]$leucine-labeled cellular apoB$_H$, apoB$_L$, apoE and apoC. Attached hepatocytes were pulsed with $[4,5-^3H]$leucine (100 µCi/dish) in the absence (●) or presence of choline (100 µM, ■) or methionine (200 µM, ▲) for 2 h, then chased for 4 h. At the times indicated, the cellular apolipoproteins were immunoprecipitated and radioactivity was determined as described in the legend to Figure 17. Each point is the average of two samples. The experiment was repeated and a similar result was obtained.
3. Synthesis of Phosphatidylcholine by Reacylation of Lysophosphatidylcholine Corrects the Impaired VLDL Secretion from Choline-Deficient Hepatocytes

We have shown in the previous investigations that impaired VLDL secretion from CD hepatocytes can be corrected by supplementation of either choline or methionine into the culture medium, suggesting that active PC synthesis via either the CDP-choline pathway or PE methylation is sufficient for normal VLDL secretion. In this work, supplementation of lysoPC to the cells was conducted to examine whether reacylation of lysoPC could satisfy the requirement of PC synthesis for VLDL secretion.

3.1. Effect of Lysophosphatidylcholine on Lipogenesis in Hepatocytes

An active synthesis of PC was observed in the hepatocytes after the addition of lysoPC to the culture medium. The cellular PC level was rapidly increased by 25 nmol per mg cell protein within 4 h of lysoPC addition, and the increased PC concentrations were maintained for up to 24 h (Fig. 19). This stimulatory effect on cellular PC levels was identical to that of choline, indicating the cells could effectively utilize the choline moiety from the exogenous lysoPC.

In order to examine whether or not the exogenous lysoPC was taken up in an intact form by the cells, \([^{3}H]\)lysoPC was used as a tracer. During a 24 h incubation period, less than 10% of the total tritium incorporated into the cells was found in water-soluble materials, indicating the degradation of lysoPC, either in the cells or in the culture medium, was insignificant. Separation of cellular chloroform-soluble materials by TLC showed that 90% of the total tritium was associated with PC, while 4% was associated with lysoPC and 4% with SPM. These results indicate the hepatocytes rapidly reacylate and do not accumulate the lysoPC transported into the cells. The incorporation of tritium into PC was almost linear during the first 12 h incubation with \([^{3}H]\)lysoPC (Fig. 19; inset of A).
Addition of lysoPC or choline had no significant effect on cellular PE levels (Fig.19; B).

Fig.19. Effect of lysophosphatidylcholine on the concentration of phosphatidylcholine and phosphatidylethanolamine in hepatocytes. Hepatocytes obtained from a CD rat were plated (3 x 10^6 cells/dish) in a choline- and methionine-free medium + 17% delipidated serum. After 4-5 h incubation, the attached cells were washed with serum-free medium and cultured in the medium in the absence (◇) or presence of 100 μM lysoPC (▲) or 100 μM choline (■). At the indicated times, the cells were harvested, and cellular lipids were extracted with chloroform/methanol (2:1, v/v). PC (A) and PE (B) were separated by TLC and quantitated. Each point is the average of two dishes with less than 10% difference. Inset: the incorporation of [3H]lysoPC (250 dpm/nmol; 100 μM) into cellular PC.
3.2. Effect of Lysophosphatidylcholine on the Secretion of Triacylglycerol and Phosphatidylcholine from Hepatocytes

The mass secretion of TG and PC from the hepatocytes supplemented with choline or lysoPC was quantitated to estimate the hepatic VLDL secretion. Results obtained clearly showed that addition of either lysoPC or choline stimulated TG (Fig.20; A) and PC (Fig.20; B) secretion by 3- and 2-fold, respectively, compared to no addition. A linear secretion of $[^3\text{H}]\text{PC}$ was also observed when cell received $[^3\text{H}]\text{lysoPC}$ as a tracer (Fig.20; B; inset), indicating secretion of newly synthesized PC from the hepatocytes upon the addition of lysoPC. These results suggest that reacylation of lysoPC can supply sufficient PC for hepatic TG and PC secretion.

Fig.20. Effect of lysophosphatidylcholine on the secretion of triacylglycerol and phosphatidylcholine from hepatocytes. The experiment was performed as described in the legend of Fig.19, except that the lipids were extracted from the culture medium (combined from 2 dishes) at the indicated times. TG (A) and PC (B) secreted from the cells supplemented with no addition (X), 100 μM lysoPC (▲) or 100 μM choline (■) were quantitated. Each point is the average of two dishes which differ by less than 10%. Inset: secretion of $[^3\text{H}]\text{PC}$ from the cells incubated with $[^3\text{H}]\text{lysoPC}$ (250 dpm/nmol; 100 μM).
3.3. Effect of Lysophosphatidylcholine on the Secretion of Lipoproteins from Hepatocytes

To determine further that the stimulated TG and PC secretion was a consequence of increased VLDL secretion, the culture medium was fractionated into VLDL, LDL, HDL and BF (D>1.18 g/ml) in a salt gradient and the lipoproteins secreted were analysed. Chromatography of lipids in each of the four fractions clearly showed that the increased secretion of PC and TG in the presence of choline or lysoPC was mainly due to stimulated VLDL/LDL secretion from the cells (Fig.21). The stimulatory effect of choline and lysoPC on VLDL lipid secretion was almost identical. It was again noted that the secretion of HDL lipids was not affected by choline deficiency (Fig.21). The secretion of \[^3H\]leucine-labeled apolipoproteins in each of the four fractions was analyzed by SDS-PAGE. The secretion of VLDL apolipoproteins (apoB\(_H\), -B\(_L\), and -E) was markedly stimulated by the addition of choline or lysoPC (Fig.22), further indicating that the reacylation of lysoPC caused resumption of normal secretion of apoB/TG-rich lipoproteins. The secretion of HDL apolipoproteins (apoA-I and apoE), albumin and other dense proteins was not affected by choline deficiency.

Fig.21. Lipids in fractionated lipoproteins secreted from hepatocytes supplemented with lysophosphatidylcholine. After plating, the attached hepatocytes were incubated with 100 μM choline (C), 100 μM lysoPC (L) or no addition (-) in a serum-free medium for 12 h. Separation of secreted lipoproteins, extraction of lipoprotein lipids and HPTLC were performed as described in the legend of Fig.13. FC, free cholesterol.
Fig. 22. Fluorography of \[^{3}H\]labeled-apolipoproteins secreted from hepatocytes supplemented with lysophosphatidylcholine. Hepatocytes were incubated with \[^{3}H\]leucine (15 \(\mu\)Ci/dish) in a leucine-free medium for 12 h. Fractionation of secreted lipoproteins, separation of apoproteins by SDS-PAGE and fluorography were performed as described in the legend of Fig. 14. The x-ray film was exposed for 72 h for VLDL and 18 h for HDL and BF. There were no visible bands in LDL samples after 2-3 days exposure. Alb, albumin; -, no addition; C, +choline; L, +lysoPC.

4. Striking Head Group Specificity in the Requirement of Phosphatidylcholine Biosynthesis for Hepatic VLDL Secretion

We have demonstrated that hepatic VLDL secretion requires active synthesis of PC via the CDP-choline pathway, PE methylation or reacylation of lysoPC. To gain further understanding of the requirement of phospholipid synthesis for hepatic VLDL secretion, we examined the effect of various choline analogs or methyl group donors on hepatic lipogenesis and lipoprotein secretion.

4.1. Effect of Phospholipid Bases on the Concentrations of Major Phospholipids in Hepatocytes

Hepatocytes obtained from CD rats were incubated with various phospholipid bases for 20 h, and the concentrations of four major cellular phospholipids (PC, PE, PMME and PDME) were determined (Fig. 23). When the cells were cultured in a medium free of choline and methionine, the cellular PC and PE concentrations maintained at about 74 and 60 nmol/mg cell protein respectively. There were no detectable amounts of PMME or PDME in these cells. The addition of
choline stimulated PC synthesis (cellular PC concentration increased about 25 nmol/mg cell protein after 20 h incubation), but had no significant effect on cellular PE levels. The addition of ethanolamine, however, did not change the cellular PE levels significantly and had no effect on cellular PC concentrations. There were also no detectable amounts of PMME and PDME in the cells supplemented with choline or ethanolamine. Exposure of the cells to $N$-monomethylethanolamine led to an active synthesis of PMME and a minor formation of PDME. The synthesis of PMME and PDME upon the addition of $N$-monomethylethanolamine were accompanied by significant decreases in the cellular PC and PE levels. Similarly, addition of $N,N$-dimethylethanolamine led to an active synthesis of PDME (but not PMME), and decreases in PC and PE concentrations in the cells. After 4 h incubation with these bases, the sum of the four cellular phospholipid concentrations (nmol/mg cell protein) was as follows: no addition, 137.9; +choline, 154.9; +ethanolamine, 137.4; +$N$-monomethylethanolamine, 128.5; +$N,N$-dimethylethanolamine, 140.6. When the cells were supplemented with $N$-monomethylethanolamine or $N,N$-dimethylethanolamine, the amounts of PMME or PDME were about 20% of the total of the four phospholipids measured.

The above results demonstrate that supplementation of various head group bases, except ethanolamine, into the culture medium leads to active synthesis of the corresponding phospholipids in the hepatocytes.
Fig. 23. Effect of phospholipid bases on the concentration of phosphatidylcholine, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine in hepatocytes. Hepatocytes from CD rats were plated in a choline- and methionine-free medium containing 17% delipidated serum. After 4-5 h, the medium was replaced with serum-free medium supplemented with no addition (X), 100 μM choline (■), 100 μM ethanolamine (▲), 100 μM monomethyl-ethanolamine (●) or 100 μM dimethylethanolamine (○). At the indicated times, lipids were extracted from cells, phospholipids were separated by two-dimensional TLC and quantitated. The experiment has been repeated three times and each point represents the average value of three independent measurements. The average standard error of the means is 15%.

4.2. Effect of Phospholipid Bases on the Secretion of Triacylglycerol from Hepatocytes

Knowing that hepatocytes could synthesize significant amounts of PMME and PDME from the corresponding bases in the absence of choline and methionine, we examined whether or not the synthesis of these partially methylated phospholipids could correct the impaired VLDL secretion from the choline- and methionine-deficient cells. The mass secretion of TG from the hepatocytes incubated with the free bases was quantitated to estimate hepatic VLDL secretion (Fig. 24). Results obtained clearly showed that the secretion of TG
from the cells was low in the presence of phospholipid bases except when choline was added to the culture medium. The addition of ethanolamine had no effect on TG secretion; \( N,N \)-dimethylethanolamine had a small stimulatory effect in the first 14 h, while \( N \)-monomethylethanolamine was inhibitory. These results are the first indication of the striking specificity of PC biosynthesis for secretion of TG-rich lipoproteins. This requirement can not be replaced by PE, PMME or PDME.

4.3. Effect of Phospholipid Bases on the Secretion of Lipoproteins from Hepatocytes

The above studies were extended to examine the effect of base supplementation on lipoprotein secretion by the choline- and methionine-deficient hepatocytes. Lipoproteins secreted from the cells in a 12-h period were separated into VLDL, LDL, HDL and BF (d>1.18 g/ml) fractions in a salt gradient by ultracentrifugation. Lipids in each of the four fractions were qualitatively analyzed by HPTLC (Fig.25). Compared to cells maintained in choline- and
methionine-free conditions, the amount of VLDL and LDL phospholipids (PC, PE and SPM) and neutral lipids (TG and free cholesterol) secreted was enhanced from the cells supplemented with choline, but the secretion of HDL and BF lipids was not changed significantly (Fig.25; C). The addition of ethanolamine again had no effect on the secretion of the lipoprotein lipids compared to no addition (Fig.25; E). N-monomethyl-ethanolamine remarkably inhibited the secretion of VLDL and LDL lipids (both phospholipids and neutral lipids), but did not alter the amounts of HDL lipids secreted (Fig.25; ME). A small amount of PMME was found in the lipids

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**Fig.25.** Lipids in fractionated lipoproteins secreted by hepatocytes supplemented with phospholipid bases. Four to 5 h after plating, hepatocytes were further cultured in serum-free medium in the absence (-) or presence of 100 μM choline (C), ethanolamine (E), N-monomethyl-ethanolamine (ME) or N,N-dimethyl-ethanolamine (DE) for 12 h. Medium combined from 8 dishes was fractionated into VLDL, LDL, HDL and BF (d>1.18 g/ml) fractions in a salt gradient by ultracentrifugation, and lipids were extracted from each of the four fraction. Aliquots of lipid samples, derived from equal volumes of medium, were analyzed by HPTLC. The TG in VLDL samples was overloaded onto the HPTLC plates in order to show phospholipids. Lipid standards are indicated on the right. FC, free cholesterol.
extracted from the whole medium (as examined by two-dimensional TLC), and the secretion of PMME was 0.38 nmol/mg cell protein after a 15 h incubation. HPTLC of the lipids from fractionated lipoproteins indicated the PMME (co-migrated with PE in the solvent system) was almost exclusively associated with the HDL fraction (Fig.25; ME). The addition of N,N-dimethylethanolamine, on the other hand, had little effect on the secretion of lipoprotein lipids, except that PDME was observed in both the secreted VLDL and HDL (Fig.25; DE). The secretion of PDME from these cells was 0.61 nmol/mg cell protein after a 15 h incubation.

The secretion of [3H]leucine-labeled lipoproteins from the hepatocytes is summarized in Table X, and the apoprotein components in each of the four lipoprotein fractions were further examined by fluorography (Fig.26). VLDL secretion from the choline-supplemented cells was stimulated by about 3-fold compared to no addition. Fluorography of the VLDL apoproteins showed the amounts of labeled apo-B\textsubscript{H}, -B\textsubscript{L} and -E were all increased (Fig.26). The addition of ethanolamine, on the other hand, had no significant effect on labeled VLDL secretion. Addition of N-monomethylethanolamine diminished the secretion of labeled VLDL by almost 60% compared to that of no addition (Fig.26 and Table X). These results indicate that synthesis of PMME in the choline- and methionine-deficient hepatocytes does not substitute PC for VLDL secretion, but inhibits it. The secretion of labeled VLDL from cells supplemented with N,N-dimethylethanolamine was stimulated by about 50% (Table X). Fluorography of the VLDL apoprotein components showed the stimulation was due mainly to an increase in apoE, and probably a small increase in apoB\textsubscript{L}, but not apoB\textsubscript{H}. There were also small increases in the secretion of labeled LDL from choline- or N,N-dimethylethanolamine-supplemented cells, and decreases from ethanolamine- or N-monomethylethanolamine-supplemented cells (Table X). Unlike VLDL and LDL, the secretion of labeled HDL (mainly apoE and A-I) and BF (mainly albumin)
proteins from the hepatocytes was less affected by the supplementation of these phospholipid bases (Fig.26 and Table X). Proteins separated by SDS-PAGE and stained with Coomassie blue exhibited a pattern similar to that shown by fluorography in Fig.26. Supplementation of different phospholipid bases had no significant effects on the total uptake or the incorporation of $[^3]H$leucine into cellular trichloroacetic acid precipitable materials in the hepatocytes.

Taken together, the above results clearly demonstrate that hepatic VLDL secretion requires specifically PC synthesis, and the synthesis of PE, PMME, or PDME can not substitute. Also, the results show that the secretion of HDL, albumin and other proteins from hepatocytes is not altered by choline deficiency or supplementation with any of the bases.

<table>
<thead>
<tr>
<th>Addition</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
<th>d&gt;1.18 g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
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<td>96</td>
<td>2322</td>
<td>5248</td>
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<tr>
<td>Choline</td>
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<td>2430</td>
<td>6353</td>
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<tr>
<td>Ethanolamine</td>
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<td>79</td>
<td>1761</td>
<td>6017</td>
</tr>
<tr>
<td>MME</td>
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<tr>
<td>DME</td>
<td>384</td>
<td>100</td>
<td>1782</td>
<td>4824</td>
</tr>
</tbody>
</table>

Table X. Secretion of $[4,5-^3]H$Leucine-Labeled Lipoproteins by Hepatocytes Supplemented with Various Phospholipid Bases

The experiment was performed as described in the legend to Figure 26. Aliquots of the lipoprotein samples were counted in 5 ml aqueous counting scintillant. MME: $N$-monomethylethanolamine; DME, $N,N$-dimethylethanolamine.
Fig. 26. Fluorography of $[^3\text{H}]$leucine-labeled apoproteins secreted by hepatocytes supplemented with phospholipid bases. The experiment was performed as described in the legend of Fig. 25, except that the cells were incubated with $[^3\text{H}]$leucine (15 $\mu$Ci/dish) in a serum-free medium for 12 h. Apoproteins were separated by SDS-PAGE and subjected to fluorography. This x-ray film was exposed for 5 days for VLDL (to see apoB$_1$), and 18 h for HDL and BF. There were no visible bands in LDL samples after a 2-day exposure. Apolipoprotein standards are indicated on the left. Alb, albumin. The culture conditions (indicated on the top) are: -, no addition; C, +choline; E, +ethanolamine; ME, +N-monomethylethanolamine; DE, +N,N-dimethylethanolamine.

4.4. Effect of Betaine and Homocysteine on the Secretion of Lipoproteins from Hepatocytes

Betaine has been reported to be a lipotropic agent which effectively reduces liver fat content caused by choline deficiency [116]. Therefore, comparative studies were conducted to examine the effect of choline, betaine and betaine + homocysteine on lipoprotein secretion and phospholipid synthesis in choline- and methionine-deficient hepatocytes. Lipids and $[^3\text{H}]$leucine-labeled apoproteins from the fractionated lipoproteins were qualitatively analyzed by
HPTLC and SDS-PAGE, respectively. Addition of 200 μM betaine to the culture medium stimulated the secretion of VLDL and LDL lipids (Fig.27) and apolipoproteins (Fig.28 and Table XI) from the cells in a 12 h period, and this stimulatory effect was further enhanced when 200 μM homocysteine was present. However, supplementation with 100 μM betaine or 100 μM betaine + 100 μM homocysteine for the same period of time did not effectively correct the impaired lipoprotein secretion from the cells. There was no effect of betaine and/or homocysteine on the secretion of HDL and lipid-free proteins (d>1.18 g/ml).

![Fig.27. Lipids in fractionated lipoproteins secreted by hepatocytes supplemented with betaine and homocysteine. The experiment was performed as described in the legend of Fig.25, except that the medium was supplemented with 200 μM betaine (B), 200 μM betaine and 200 μM homocysteine (B/H), 100 μM choline (C) or no addition (-). Lipid standards are indicated on the right. FC, free cholesterol.](image-url)
Fig. 28. Fluorography of $[^3H]$leucine-labeled apoproteins secreted by hepatocytes supplemented with betaine and homocysteine. The experiment was performed as described in the legend of Fig. 26, except that the culture medium was supplemented with 200 μM betaine (B), 200 μM betaine and 200 μM homocysteine (B/H), 100 μM choline (C) or none (-). The x-ray film was exposed for 18 h.

Table XI. Secretion of L-$[4,5-^3H]$Leucine-Labeled Lipoproteins by Hepatocytes Supplemented with Betaine and/or Homocysteine

The experiment was performed as described in the legend to Figure 28. Lipoproteins in the medium were fractionated and aliquots of the samples were counted in 5 ml aqueous counting scintillant.

<table>
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<th>Addition</th>
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<th>LDL</th>
<th>HDL</th>
<th>$d&gt;1.18$ γ/ml</th>
</tr>
</thead>
<tbody>
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<td>1. No addition</td>
<td>463</td>
<td>33</td>
<td>2169</td>
<td>6162</td>
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<tr>
<td>2. 200 μM betaine</td>
<td>638</td>
<td>56</td>
<td>2342</td>
<td>5984</td>
</tr>
<tr>
<td>3. 200 μM betaine +</td>
<td>887</td>
<td>95</td>
<td>2195</td>
<td>5807</td>
</tr>
<tr>
<td>200 μM homocysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. 100 μM choline</td>
<td>1181</td>
<td>85</td>
<td>2167</td>
<td>5839</td>
</tr>
</tbody>
</table>
Homocysteine has been shown to be readily taken up by hepatocytes in the perfused rat liver [315]. We examined the utilization of betaine by cultured hepatocytes. In the absence of homocysteine, a nearly linear uptake of \([\text{methyl-}^{3}\text{H}]\)betaine (25 μM, 100 μCi/μmol) was observed when the cells were incubated for 4 h (Fig.29;A), and about 3% of the methyl groups were integrated into cellular PC (Fig.27;B). TLC of the labeled water-soluble compounds in the cells showed that tritium exclusively co-migrated with betaine, indicating that betaine was taken up by the cells in an intact form (inset of Fig.29;A). When the cells received 100 μM homocysteine, the accumulation of \([^{3}\text{H}]\)betaine in the cells was about 50% less than those of cells cultured without homocysteine in a 4 h incubation period (Fig.29;A). However, the incorporation of the methyl groups into PC was increased by 4.5 fold and about 25% of the incorporated methyl groups was integrated into cellular PC at the end of a 4 h incubation (Fig.27;B). PC was the major labeled phospholipid in the cells, accounting for about 75% of the total tritium in the cellular chloroform-soluble materials after 4 h, whereas 3%, 2% and 3.5% of the chloroform-soluble tritium was associated with lysoPC, PS and PE, respectively (inset of Fig.27;B). The remaining unidentified materials (about 12.5% of total tritium in the chloroform-soluble materials) migrated at the solvent front. In parallel experiments, exposure of the hepatocytes to betaine (200 μM) for 12 h did not significantly change cellular PC concentrations compared to no addition (70.9 ± 5.0 vs. 66.0 ± 4.0 nmol/mg cell protein; p>0.1; n=4). However, the PC level was remarkably increased in the cells when both betaine (200 μM) and homocysteine (200 μM) were added to the medium (80.5 ± 3.8 vs. 66.0 ± 4.0 nmol/mg cell protein; 0.01<p; n=4).

These data suggest that the lipotropic effect of betaine [116] most likely resulted from a stimulated VLDL secretion due to the active synthesis of PC by PE
methylation. This in turn is a consequence of increased synthesis of methionine by betaine:homocysteine methyltransferase.

**Fig 29.** Uptake of [methyl-3H]betaine and incorporation of labeled-methyl group into phosphatidylcholine. Hepatocytes were incubated with [methyl-3H] betaine (100 μCi/μmol; 5 μCi/dish in 2 ml) in the absence (□,△) or presence (■,▲) of homocysteine (100 μM). At the indicated times, a dish of cells was harvested in 1 ml methanol, and mixed with 1 ml H2O and 2 ml chloroform. After phase separation, aqueous choline derivatives (inset of A) and phospholipids (inset of B) were separated by TLC. Sample bands were scraped into scintillation vials, mixed with 0.5 ml H2O and 5 ml aqueous counting scintillant, and the radioactivity was determined after 2 days. A, [3H]betaine. B, [3H]PC. Each point is the average of 3 dishes, and the standard errors of the means are less than 10%.
5. **Microsomal CTP:Phosphocholine Cytidylyltransferase Activity is Increased in Rat Hepatocytes Deficient in Choline**

Hepatic PC synthesis is mainly (60-80% of total synthesis) via the CDP-choline pathway [42], and the rate-limiting step in this pathway is thought to be the formation of CDP-choline catalyzed by cytidylyltransferase [41,54]. Schneider and Vance [91] determined the effect of choline deficiency on the activities of enzymes involved in the CDP-choline pathway and found the *in vitro* activities of choline kinase and cholinephosphotransferase were virtually unchanged during choline deficiency, while the cytosolic cytidylyltransferase activity was reduced by 40%. However, the activity of cytidylyltransferase in microsomal membranes was not assessed in their work [91]. More recently, it has been shown that *in vivo* the membrane-associated cytidylyltransferase is the active species, while the cytosolic form of the enzyme serves as a reservoir [59,60]. Thus, it was of interest to examine the effect of choline deficiency on the activity of the membrane-bound enzyme in rat hepatocytes.

5.1. **Subcellular Localization of Cytidylyltransferase Activity in the Livers of Choline-Deficient and Choline-Supplemented Rats**

The *in vitro* activity of cytidylyltransferase was assayed in the presence of exogenous PC/oleate vesicles, the optimal condition for the enzyme assay, as described by Weinhold *et al.* [57]. Three-day choline deficiency had no effect on the specific activity of cytidylyltransferase in total liver homogenate (2.36 nmole CDP-choline formed per min per mg protein). As reported previously [91], the specific activity of the cytosolic cytidylyltransferase was decreased by about 40% after 3 days of choline deficiency (CS, 5.00±0.81, CD, 3.14±0.09; n=4, p<0.01). On the other hand, the specific activity of the microsomal cytidylyltransferase was increased by 80% (CS, 1.41±0.08, CD, 2.53±0.21, n=4, p<0.001). The recovery of microsomes from the total liver homogenates, evaluated by measuring NADPH:cytochrome c reductase activity, was similar in both CD and CS rats (CS,
Liver microsomes were further fractionated into ER and Golgi, and the activity of cytidylyltransferase in the two organelles was assayed. The recovery of ER and Golgi proteins in CD and CS livers, and the cross-contamination between these two subcellular fractions were estimated by measurement of marker enzymes (see Table VIII). The contamination of the Golgi proteins by ER was 35-50% and the ER proteins contaminated by the Golgi was 4-6%. There was no significant difference in the recovery of ER and Golgi or the extent of cross-contaminations between CD and CS rats. Table XII summarizes the distribution of cytidylyltransferase activity. The specific activity of cytidylyltransferase in the Golgi fractions was not affected by choline deficiency. However, the specific activity of the enzyme in the ER fractions was increased by more than 2-fold in choline deficiency compared to those of CS samples. These results indicate that the increased microsomal enzyme activity in choline deficiency is mainly due to the increase in the ER, but not in the Golgi, fractions.

Table XII. Subcellular Distribution of Cytidylyltransferase in the Liver of Choline-Deficient and Choline-Supplemented Rats

ER and the Golgi apparatus were isolated from the livers of CD and CS rats according to Croze and Morré [300]. Cytidylyltransferase activity was assayed in the presence of PC/oleate vesicles according to Weinhold et al. [57]. The specific activity of enzyme is expressed as nmole CDP-choline formed per min per mg microsomal protein. Values are averages ± S.D. from four independent experiments. The significance of differences was determined by a t-test.

<table>
<thead>
<tr>
<th></th>
<th>CD</th>
<th>CS</th>
<th></th>
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<tbody>
<tr>
<td>Golgi</td>
<td>1.92±0.95a</td>
<td>1.70±0.82a</td>
<td>n.s.</td>
</tr>
<tr>
<td>ER I</td>
<td>2.36±0.58</td>
<td>0.85±0.48</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>ER II</td>
<td>1.55±0.47</td>
<td>0.68±0.39</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

n.s. not significant.
a. Enzyme activities are not corrected for ER contamination.
5.2. Effect of Supplementation of Choline on the Distribution of Cytidylyltransferase Activity in Hepatocytes

Hepatocytes prepared from a single liver from a CD rat were cultured in medium with or without choline and the distribution of cytidylyltransferase between cytosol and membranes was investigated. Supplementation of choline to the culture medium rapidly (within 2 h) increased the specific activity of the cytosolic cytptylyltransferase (Fig.30, A). Concomitantly, the membrane-associated enzyme activity was decreased after the addition of choline (Fig.30, B). Prolonged incubation of the hepatocytes with choline showed that the elevated cytosolic and the lowered membrane-bound cytptylyltransferase activities were maintained for at least 20 h. The redistributed cytptylyltransferase activity between cytosol and membranes after the addition of choline was similar to the enzyme distribution observed in hepatocytes from CS rats (cytosol, 3-4 unit/mg protein; membranes, about 1.2 unit/mg protein).

The redistribution of cytptylyltransferase activity between membranes and cytosol in the hepatocytes upon the addition of choline was further confirmed by digitonin permeabilization. Digitonin disrupts cell membranes and renders the release of cytosolic components from cultured hepatocytes [302]. A nearly linear release of cytosolic cytptylyltransferase activity from the cells was observed during a period of 8 min incubation with digitonin (Fig.31). The rate of release of the cytosolic enzyme was increased in the cells supplemented with choline and the maximal rate was achieved after 2-4 h of the choline addition (Fig.31). This result agrees with the fractionation experiments where the cytosolic activity reached a plateau 2 to 4 h after the addition of choline (Fig.30). The rate of release of lactate dehydrogenase (LDH), a well characterized cytosolic enzyme, was determined to assess the specific effect of choline deficiency on cytptylyltransferase. The release of LDH from the choline-deficient hepatocytes
Fig. 30. Effect of choline on the distribution of cytidylyltransferase activity in cultured hepatocytes. Hepatocytes were prepared from a single liver of a rat fed a CD diet for 3 days, and plated in a choline- and methionine-free medium + 17% delipidated serum. Four h after plating, the medium was replaced with a serum-free medium in the absence (■) or presence (□) of choline (100 μM). At the indicated times, the cells combined from 4 dishes were homogenized in 2 ml homogenization buffer and cytosol (A) and membrane (B) fractions were isolated. The specific activity of the enzyme in these two fractions was assayed in the presence of PC/oleate vesicles. Each point is the average of four assays from 2 independent experiments. The standard deviations are determined by t-test.

Fig. 31. Digitonin-mediated release of cytidylyltransferase activity by cultured hepatocytes supplemented with choline. Hepatocytes were isolated from CD rats and cultured in a medium ± choline as described in the legend to Figure 30. After being incubated with or without choline (100 μM) for 1, 2 and 4 h, the culture medium was removed, and the cells were washed with ice-cold PBS and incubated with 1 ml digitonin solution. The digitonin solution was collected at 2, 4 and 8 min and aliquots of the samples were used for the enzyme assay. Each point is the average of two independent experiments, which do not differ by greater than 20%. The enzyme activities released from the cells incubated with no choline for 1, 2 and 4 h were identical (×).
displayed the same rate as that from choline-supplemented cells, indicating the increased rate of cytidylyltransferase release from the cells supplemented with choline was specific.

As reported previously, supplementation of choline stimulated PC synthesis (Fig. 15). After the addition of choline to the medium, an almost linear increase in the hepatic PC levels was observed (Fig. 32). In the hepatocytes cultured in a choline-free medium, the low PC levels were maintained (Fig. 32). The concentrations of other major cellular phospholipids (i.e. PE, PI and PS) were unchanged in the CS cells compared to those of CD cells (Fig. 32).

Fig. 32. Cellular phospholipid concentrations in cultured hepatocytes supplemented with choline. The experiment was performed as described in the legend of Fig. 30. Lipids were extracted from the cells with chloroform/methanol (1:1; v/v) at the indicated times. Phospholipids were separated by TLC and quantitated by phosphorus assay.
5.3. Effect of Supplementation of Lysophosphatidylcholine on the Distribution of Cytidylyltransferase Activity in Hepatocytes

The diminished membrane-bound cytidylyltransferase activity and elevated cytosolic enzyme activity induced by choline supplementation (Fig. 30) may result from the changes in membrane phospholipid composition (mainly increase in PC) after the addition of choline (Fig. 32). To test this, hepatocytes were supplemented with lysoPC and the subcellular localization of cytidyltransferase, before and after the addition of lysoPC, was determined. It is known that supplementation of lysoPC to CD hepatocytes stimulates hepatic PC synthesis and increases cellular PC content (Fig. 19). Thus, a redistribution of cytidylyltransferase between cytosol and membranes upon the addition of lysoPC was expected. Results from the digitonin permeabilization experiment, however, showed that the addition of lysoPC to the culture medium had no effect on the distribution of cytidylyltransferase (Fig. 33). The lack of effect of lysoPC supplementation on the enzyme activity distribution was also observed in the fractionation experiments in which the cytosol and membranes were isolated. These results suggest the activity of membrane-associated cytidylyltransferase may not be solely determined by the abundance of cellular PC.

Fig. 33. Digitonin-mediated release of cytidylyltransferase activity by cultured hepatocytes supplemented with lysophosphatidylcholine. The experiment was performed as described in the legend of Fig. 31, except that lysoPC (100 μM) was added to the medium of one group of cells. Each point represents released enzyme activity at the end of an 8-min incubation with digitonin. Data are the average of two independent experiments.
5.4. Changes in Cellular [\(^{14}\text{C}\)]Fatty Acid and [\(^{14}\text{C}\)]Diacylglycerol in Hepatocytes Supplemented with Choline or Lysophosphatidylcholine

Free fatty acids have been shown to promote binding of cytidylyltransferase to membranes of hepatocytes [90]. In the present studies, the concentrations of free fatty acid in the livers of CD and CS rats were determined. Preliminary results showed that the free fatty acid level in CD livers was 82.0 µg/g liver (the average of three CD livers), while in CS livers the free fatty acid level was barely detectable. Hence, the increased membrane-bound activity of cytidylyltransferase in the livers might result from the elevated free fatty acid concentration during choline deficiency. To test this, we incubated hepatocytes from CD rat livers with or without choline, and examined whether or not the supplementation of choline would affect cellular free fatty acid levels. Since the amounts of free fatty acids were too small to quantitate in the cultured hepatocytes, we pre-labeled the cells with [\(^{14}\text{C}\)]acetate and monitored the decay of radioactivity from the labeled free fatty acids. If the dissociation of cytidylyltransferase activity from the membranes after the addition of choline resulted from diminished free fatty acid concentrations, a faster disappearance of the radioactive-labeled free fatty acids should be expected. Results obtained from the [\(^{14}\text{C}\)]acetate-labeling experiments showed the decay of radioactivity from the labeled fatty acid was independent of the addition of choline (Fig.34). A turnover rate of free fatty acids at \(t_{1/2} = 30\) min was observed regardless of the presence or absence of choline. In a parallel experiment, hepatocytes obtained from the CD livers were incubated with albumin (fatty acid free) and the effect on cytidylyltransferase activity and location was determined. Results obtained from digitonin-permeabilization and from cell homogenization all showed that albumin did not cause dissociation of the enzyme from membranes. Taken together, these
data suggest that the increased membrane-bound cytidylyltransferase activity in choline deficiency is unlikely a result of elevated cellular free fatty acid levels.

Endogenous DG is thought to stimulate cytidylyltransferase activity by promoting the association of the enzyme to membranes [316]. The different response of cytidylyltransferase activity to the addition of choline and lysoPC may reflect the difference in cellular DG levels, considering that 1,2-DG is a requisite substrate in the CDP-choline pathway. The concentration of 1,2-DG in the total liver homogenate extract was increased 3-fold after 3 days of choline deficiency (CS, 74.7 µg fatty acid/g liver; CD, 178.2 µg fatty acid/g liver). This result is similar to the data reported by Tinoco et al. [135] which showed a 6-fold elevation of 1,2-DG in rat livers (CS, 96.4±42.3 µg fatty acid/g liver; CD, 517±96 fatty acid/g liver) after 7 days of choline deficiency. In [14C]acetate-labeling experiments, we examined the effect of choline on the cellular levels of [14C]acetate-labeled 1,2-DG. The decay of radioactivity from the pre-labeled 1,2-DG in the hepatocytes was stimulated by choline, while the decay of radioactivity from 1,3-DG was not affected by choline (Fig.35). The stimulated turnover of [14C]1,2-DG possibly reflects an active synthesis of PC in the presence of choline. In a similar experiment where lysoPC was used, the increased decay of radioactivity from the 1,2-DG was not observed (Fig.35). The reduced [14C]1,2-DG levels upon the addition of choline, but not lysoPC, may be the cause of the changed cytidylyltransferase activity observed in the cells.
Fig. 34. Effect of choline on the cellular \[^{14}C\] acetate-labeled free fatty acid levels in hepatocytes. Hepatocytes from a single CD liver were plated in a choline- and methionine-free medium containing 17% delipidated serum and \[^{14}C\] acetate (5 μCi/dish). Five h after plating, the labeling medium was replaced with a serum-free medium with (■) or without (□) choline (100 μM) and contained no labeled acetate. At the indicated time points, the culture medium was removed and the cells were immediately harvested into 1 ml methanol. Cellular lipids were extracted by the addition of 2 ml chloroform and 1 ml H₂O, and separated by TLC using oleic acid as a carrier for free fatty acids. The radioactivity in the fatty acid bands was determined in 5 ml aqueous counting scintillant. Each point is the average of two dishes, which do not differ more than 10%.

Fig. 35. Effect of choline on the cellular \[^{14}C\] acetate-labeled 1,2-diacylglycerol levels in hepatocytes. The experiment was performed as described in the legend of Fig. 24. Diolein was used as 1,2-DG carrier for TLC. Each point is the average of two dishes, which do not differ more than 10%. The decay of \[^{14}C\]1,2-DG in the presence of lysoPC (100 μM) was identical to that of no addition (□).
6. The Mass of Phosphatidylethanolamine \(N\)-Methyltransferase in Rat Hepatocytes is not Altered during Choline Deficiency

Methylation of PE is of quantitative importance for PC synthesis in the liver; accounting for 20-40% of the total hepatic PC formation [42]. It is well known that a diet low in methionine has to be used to produce changes in liver lipid compositions characteristic of choline deficiency [317]. Previous studies have clearly shown that supplementation of methionine effectively stimulates hepatic PC synthesis and corrects the impaired VLDL secretion, implying that hepatic PE methylation can compensate for the defect of PC synthesis via the CDP-choline pathway in choline deficiency. In the present work, the amount of PE \(N\)-methyltransferase and its \textit{in vitro} activity was examined to assess whether there was an adaptive change in the enzyme level during choline deficiency.

6.1. Activity and Mass of Phosphatidylethanolamine \(N\)-Methyltransferase in Choline-Deficient and Choline-Supplemented Livers

Hepatic PE \(N\)-methyltransferase activity is largely associated with microsomal membranes [78]. Fractionation of the liver microsomal membranes revealed that the ER possessed the majority of the enzyme activity. Golgi had minimal PE \(N\)-methyltransferase activity (Table XIII). Since the contamination of Golgi proteins by ER in our experiments was 35-50%, the small amount of PE \(N\)-methyltransferase activity in the Golgi might result from ER contamination. There was an about 2-fold increase in the enzyme activity in the ER, but not the Golgi, fractions of rat livers after 3 days of choline deficiency (Table XIII). Reports from other investigators showed the \textit{in vitro} PE \(N\)-methyltransferase activity was increased in choline deficiency using endogenous microsomal PE as a substrate [91, 150-152]. The present data confirmed this observation.

The characteristic increase in PE/PC ratio in choline deficiency was also observed in microsomal membrane subfractions, which were separated from microsomes by the treatment of \(\text{Na}_2\text{CO}_3\) at pH 11.0 [203]. PE content in the
microsomal membranes obtained from CD rat livers was 30% higher than that of CS (CS, 209.2±24.4, CD, 273.2±34.9 nmol/mg membrane protein; n=4, p<0.05), while PC content in the microsomal membranes obtained from CD rat livers was 20% lower than that of CS (CS, 571.6±52.0, CD, 461.0±43.7 nmol/mg membrane protein; n=4, p<0.05). Thus, the increased PE N-methyltransferase may reflect the elevated endogenous PE concentrations in the CD microsomal membranes. The new knowledge acquired from the purification of rat hepatic PE N-methyltransferase indicated that the three-step PE methylation is catalyzed by a single enzyme protein, and the enzyme exhibits the highest specific activity using PMME as a methyl group acceptor [66]. Since PMME barely exists in microsomal membranes, using PMME as a methyl group acceptor should eliminate any variance in the enzyme assay due to endogenous substrate levels. In the enzyme assay using PMME, no significant difference in PE N-methyltransferase activity was observed.

**Table XIII. Activity of Phosphatidylethanolamine N-Methyltransferase in Endoplasmic Reticulum and the Golgi Apparatus from Choline-Deficient and Choline-Supplemented Rat Livers**

ER and Golgi were isolated from the livers of CD and CS rats according to Croze and Morré [300]. PE N-methyltransferase activity was assayed using either endogenous microsomal PE or exogenous PMME as acceptors of methyl groups from AdoMet [66]. The specific activity of the enzyme is expressed as nmole methyl groups transferred per min per mg microsomal protein. Values are averages ± S.D. from four independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>Endogenous PE</th>
<th>Exogenous PMME</th>
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<tbody>
<tr>
<td></td>
<td>CD</td>
<td>CS</td>
</tr>
<tr>
<td>ER I</td>
<td>1.45±0.34</td>
<td>0.74±0.19</td>
</tr>
<tr>
<td></td>
<td>0.01&lt;p&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>ER II</td>
<td>1.09±0.17</td>
<td>0.61±0.15</td>
</tr>
<tr>
<td></td>
<td>0.001&lt;p&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Golgi</td>
<td>0.33±0.07a</td>
<td>0.23±0.04a</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td></td>
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n.s., not significant by *t*-test.
a Enzyme activities are not corrected for ER contamination.
These results suggest that the increased PE N-methyltransferase activity in CD rat livers most likely results from elevated endogenous PE levels, rather than an adaptive change in the enzyme mass.

To confirm further that PE N-methyltransferase mass was not affected by choline deficiency, a comparative experiment was conducted by immunoblotting using a rabbit polyclonal antibody against the enzyme protein. Fig.36 shows an autoradiogram of an immunoblot of microsomal proteins from CD and CS rat liver microsomes, and a purified PE N-methyltransferase standard. The blot showed nearly identical intensities for the microsomal methyltransferase (18.3 Kd), which co-migrated with the authentic enzyme standard, clearly indicating that there was no mass change in PE N-methyltransferase in the livers 3 days after choline deficiency.

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**Table XIII.**

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![Immunoblotting of phosphatidylethanolamine N-methyltransferase in liver microsomes of choline-deficient and choline-supplemented rats.](image)

Microsomal proteins (100 µg for the right two lanes and 300 µg for the middle two lanes) from CD and CS livers were separated by electrophoresis on a 5-15% gradient polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated successively with rabbit anti-PE N-methyltransferase antibody and 125I-labeled protein A and subjected to autoradiography. Purified PE N-methyltransferase was used as a standard (the left lane).
6.2. Effect of Supplementation of Choline and Methionine on Phosphatidylethanolamine N-Methyltransferase Activity in Hepatocytes

Hepatocytes obtained from a single CD rat liver were cultured in a methionine-free medium with or without choline and the activity of PE N-methyltransferase was determined. In vitro activity of the methyltransferase, using either endogenous microsomal PE or exogenous PMME as substrate, was unchanged over a 4 h incubation with choline (Fig.37, A), although active PC synthesis was initiated upon the addition of choline (Fig.37, B). Thus, active synthesis of PC did not seem to affect the activity of PE N-methyltransferase in vitro.

![Fig.37](image)

Fig.37. Effect of choline on the activity of phosphatidylethanolamine N-methyltransferase and cellular phosphatidylcholine and phosphatidylethanolamine levels in hepatocytes. Hepatocytes were plated in choline- and methionine-free medium + 17% delipidated serum. Four h after plating, the medium was replaced with a serum-free medium ± choline (100 μM). At the indicated times, the hepatocytes combined from 4 dishes were homogenized in 2 ml homogenization buffer, and membrane fractions were obtained by centrifugation. A, PE N-methyltransferase activity, assayed using endogenous PE (Δ,▲) or exogenous PMME (□,■) as substrates, in the cells incubated with (closed symbols) or without (open symbols) choline. B, concentrations of PC and PE in the membranes of the hepatocytes.
Choline supplementation has been shown to have no effect on the hepatic PE levels, while the addition of methionine diminished the elevated cellular PE contents caused by choline deficiency (Fig.15). Thus, if the lack of effect of choline on methyltransferase activity reflects the unchanged cellular PE concentration, a corresponding decrease in the enzyme activity is to be expected when the cells are incubated with methionine. Fig. 38 shows the activity of the methyltransferase and levels of cellular PC and PE in hepatocytes incubated with methionine. At the end of a 12 h incubation with methionine, the activity of methyltransferase using endogenous PE as a substrate was reduced by 50%, but activity using exogenous PMME as a substrate was unchanged compared to the cells incubated without methionine (Fig.38, A). Correspondingly, PE content in the membranes was reduced by 40 nmole/mg membrane protein (Fig.38, C), while PC content in the membranes was increased by 40 nmole/mg membrane protein (Fig.38, B). The amount of PE N-methyltransferase in the hepatocytes supplemented with methionine was estimated by immunoblot. Fig. 39 shows that the amount of enzyme protein was unchanged regardless of the changes in membrane PC and PE levels caused by methionine supplementation. Taken together, these results clearly demonstrated that the activity of PE N-methyltransferase was mainly influenced by the availability of methyl group acceptors (i.e. PE) rather than enzyme mass.
Fig. 38. Effect of methionine on the activity of phosphatidylethanolamine N-methyltransferase and cellular phosphatidylcholine and phosphatidylethanolamine levels in hepatocytes. Hepatocyte preparation and plating were described in the legend of Fig. 37. After plating, the cells were incubated in a serum-free medium ± 200 μM methionine. Hepatocytes were homogenized, and the membrane fractions were isolated at the indicated time points. The microsomal PE N-methyltransferase (A) was assayed using endogenous PE (Δ,▲) or exogenous PMME (■,■) as methyl group acceptor after the cells were cultured in the absence (open symbols) or presence (closed symbols) of methionine. Unit of the enzyme is: nmole methyl group transferred/min/mg microsomal protein. The concentrations of PC (B) and PE (C) in the membranes of hepatocytes before (open symbols) and after (closed symbols) incubation with methionine were quantitated.
Fig. 39. Immunoblotting of phosphatidylethanolamine \( N \)-methyltransferase in cultured hepatocytes supplemented with methionine. The experiment was performed as described in the legend of Fig. 38. Membrane proteins (200 \( \mu \)g) from the cells incubated with (+) or without (-) methionine (200 \( \mu \)M) for various periods of time were separated by SDS-PAGE and subjected to immunoblotting using rabbit anti-PE \( N \)-methyltransferase antibody as described in the legend of Fig. 36.
DISCUSSION

1. Hepatic Lipid Metabolism in Choline Deficiency

The earliest characteristic features of choline deficiency in the liver are reduction of hepatic PC/PE ratio and accumulation of TG [7,23]. In the present work, these two features were confirmed. Choline and methionine deficiency diminished PC concentration in the liver (especially in the liver microsomal membranes), and this reduction results most probably from the defect in PC biosynthesis. Supplementation of choline, methionine, lysoPC or betaine and homocysteine to the deficient cells rapidly brought the decreased cellular PC levels to normal. The lack of sufficient substrates (choline head groups and methyl groups) appears to be the main cause of the defect in PC synthesis. Other lines of evidence to support this viewpoint were obtained from the activity assay and mass determination of the hepatic PC synthetic enzymes. Our data strongly suggest that in the CD livers, the amount of cytidylyltransferase and PE N-methyltransferase is preserved (discussed below).

There may be at least two explanations for the elevated cellular PE concentrations during choline and methionine deficiency. One is an inhibited PE methylation due to the lack of methyl group donors. The result that supplementation with methionine rapidly diminished the elevated PE levels in the CD hepatocytes (Fig. 15) supports this point of view. The other is an elevated cellular concentration of PE precursors in CD livers [142,147]. However, the sources of the elevated PE precursors in choline deficiency are not known. Several reports have suggested that the rate of hepatic PE synthesis from ethanolamine is not altered in choline deficiency and the in vitro activities of PE synthetic enzymes are unchanged [91,135]. Thus, the elevated PE levels probably result from blocked hepatic PE methylation, rather than from an increased rate of PE synthesis. Decarboxylation of PS also contributes to de novo PE synthesis.
Yamamoto et al. [153] have shown in a rat hepatoma cell line that the synthesis of serine-derived PS or serine-derived PE is not changed during choline deficiency.

Choline deficiency causes a tremendous accumulation of TG in the liver. We have found that the initial rate of radiolabeled-oleate incorporation into cellular TG in CD hepatocytes is almost identical to that in choline- or methionine-supplementated cells (Fig. 16). Our data is consistent with the previous results that choline deficiency does not alter the rate of hepatic TG synthesis [8, 134]. The active synthesis of TG in choline- and methionine-deficient cells clearly indicates that hepatic TG synthesis is independent of PC synthesis and VLDL secretion. Apparently, failure in the assembly of de novo synthesized TG into VLDL will lead to accumulation of the lipid in the storage pool in the cytoplasm of hepatocytes. Lombardi et al. [8] have shown that the TG content in microsomes is normal in CD rat livers. Similar results were obtained in our laboratory. The TG levels in microsomal membranes (after separating microsomal contents by Na₂CO₃ treatment) of CD livers were nearly identical to that of CS livers (CD, 31.1±4.4, CS, 27.9±2.0 nmol/mg membrane protein, n=4). Thus, it is clear that the TG accumulated in CD livers is entirely located in the cytoplasmic storage pool. Whether or not the abnormally high levels of TG will disturb cell functions is not clear. In our hepatocyte cultures, cell viability and cellular protein synthesis does not seem to be affected by the accumulation of TG.

2. **Hepatic VLDL Apolipoprotein Metabolism in Choline Deficiency**

Lombardi and his co-workers [154, 155] have suggested that in intact livers, hepatic protein synthesis is not altered by choline deficiency. Our data from cultured hepatocytes are consistent with their observations. In all the supplementation experiments performed, overall hepatic protein synthesis is independent of the addition of choline, methionine, betaine and homocysteine or other phospholipid head groups. Thus, hepatic protein synthesis is not affected
by defective PC synthesis in choline deficiency. Using radioimmunoassays, we have investigated hepatic metabolism of VLDL apolipoproteins, and demonstrated that the synthetic rates of apo-B\(\text{H}\), -B\(\text{L}\), -E and -Cs are not altered in choline deficiency. Although choline deficiency caused a severe inhibition on VLDL secretion, the rates of disappearance of radioactivity from labeled apo-B\(\text{H}\), -B\(\text{L}\), -E and -Cs in hepatocytes are not affected. Furthermore, immunoblotting whole liver homogenates showed no accumulation of VLDL lipoproteins in CD livers. These results suggest that choline deficiency may have little or no effect on VLDL apolipoprotein metabolism. Borchardt and Davis [230] have reported that in cultured rat hepatocytes, only 36% of apoB\(\text{H}\) and 60% of apoB\(\text{L}\) synthesized are secreted, while the remainder are degraded intracellularly. We have found that in the hepatocytes obtained from CD rats, secreted VLDL apolipoproteins (in the presence of choline or methionine) account for only 10% of the total apolipoproteins synthesized, and a significant amount of VLDL apolipoproteins are degraded within the cells (data not shown). The hepatocytes defective in PC synthesis might degrade non-secreted VLDL apolipoproteins more rapidly than normal cells to prevent accumulation of these proteins in the cells.

In orotic acid-treated rats, a lack of immature VLDL apolipoproteins in the Golgi apparatus and accumulation of these particles in the ER has been observed [277]. Thus, orotic acid seems to block the transit of VLDL from ER to Golgi. In choline-deficient livers, we have attempted to examine the possible inhibition site in the transit of VLDL. Immunoblot of proteins from the liver ER and Golgi showed moderately increased levels of apoE and apoCs in the ER and decreased levels of these apolipoproteins in the Golgi (Fig.10). In preliminary experiments, we have observed that TG levels in the microsomal contents (separated from microsomal membrane by Na\(_2\)CO\(_3\) treatment) of CD livers were 80% higher than normal (CS, 15.6, CD, 28.4 nmol/mg microsomal content protein). These results
may suggest that there is a slowdown in VLDL transport out of the ER in choline deficiency. However, since the contamination of the Golgi fractions by endosomes was not determined, we are uncertain whether the decreased VLDL apolipoproteins observed in the Golgi preparations from CD liver is due to the inhibited transit, or due to the decreased plasma VLDL in choline deficiency. Distribution of apoB_L between ER and Golgi appeared to be normal in choline deficiency. ApoB_L has been shown to be responsible for rat hepatic VLDL secretion and apoB_H is not \([271-273]\). Thus, the normal intracellular distribution of apoB_L may suggest that the inhibitory effect of choline deficiency on VLDL secretion probably is not due solely to a block in the transit between ER and Golgi.

3. Reduction in plasma VLDL, but not HDL, in Choline Deficiency may Reflect Differential Mechanisms for Their Secretion

Choline deficiency-caused hypolipidemia has also been well demonstrated \([138,154]\), and was confirmed by the present results. Our data clearly indicated that in CD rat plasma, the decrease in lipid and apolipoprotein levels results mainly from the lowering in VLDL and LDL concentrations, while HDL and lipid-free plasma proteins are less or not affected by choline deficiency. Several lines of evidence, obtained from analysis of whole plasma by agarose gel electrophoresis, from analysis of lipoprotein lipids by HPTLC and from analysis of apolipoprotein components by SDS-PAGE, support the result that choline deficiency diminishes plasma VLDL, but not HDL. Mookerjea *et al.* \([9, 138]\) have reported that there is a 30% decrease in the amount of total VLDL + LDL proteins in choline deficiency, but no change in protein contents in chylomicron, HDL and d>1.21 fractions. Our data on plasma lipoprotein profiles confirm and extend the previous reports. Plasma VLDL mainly originates from the liver, and to a lesser degree from the absorptive cells of the small intestine \([2]\). The impaired VLDL secretion observed in the cultured CD hepatocytes is most likely a cause of the reduction in plasma VLDL.
The liver is a major source of HDL apolipoproteins [16]. Steinberg's group [279] reported that hepatic secretion of HDL, like VLDL, could be blocked by monensin, and suggested that the secretion of these two particles might share similar routes. In the present studies, the findings of normal plasma HDL levels and normal hepatic HDL secretion in choline deficiency are contrary to this view. We found secretion of HDL lipids and apolipoproteins by the cultured hepatocytes deficient in choline and methionine is unaffected, strongly suggesting that the route of HDL secretion differs from that of VLDL. Mookerjea [156], in perfused livers using [1-14C]glucosamine as a plasma glycoprotein tracer, demonstrated that while the incorporation of [1-14C] glucosamine into VLDL, LDL and protein of d>1.21 g/ml was inhibited in choline deficiency, secretion of labeled HDL was normal. In recent orotic acid-feeding studies, Hamilton et al. [277] observed that in perfused livers orotic acid only blocked VLDL secretion, while the accumulation of HDL (resembling the typical discoidal conformation) in the perfusate was normal. The possibility that liver (and intestine) cells secrete HDL apolipoproteins (i.e. apoA-I) that become associated with phospholipids derived from cell membranes or other lipoproteins after secretion has been proposed [285]. An increased amount of lipid-free apoA-I was observed in the plasma in choline deficiency (Table VII). We have also observed, in media from cultured hepatocytes, significant amounts of HDL proteins (i.e. apoE and apoA-I) in the d>1.18 g/ml fraction. These proteins could be secreted directly in a lipid-free form from hepatocytes, or generated as an artifact of ultracentrifugation.

Circulating HDL apolipoproteins also originate from the intestine. Bearnot et al. [182] have shown that intestinal secretion of apoA-I and HDL is not affected by bile diversion (to block the supply of biliary lysoPC for intestinal reacylation), while the secretion of lymph chylomicrons and VLDL are reduced. The differential response between intestinal HDL and apoB-containing lipoprotein
secretion to PC synthesis also suggests there might exist two independent routes for their secretion.

4. Specific Requirement of Active Phosphatidylcholine Synthesis for Hepatic VLDL Secretion

The present information unambiguously supports the viewpoint that reduced plasma VLDL levels in the rats deficient in choline directly resulted from impaired hepatic secretion of VLDL. Quantitative analysis revealed the amounts of TG and PC secreted by hepatocytes deficient in choline and methionine were only one-third and one-half, respectively, of those in hepatocytes supplemented with choline, methionine or lysoPC. Since TG secreted from hepatocytes is primarily associated with VLDL, the reduced TG secretion observed in CD cells indicates an impairment in VLDL secretion. Qualitative analysis of lipid and apolipoprotein compositions in the fractionated lipoproteins confirmed the defect in VLDL secretion in choline and methionine deficiency. In the cells supplemented with choline, methionine or lysoPC, the stimulated secretion of TG and PC is always accompanied by an increase in cellular PC concentrations, implying that active synthesis of PC is required for VLDL secretion.

It appears that the requirement of PC synthesis for VLDL secretion can be equally satisfied by the CDP-choline pathway, PE methylation or reacylation of lysoPC. Whether there is a selection among the molecular species of PC synthesized via these different pathways in the process of VLDL assembly has not been studied. In methionine supplementation experiments, the concurrent decrease in PE and increase in PC implies that the increased cellular PC results from active PE methylation (Fig.15). Although the molecular species of PC secreted by the methionine-supplemented cells were not determined, Ridgway and Vance [67] have recently shown a rapid (within 12 h) remodeling of the PE-derived PC species within cultured hepatocytes. In previous reports, hepatic methylation of PE was suggested not to be essential for lipoprotein secretion when
the CDP-choline pathway was functional [207]. The present investigation has extended this result and further proved that hepatic PE methylation pathway can effectively substitute for the CDP-choline pathway if the latter were blocked. Our data also entirely agree with the results of Pascale et al. [209] that supplementation of AdoMet to cultured hepatocytes deficient in choline and methionine stimulates TG secretion.

Our result is the first to demonstrate in cultured hepatocytes that reacylation of exogenous lysoPC can supply sufficient PC for hepatic VLDL secretion. LysoPC used in the supplementation studies is derived from egg PC and contains mainly palmitic acid (16:0) and stearic acid (18:0). The nearly identical stimulatory effect caused by choline and lysoPC on the secretion of VLDL lipids and VLDL apolipoproteins showed that hepatic reacylation of the egg lysoPC could effectively substitute for the CDP-choline pathway. Formation of PC from lysoPC can be achieved by either the Lands' pathway [70] or by the condensation of two molecules of lysoPC described by Marinetti et al. [74]. Which, if either, pathway is responsible for the observed PC synthesis in the hepatocytes was not studied. Our data show that hepatocytes rapidly convert lysoPC to PC and virtually no lysoPC accumulated in the cells. This is consistent with the viewpoint that high concentrations of lysoPC are toxic to cell membrane functions [71]. Acquiring the choline moiety from exogenous lysoPC (bound to albumin) for cellular PC synthesis has also been observed in cultured CHO cells [40]. Our results show that liver cells can make PC from plasma lysoPC, at least in choline deficiency.

The lipotropic effect of betaine reported by Best and his co-workers in the 1950's [116] led us to investigate the mechanism by which betaine compensates for choline and methionine deficiency. It has been shown in cultured hepatocytes that betaine is rapidly produced by choline oxidation and released into the culture medium [90]. The hepatic oxidation of choline is thought to be the major route for
reducing free choline levels [45]. Hepatic uptake of betaine, on the other hand, has rarely been studied. Our results showed for the first time that betaine can be taken up in an intact form by the cultured hepatocytes. In addition, we have shown that betaine combined with homocysteine, but not betaine alone, can effectively stimulate hepatic PC synthesis and correct the impaired VLDL secretion caused by choline and methionine deficiency. These results suggest to us that the lipotropic effect of betaine is most likely a consequence of stimulated methionine synthesis. In the liver, formation of methionine is catalyzed by methylation of homocysteine with either betaine or \(N^5\)-methyltetrahydrofolate as methyl group donor [93,94]. Although which methylation reaction is more important for methionine synthesis in rat liver remains to be defined [318], homocysteine is an indispensible substrate for either reaction (Fig.3). That betaine corrected impaired VLDL secretion and also increased cellular PC levels in the presence of homocysteine suggests that hepatic synthesis of methionine from betaine depends on the availability of the methyl group acceptor, homocysteine. Thus, our data have confirmed that betaine is a lipotropic compound by donating its methyl groups for the formation of methionine.

Our present work also shows that hepatic synthesis of PE and partially methylated intermediates (PMME and PDME) do not substitute for PC in VLDL secretion, indicating the requirement of active PC synthesis is specific. The reason for this specificity is presently unknown. A new hypothesis for the assembly of apoB\(_{100}\)-containing lipoproteins is that the monolayer of phospholipid on the surface of TG-rich particles is acquired from the luminal leaflet of the endoplasmic reticulum bilayer [276b]. The highly hydrophobic nature of apoB [210,211] and its association with ER membranes in human HepG 2 cells [267] and rat hepatocytes [276] all support this hypothesis. Thus, the less effective incorporation of PE, PMME and PDME into VLDL might be explained by
l lipid topogenesis; the phospholipids synthesized on the cytosolic leaflet of the endoplasmic reticulum bilayer might undergo a slow transbilayer movement. It has been shown that the transbilayer movement of PC on endoplasmic reticulum is facilitated by a protein called "flipase" [198,200]. However, little is known about the rates of transbilayer movement of PE and the partially methylated intermediates on endoplasmic reticulum, nor has the asymmetric distribution of phospholipids across the bilayer of ER been shown.

PE is a poor lipid component for lipoproteins, and accounts for only 2-3% of the total phospholipids in the plasma (Table III). Vance and Vance [33] have shown that in cultured rat hepatocytes there is very little secretion of \[^{3}\text{H}]\text{ethanolamine-derived PE, while the cellular PE pool is heavily labeled. Analysis of lipid distribution between microsomal membranes and microsomal contents also indicates that the exchange of PE, if it exists, between these two fractions is slow (Table IV). A slow rate for intravesicular transfer of PE (5- to 7-fold slower than the rates for PC) has been reported in model membranes [319]. Thus, an inability to transfer from ER membranes to lipoprotein particles may be an alternative explanation for the failure of PE (probably PMME as well) to replace PC in the VLDL assembly process.

PDME, associated with VLDL and HDL, has been found to be released from cultured hepatocytes, although the synthesis of PDME does not correct effectively the impaired secretion of TG (Fig.24). The VLDL secreted by \(N,N\)-dimethylethanolamine-supplemented cells contains more apoE and apoB\(_L\) (not apoB\(_H\)), but not TG and CE, compared to cells with no addition (Fig.24-26). Apparently, PDME is not sufficient for the assembly of TG-rich lipoproteins. PDME could be released by means of either lipoprotein secretion or rapid lipid exchange on the outer surface of plasma membrane. Which, if either, mechanism is responsible for the release is obscure. It was noted that the secretion of VLDL
from N-monomethylethanolamine-supplemented cells was remarkably inhibited, and the cellular PC concentration was also rapidly decreased when the active synthesis of PMME occurred. Why the addition of N-monomethylethanolamine inhibits VLDL secretion is not understood. One explanation is an altered structure and function of biomembranes due to accumulation of PMME. However, studies conducted in mouse fibroblast cells showed that the accumulation of PMME on microsomes, mitochondria and plasma membranes (up to 37% of total membrane phospholipid) did not affect the activities of some membrane-bound enzymes [85] or the characteristic temperatures (transition temperature) of plasma and intracellular membranes [87]. In addition, our data have showed that the secretion of HDL and lipid-free proteins (such as albumin) is not altered by the accumulation of PMME (20% of four major cellular phospholipids), indicating general cell functions are maintained. An alternative explanation for the inhibited VLDL secretion by the cells cultured with N-monomethylethanolamine could be a consequence of the reduced cellular PC levels (Fig.23). However, a similar decrease in the cellular PC levels was also observed in N,N-dimethylethanolamine-supplemented cells (Fig.23), while the basal rate of VLDL secretion from these cells was not altered compared to the cells with no addition (Fig.24). Thus, the inhibited VLDL secretion by the cells after the addition of N-monomethylethanolamine might not simply result from lowered cellular PC concentrations.

Our data clearly points to a specific function of PC in VLDL secretion; and this result is interesting in the context of the question of why there is diversity in lipid structure [320].

Where and how PC is integrated into VLDL particles during the course of assembly is not clear. The present results suggest that active synthesis of PC is essential for VLDL secretion, but the pre-existing PC is not utilized. Several
laboratories have shown that both ER and Golgi contain the activities for PC synthetic enzymes such as cytidylyltransferase, cholinephospho-transferase and PE $N$-methyltransferase [78,190,191]. These enzymatic studies strongly support the viewpoint that VLDL can acquire PC in both ER and Golgi. PC "flippase" may facilitate the transbilayer movement of newly synthesized PC into the lumen of ER and Golgi where VLDL is assembled. In cultured hepatocytes from rats [206] or estrogen-treated chickens [205], a rapid incorporation of labeled phospholipids into VLDL in Golgi was observed. Higgins and Huston [206] have even suggested in rat hepatocytes that as much as 80% of the secreted PC is incorporated into lipoproteins in Golgi.

Choline and methionine deficiency effectively block active PC synthesis. We anticipated that the choline-deficiency model might allow us to elucidate the process of lipid/protein assembly in VLDL formation. Preliminary results from the subcellular fractionation studies (Fig.10) have shown there may be accumulation of TG, apoE and apoCs in the ER during choline deficiency. Considering the models of VLDL assembly proposed by Norum et al. [131] and Olofsson et al. [276b], we speculate that the active synthesis of PC in the ER may be a driving force for the extrusion of apoB into the lumen of the ER. Newly synthesized apoB is cotranslationally associated with ER membranes. A defect in PC synthesis in the ER might result in the failure to discharge apoB/core lipid complex out of the inner leaflet into the cisternal space of the ER. Recent pulse-chase studies in cultured rat hepatocytes has shown the rate of transport out of the ER determines rate of VLDL secretion [230]. Therefore, it might be logical to propose that most of the PC required for VLDL particles to move to the Golgi is supplied from ER. However, this proposal does not eliminate the possibility that secretory lipoprotein particles might acquire some PC at the Golgi and/or somewhere on the route from Golgi to plasma membrane.
5. **Synthesis of Phosphatidylmonomethylethanolamine, Phosphatidyldimethylethanolamine and Phosphatidylethanolamine in Choline-Deficient Hepatocytes**

Synthesis of PC, PE, PMME or PDME in the presence of free bases has been studied in many cultured cells [85-87], including rat hepatocytes [42]. However, the *in vivo* synthetic pathways through which PMME and PDME are formed from the corresponding free bases are unknown. Our data showed that cultured hepatocytes from CD rats could synthesize and accumulate (in the absence of methionine) PMME and PDME from the corresponding bases, and the formation of these lipids was accompanied by significant decreases in cellular PC and PE levels. We do not know whether the reduction in cellular PC and PE is a consequence of inhibited synthesis or stimulated degradation or both. It has been shown in rat liver that *in vitro* formation of PMME and PDME from the corresponding bases share the cytidine diphosphate pathways used for PC and PE synthesis[84,87]. Possibly, the phosphate esters of mono- and di-methylethanolamine compete with phosphocholine and phosphoethanolamine for the synthesis of CDP-esters. Preliminary results from this laboratory have shown that supplementation of *N*-monomethylethanolamine to the culture medium inhibits the conversion of phospho[3H]choline to PC in cultured rat hepatocytes (Szarka, R., Yao, Z. and Vance, D.E., unpublished observation). How phosphate esters of mono- and di-methylethanolamine are formed is also unknown. It has been demonstrated in rat that choline kinase also phosphorylates ethanolamine, and these two kinases are the same protein [53]. Thus, phosphorylation of mono- and di-methylethanolamine is presumably catalyzed by choline/ethanolamine kinase. However, direct evidence for these reactions is lacking. Formation of CDP esters of phosphomonomethylethanolamine and phosphodimethylethanolamine by purified cytidylyltransferase needs to be demonstrated.
The present work has shown that hepatic PE concentrations are not altered upon the addition of ethanolamine. A lack of effect of ethanolamine on cellular PE concentrations might be due to a pre-existing elevated PE caused by choline deficiency (Table VI). Similar to our results, data obtained by Yorek et al. [79] in a human tumor cell line also have shown that ethanolamine does not affect cellular PE levels or PE \( N \)-methyltransferase activity. Stimulated incorporation of \(^3\text{H}\)glycerol into PE, but not PC, has been observed in cultured normal rat hepatocytes with the addition of exogenous ethanolamine [42]. However, there is little evidence showing PE mass is increased by ethanolamine supplementation. An alternative explanation for the lack of effect of ethanolamine might be that synthesis of PE in hepatocytes is mainly via phosphatidylserine decarboxylation rather than the CDP-ethanolamine pathway, as observed in cultured baby hamster kidney cells [83].

6. The Increased Microsomal Cytidylyltransferase Activity may Compensate for the Lack of Choline Supply during Choline and Methionine Deficiency

Enzymatic studies suggest that the total activity of cytidylyltransferase in rat liver appears to be preserved in choline deficiency, but the specific activity of the enzyme in cytosol is decreased, while microsomal activity is increased. The reason for this reciprocal change in cytidylyltransferase activity in these two subcellular fractions during choline deficiency is unclear, although supplementation of choline to the deficient hepatocytes has been clearly shown to reverse the changes in the distribution of enzyme activity.

Several possible factors which may cause the change in cytidylyltransferase activity in choline deficiency have been examined. Since free fatty acids promoted cytidylyltransferase association to membranes [90,99,100], we first examined hepatic free fatty acid concentrations in choline deficiency. Quantitative analysis showed that liver fatty acid level was 82 \( \mu \)g/g liver in
choline deficiency (this is about 0.3 mM, assuming the average molecular weight of the fatty acid is 270 and 1 g liver is equivalent to 1 ml), while in normal liver free fatty acid is below the level of detection. These data are reminiscent of the results obtained from HeLa cells that 0.35 mM free fatty acids effectively caused cytidylyltransferase association to membranes [99]. In culture hepatocytes, we performed $[^{14}\text{C}]$acetate-labeling experiments in order to examine whether or not supplementation of choline would reduce cellular free fatty acid levels and therefore cause the change in cytidylyltransferase activity and its location. Results obtained show that the rate of fatty acid turnover appears to be independent of choline supplementation. It is possible, however, that the labeled free fatty acids underwent rapid turnover and represented only the metabolically active fatty acid pools, while the bulk of free fatty acids accumulated in hepatocytes in choline deficiency was inert. However, the lack of an effect of albumin supplementation on cytidylyltransferase activity and its location in CD hepatocytes suggests that cellular fatty acid levels are unlikely to be involved. Therefore, even though choline deficiency causes an accumulation of free fatty acids in the liver, the change of cytidylyltransferase activity in cytosol and microsomes may not be a direct consequence of the elevated fatty acids.

Membrane PC content has been suggested to be important for the membrane-association of cytidylyltransferase [103-105]. Our data, however, do not support this point of view. We found that the decreased membrane-associated cytidylyltransferase activity upon the choline supplementation is not simply a consequence of elevated microsomal PC levels, because the addition of lysoPC also stimulated PC synthesis and increased cellular PC concentration, but had not effect on the subcellular localization of cytidylyltransferase activity (Fig.33). Therefore, changes in the location of cytidylyltransferase activity after the addition of choline most probably result from some factors produced during the
process of PC synthesis via the CDP-choline pathway rather than the level of PC itself.

Liver 1,2-DG levels are also increased in choline deficiency. $^{14}$C$^{1,2}$-DG concentration was diminished when active PC synthesis via the CDP-choline pathway is regained after the addition of choline (Fig.35). It has been suggested that endogenous DG will enhance the membrane binding of cytidylyltransferase [316]. Possibly, the reduced 1,2-DG levels during choline supplementation cause the dissociation or inhibition of membrane-bound enzyme. Since the formation of PC via reacylation of lysoPC does not require 1,2-DG, the lack of a change in cytidylyltransferase activity after the addition of lysoPC may reflect the unchanged 1,2-DG levels. However, if cellular 1,2-DG levels do play an important role in mediating cytidylyltransferase activity, how 1,2-DG acts reciprocally on the cytosolic and membrane-bound enzyme activities is unclear. Involvement of phosphorylation of cytidylyltransferase in the changes of enzyme activity during choline deficiency has not been investigated.

Although the mechanism(s) involved is not clear, the increase in hepatic microsomal cytidylyltransferase activity during choline deficiency may be of importance to cell PC metabolism. Choline deficiency does not cause a dramatic decrease in hepatic PC levels (Table VI). While hepatic choline and phosphocholine concentrations are severely reduced in choline deficiency (142-145), hepatic CDP-choline levels are virtually unaffected [146,146b]. Apparently, the CD livers could more effectively utilize choline and phosphocholine to maintain their normal CDP-choline levels. This is most likely achieved by the increased activity of microsomal cytidylyltransferase, the active species of the enzyme in liver cells. Several lines of evidence support this point of view. In CHO cells, PC turnover is reported to be supressed by choline deficiency, and utilization of the free choline generated during phospholipid turnover is
enhanced [102]. In the choline-deficient lung, both CDP-choline and PC levels are maintained and the microsomal cytidylyltransferase activity is stimulated [101,176,177]. Therefore, increase in the activity of microsomal cytidylyltransferase may reflect the capability of liver (lung as well) to maintain relative normal cellular PC levels in choline deficiency.

It is worthwhile to note that a clear demonstration of the movement of cytidylyltransferase protein between cytosol and microsomal membrane has not been provided. Choy et al. [148] have reported in CD livers that the decrease in cytosolic cytidylyltransferase activity was not due to a diminished enzyme protein. Thus, the question whether or not there is a cellular modulator which governs the activity of cytidylyltransferase in choline deficiency remains to be answered.

7. Hepatic Phosphatidylethanolamine Methylation in Choline Deficiency

Previous studies on the effect of choline deficiency on hepatic PE methylation could not generate a consensus, mainly due to the lack of knowledge about the enzyme which catalyzes PE methylation. It was thought that hepatic PE methylation was catalyzed by two separate enzymes: the first converted PE to PMME and the second catalyzed conversions of both PMME to PDME and PDME to PC [321]. In the previous studies on PE N-methyltransferase in choline deficiency, an increased conversion of PE to PMME and a decreased PDME to PC were reported [149,151]. However, since choline deficiency caused an elevation of cellular PE levels, the observed increase in PE N-methyltransferase activity using endogenous PE as the methyl group acceptor most likely resulted from the variance in enzyme substrate.

Recent purification of PE N-methyltransferase from rat liver has shown PE methylation is catalyzed by a single enzyme [66]. Having new knowledge of hepatic PE N-methyltransferase, we re-evaluated the effect of choline deficiency
on PE methylation using both PE and PMME as substrates. While the activity of PE $N$-methyltransferase activity was increased in CD livers using endogenous PE as a substrate, the difference in its activity between CD and CS livers was diminished when exogenous PMME replaces PE as the methyl group acceptor. Supplementation studies conducted in cultured hepatocytes have demonstrated that hepatic PE $N$-methyltransferase activity is most possibly regulated by cellular PE levels. Active synthesis of PC, resumed by the addition of choline, does not seem to affect PE methylation. Furthermore, immunoblotting studies have provided more supportive information on the amount of PE $N$-methyltransferase during choline deficiency. The immunoblots of liver and hepatocyte proteins (Fig.36 & 39) fully agreed with the enzyme activity assays using PMME as a substrate; that PE $N$-methyltransferase does not undergo adaptive changes in choline deficiency. The preserved PE $N$-methyltransferase mass is also reflected by the capability of hepatocytes to rapidly compensate for choline deficiency by PE methylation when methionine is supplemented.

8. Conclusions and Future Considerations

Direct studies on the role of active PC synthesis in the process of hepatic lipoprotein secretion is the main theme of this thesis. Using cultured choline- and methionine-deficient hepatocytes, we have demonstrated that: (1) the active synthesis of PC is required for hepatic VLDL, but not HDL, secretion; (2) there is no requirement for a specific PC synthetic pathway in hepatic VLDL secretion; one of the three pathways, the CDP-choline pathway, methylation of PE or reacylation of lysoPC, is sufficient; (3) there is a striking head group specificity in the requirement of PC synthesis for hepatic VLDL secretion; synthesis of PE, PMME or PDME can not substitute for PC; and (4) hepatic synthesis and degradation of apoproteins B, E and Cs is not affected by choline and methionine deficiency.
In studies on the effect of choline deficiency on the activities and the amount of hepatic PC synthetic enzymes, we have shown that: (1) choline deficiency caused an increase in the microsomal cytidylyltransferase activity and a decrease in the cytosolic cytidylyltransferase; (2) supplementation with choline rapidly diminished the elevated microsomal cytidylyltransferase activity and increased the cytosolic cytidylyltransferase activity; and (3) there is no adaptive change in hepatic PE N-methyltransferase during choline deficiency.

We now know that active PC synthesis is essential for hepatic VLDL secretion, but the reason for this rather specific requirement is not explained. Based on the discoveries presented in this thesis, we believe that cultured hepatocytes from a single liver of a CD rat has great potential as an experimental system for the investigation of VLDL assembly, intracellular transit between ER and Golgi and secretion. Further studies will be directed to elucidate the site(s) of choline phospholipid integration into VLDL, the site(s) of the defect in the intracellular transit of VLDL particles during choline deficiency, and the source(s) of choline phospholipids for hepatic HDL.

That choline deficiency caused changes in cytidylyltransferase activity and its subcellular localization is also of interest, since it provides a model to study the regulation of PC synthesis via the CDP-choline pathway. Regulatory mechanism(s) involved in controlling cytidylyltransferase activity and its location, for example phosphorylation and dephosphorylation, may be revealed in this experimental system.
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